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ANALYSIS OF PLATELET PHOSPHOLIPIDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Analysis of platelet phospholipids was attempted by means of high performance liquid chromatography (HPLC). In order to separate phosphatidic acid (PA) in addition to phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC), the mobile phase of Chen and Kou (J. Chromatogr., 227, 25-31, 1982) was modified. For the quantitative analysis, fluorescein was found to be a suitable internal standard. With these inventions, the amount of phospholipids could be determined within 30 min after lipid extraction and 10^7 platelets were required for one assay. Then, stimulus linked phospholipids breakdown was studied using HPLC and the results were compared with those by conventional ^{32}P-thin layer chromatography (TLC) method. Similar results were obtained except that the amount of PA determined by TLC was much higher, probably due to active phosphorylation process. These observations suggest that the quantitative analysis of phospholipids could be achieved by our method with HPLC, which is advantageous over conventional methods in rapidity, no requirement of isotopes and determination of absolute amount of phospholipids.

INTRODUCTION

Blood platelets undergo secretion of granular contents upon physiological stimulation such as collagen or thrombin. And as seen in other secretory cells, this secretory reaction is coupled with a very rapid metabolism of membrane phospholipids in part to supply arachidonate for cellular synthesis of prostaglandins and thromboxanes. These reactions have been studied using

Key words: human platelet, phospholipid, TLC, HPLC
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radioisotopes and conventional TLC. In most of reports, radioisotopes tagged with arachidonate (1-7), glycerol (1,2,7,8), inositol (4) or ATP (3-5) were incorporated in living cells by means of incubation. These labelled compounds might be selectively incorporated into phospholipids with active turnover in resting state and less amount of isotopes into less active turnover pool of phospholipids, which would play a vital role upon stimulation. Therefore, it seems rather difficult to study actual turnover of phospholipids in stimulated platelets using labelled compounds. As an alternative method, measurement of phosphorus has been used (9,10) but this method is time consuming and with inadequate accuracy. Therefore, we have attempted to separate major phospholipids of platelets by means of HPLC and to study active metabolism of platelet phospholipids upon stimulation. We have originally applied the method of Chen and Kou (11) and modified it to be able to detect PA, an important intermediate in PI-cycle in addition to PI, PS, PE, PC and we have also found fluorescein as a suitable internal standard to quantitate the amount of phospholipids.

MATERIALS AND METHODS

Preparations of washed platelet suspension.....Fresh human blood anticoagulated with 0.38% trisodium citrate was obtained from healthy subjects who had not received any medication in the previous two weeks. Platelet rich plasma (PRP) was then obtained by centrifugation of the citrated blood at 120 g for 13 minutes. Though all the preparations were done at room temperature, using siliconized or plastic tubes to avoid the activation of platelets, further precaution was considered employing PGI_2 for cytoprotection according to the method of Moncada et al. (12). PGI_2 (5 ng/ml) was added to PRP and the mixture was centrifuged at 850 g for 15 minutes. Platelet pellets derived from 20 ml of PRP were resuspended in 5 ml of a modified HEPES buffer (142 mM NaCl, 6.2 mM KCl, 2.4 mM $MgSO_4$, 6.5 mM HEPES, pH 7.4). Then after the second addition of PGI_2 (300 ng/ml) to the suspension, platelets were pelleted by centrifugation at 670 g for 10 minutes. The same washing procedure was repeated once more and resuspended in the HEPES buffer without PGI_2. The final platelet count was adjusted to be 5×10^8/ml. The suspension thus obtained was found to be free of any contaminants by a microscopic examination and the viability of cells was repeatedly confirmed by the presence of ATP secretion upon addition of an agonist using Lumi-aggregometer® (CHRONO-LOG, U.S.A.).

Labelling procedure.....In order to label inorganic ^{32}P to platelet phospholipids, 300 μCi of ^{32}P (acid free, Amersham, JAPAN) was incubated at 37°C for 60 minutes with washed platelet suspension under gentle shaking, and the same washing procedure was repeated twice as described earlier to remove free ^{32}P from the suspension. Samples for HPLC was also incubated in the same way.

Lipid extraction.....The following procedure was performed according to the modified method of Bligh and Dyer (13). Washed platelet suspension with or without ^{32}P was preincubated at 37°C for 2 minutes in glass tubes and then platelets were stimulated under gentle shaking with bovine thrombin (0.5 U/ml) or calcium ionophore A_{23187} (4 μM). And at designated times, the reaction was terminated by adding 2 ml of cold extraction medium of Bligh and Dyer [Chloroform / methanol / 10 N HCl / fluorescein (200 μg/ml in methanol, added only for the samples for HPLC analysis) 100:200:2:1 (v/v)] and the following procedures were done at 0°C. After vigorous mixing for 5 seconds, 600 μl of cold chloroform and 600 μl of 2 M KCl were added in succession under shaking for 30 seconds and then the mixtures were kept

still for 5 minutes. After gentle mixing for 5 seconds, the mixtures were centrifuged at 1,700 g for 10 minutes to separate the aqueous and organic phase. In order to analyze by TLC, 1 ml each of solution was taken from organic phase, and for HPLC analysis, 0.5 ml each was taken. Then the solution was dried under N_2 flush and sealed off from the air and the samples were kept frozen at -20°C until assays.

TLC.....Separation of platelet phospholipids was performed by single-dimension TLC according to the method of Jolles et al (14). TLC plates (LK6D, Whatman, U.S.A.) were impregnated with 1% potassium oxalate and just prior to application of samples, they were activated 60 minutes at 110°C. The samples were redissolved with 35 µl of chloroform / methanol (9:1 (v/v)) under gentle shaking for 15 seconds. Twenty microliter of aliquots was applied on the plate. Then, chromatography was performed at room temperature using the solvent system [chloroform / acetone / methanol / glacial acetic acid / water (40:15:13:12:8 (v/v))]. The lipids separated were visualized with iodine vapor and the plate was autoradiographed on SAKURA ^{3}H-film (Konishiroku, JAPAN). Each spot was identified by comigrated authentic samples and the spots were scraped off and the radioactivity was measured in a liquid scintilation counter.

HPLC.....The equipments used comprised the following units: solvent delivery system BIP-I®, injector VL-614®, variable wavelength UV detector UVIDEC-100-V® from JASCO, JAPAN, integrating recorder CR1A-CHROMATOPAC® from Shimadzu, JAPAN. The stainless-steel columns prepacked with silica, LS-320®, pore size 5-8 µm, were purchased from TOYO-SODA, JAPAN. For column elution, different solvent mixtures of acetonitrile, methanol, and 85% phosphoric acid were used. The solvent was delivered to the column at a flow-rate of 1 ml/min at a pressure of approximately 50 kg/cm^2 at room temperature. Absorption determination was done at 203 nm and absorbance range was 0.064. The samples, platelet lipid extracts and authentic phospholipids, were dissolved in 10 µl of methylenechloride. After mixing vigorously for 15 seconds, an aliquot (3 µl) was applied to HPLC.

Miscellaneous.....The following authentic phospholipids were purchased from SERDARY, CANADA; PA from egg, PI, PE and PC from pig liver, PS from beef brain. All the chemicals for HPLC were of highest grade available and obtained from NAKARAI, JAPAN. PGI_2 was kindly donated by ONO, JAPAN. Bovine thrombin was also donated by MOCHIDA, JAPAN. And A_{23187} was purchased from CALBIOCHEM, U.S.A..

RESULTS

Employing the HPLC instruments mentioned above, it was possible to separate PI, PS, PE and PC of human platelets, although PI was eluted very close to the solvent front (SF). In order to study the active metabolism of membrane phospholipids, separation of additional phospholipids and metabolites were desired, such as PA and diacylglycerol (DG). With repeated preliminary experiments, it was found possible to separate and identify PA by reducing the amount of phosphoric acid in the original solvent system. With this modification, PI was also clearly separated from SF. However, it was unable to identify DG with these solvent systems. Identification of respective peaks of phospholipids was carried out by comparison of retention time with that of authentic lipids. Then, phospholipids from resting platelets and from thrombin stimulated platelets (0.5 U/ml) were separated and compared using these two different solvent systems. A typical pattern was shown

in FIG. 1. One minute after the stimulation, the amount of respective phospholipids was apparently decreased and the concomitant formation of PA was detected.

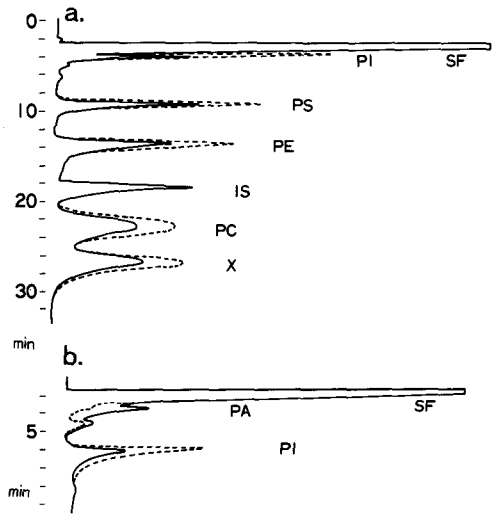

FIG. 1

HPLC pattern of lipid extracts from resting and stimulated platelets. Lipids were extracted as described in MATERIALS & METHODS from 2.5×10^8 resting platelets and from the same number of platelets stimulated by 0.5 U/ml thrombin for 1 min at 37°C. Each extract was dissolved in 10 µl of methylenechloride. After mixing for 15 sec, an aliquot (3 µl) was applied to HPLC. Chromatographic conditions; flow rate—1 ml/min, UV detection at 203 nm, absorbance range 0.064, mobile phase—acetonitrile / methanol / 85% phosphoric acid (FIG. 1-a 130:5:1.3 (v/v); FIG. 1-b 130:5:0.006 (v/v)); SF—solvent front, IS—internal standard, X—unknown peak; ----- resting, ——— stimulated

Calibration curves.....In order to quantify the amount of phospholipids separated by HPLC, several calibration curves were obtained, using authentic samples of known amount with or without extraction procedures. With the modified solvent system, calibration curves for PI and PA were obtained as shown in FIG. 2-a, b. Without extraction procedure (FIG. 2-a), as low as 10 ng of lipids were found to be determined, while microgram order of lipids were required for the assay with extraction procedure (FIG. 2-b). Calibration curves for PI, PS, PE and PC were also obtained in the same way using the original solvent system (11) (FIG. 2-c, d, e). Without extraction (FIG. 2-c, d), it was found that as low as 10 ng of PI, PS or PE can be quantified whereas a minimum amount of 150 ng was required for quantitation of PC. With lipid extraction procedure, microgram order of phospholipids were required for quantitation by HPLC. The final amount of authentic phospholipid into sample was about 10% (PI: 12.51±0.01%, PS: 10.84±0.01%, PE: 12.39±0.01%, PC: 7.90±0.01%, PA: 14.06±0.02%; mean±S.D., n=4) due to dilution and lipid extraction.

Internal standard.....Since a considerable amount of phospholipids of original sample was lost due to extraction procedure and dilution, a suitable internal standard substance was searched for. Among several candidates, it was found that fluorescein may be used as an ideal internal standard because it possesses sharp spectrum at 203 nm, dissolves easily in organic compounds and does not overlap with other peaks.

Then, analysis of phospholipids of resting platelets was compared with or without the internal standard. The results from 2.5×10^8 cells were listed in TABLE 1 and the values with internal standard were apparently with less deviation.

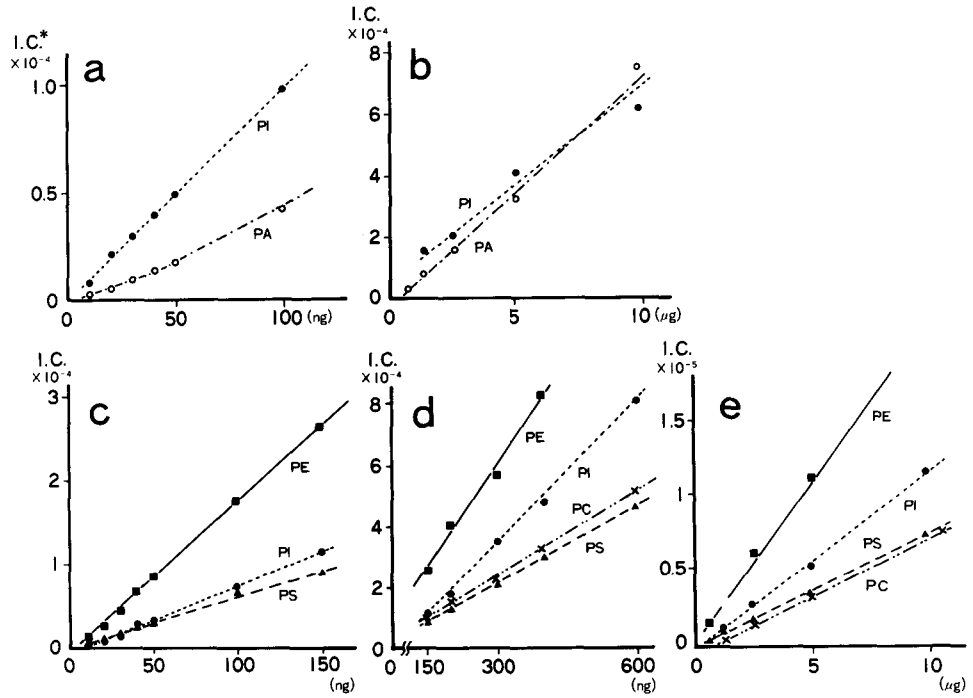

FIG. 2

Calibration curves for authentic PA (○), PI (●), PS (▲), PE (■) and PC (×). Extraction was performed in samples of FIG. 2-b, e, as described in MATERIALS & METHODS, while samples dissolved in methylenechloride were directly applied on HPLC, FIG. 2-a, c, d. Chromatographic conditions were same as in FIG. 1 except that internal standard was omitted. I.C.—integrated count

TABLE 1. Values of integrated counts of respective peaks of phospholipids derived from 2.5×10^8 resting human platelets with or without internal standard.

	I.C.*	I.C.* with I.S.**
PI	57,712 ± 8,146	57,638 ± 888
PS	135,523 ± 17,285	135,556 ± 3,546
PE	211,576 ± 31,625	211,244 ± 5,199
PC	296,859 ± 45,017	296,319 ± 7,797
I.S.	49,588 ± 6,353	(49,588)

* I.C. integrated count ($\bar{X}$ ± S.D., n=3)
** I.S. internal standard

Constituent phospholipids of human platelets.....With the present HPLC method, the composition of phospholipids per 10^9 cells was calculated using the results in Fig. 2 and TABLE 1 as follows; PI: 19.9±2.7 μg, PS: 71.0±9.0 μg, PE: 38.2±5.9 μg and PC: 138.1±20.2 μg (mean±S.D., n=3). The comparative results on similar studies by different methods such as TLC with phosphorus determination and gas liquid chromatography were summarized in TABLE 2 together with the present results expressed both in weight percentage and in I.C. percentage. Although there were slight differences between the present results expressed in weight percentage and values reported by others, values were very similar when the present results expressed in I.C. percentage were compared with the reported results.

TABLE 2. Constituent phospholipids of human platelets.

METHOD	UNIT	PI	PS	PE	PC	Ref.
HPLC	I.C. %	8.2*1	19.4	30.2	42.3	This work
	Wt*2 %	7.4	26.6	14.3	51.7	This work
GLC*3	mol %	7.6	13.8	29.0	49.6	(15)
TLC	P*4 mol %	5.0	13.3	35.2	46.5	(9)
TLC	P mol %	3.9	11.1	33.5	51.6	(16)

*1. PI + PS + PE + PC = 100%
*2. Wt % — weight percentage
*3. GLC — gas liquid chromatography
*4. P — phosphorus

Time dependent changes in phospholipids of stimulated platelets.....Time dependent changes in phospholipids was analyzed by using thrombin (0.5 U/ml) or A_{23187} (4 μM) as a stimulant, since each agent activates platelets well even in the absence of plasma proteins. When washed platelets were stimulated by 0.5 U/ml thrombin, as shown in FIG. 3-a, rapid decrease in the amount of PI was observed. On the other hand, with 4 μM A_{23187} stimulation (FIG. 3-b), a considerable amount of PI, PE and PC was decreased in the similar rate.

Stimulus linked hydrolysis of PI and formation of PA were analysed by both HPLC and ^{32}P-TLC.....In addition, secretion of ATP was monitored in Lumi-aggregometer® in parallel with lipid analysis. As in FIG. 4-a, thrombin stimulated hydrolysis of PI was observed by both methods. The gradual formation of PA was detected by HPLC, while very rapid formation of ^{32}P-PA was observed by TLC. Thrombin stimulated secretion of ATP was very rapid and reached plateau within 15 seconds. When similar study was performed with A_{23187} as a stimulant, which evoked slow and gradual ATP secretion as in FIG. 4-b, slight decrease in PI without any formation of PA was observed by HPLC, although ^{32}P-TLC analysis revealed sharp decrease in the amount of PI and considerable formation of ^{32}P-PA.

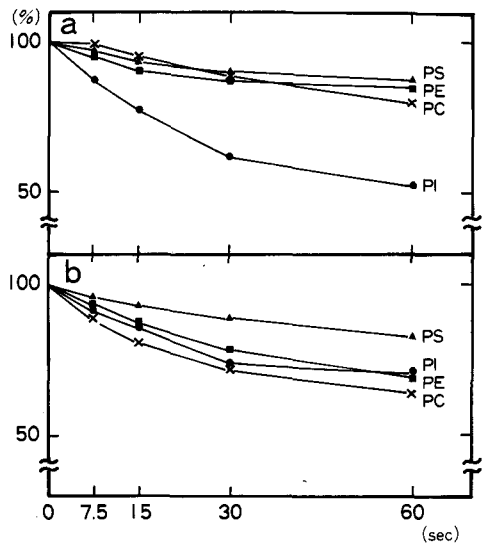

FIG. 3

Time dependent changes in phospholipids of human platelets stimulated by 0.5 U/ml thrombin (a) or by 4 μM A_{23187} (b). Extraction of lipids and HPLC were performed as in the text. The results were expressed as percentage of the amount in resting platelets.

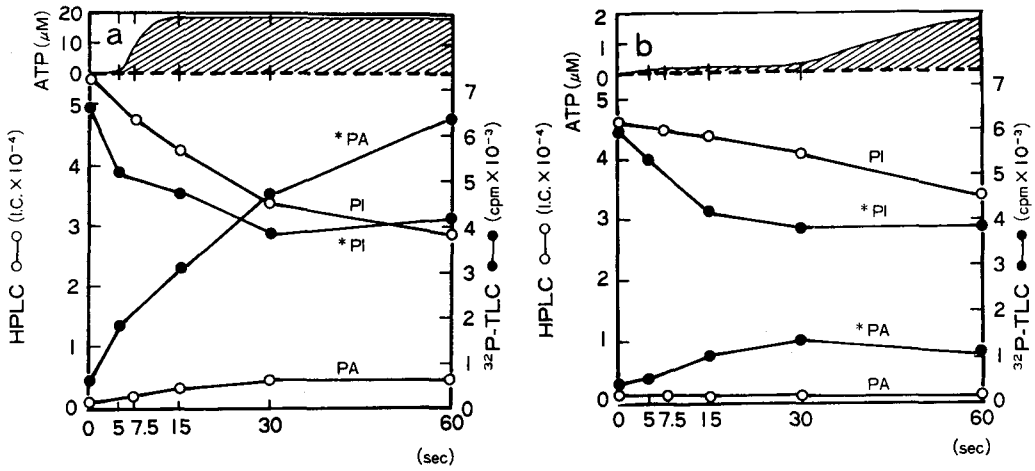

FIG. 4

Analysis of hydrolysis of PI and formation of PA in 0.5 U/ml thrombin (a) or 4 μM A_{23187} (b) stimulated platelets. Analysis was performed by HPLC (o——o) and by TLC (●——●) with ^{32}P-incorporated platelets ATP secretion was monitored simultaneously by Lumi-aggregometer®.

DISCUSSION

With recent advances in cell biology, the significance of phospholipid metabolism has been entertained, which are the major constituents of cellular membranes. However, it has been rather difficult to quantify species of phospholipids by conventional methods. Since the recent improvement on HPLC

systems, several attempts were made to apply HPLC in the analysis of phospholipids. The earlier studies using silica gel columns (17-20) or ion exchange columns (21-24) were not satisfactory to obtain rapid and reproducible resolution of phospholipids because water rich mobile phases were used which interfered the separation of PI and PS. In 1982, Chen and Kou (11) reported successful separation of major phospholipids employing a novel solvent system which was essentially devoid of water and use phosphoric acid as ion suppression. In the present study, we have modified their solvent system in order to separate PI and PA sharply, which are the major components of PI cycle. With the modified solvent system, it became possible to analyze PI cycle to some extent, though further modifications are necessary to identify and quantify DG and polyphosphoinositides.

Since the value in UV (190-210 nm) detection of HPLC is influenced mostly by double bonds, and slightly by carbonyl, carboxyl residue and phosphate (17,18), quantitation should be performed by comparing integrated count of known amount of authentic phospholipids (17,20,21,24). Although the constituent phospholipids per certain number of platelets was calculated for the first time as in RESULTS using standard curves obtained with authentic substance, the values might not reflect the actual composition because it was impossible to obtain calibration curves using authentic samples of platelet origin. Especially the curve of PE was apparently deviated from those of other phospholipids, which could explain the difference in the amount of PE assayed by different methods. Nissen and Kreysel (20) in their HPLC analysis obtained almost identical standard curves for PE from authentic substances of different species, and Briand (24) reported also identical curves for four major phospholipids by his HPLC analysis. The reason for these discrepancies should be elucidated to establish quantitative HPLC analysis of phospholipids from biological specimens by performing phosphorus determination of respective peak in HPLC. In our present method, however, it was impossible to do so because our mobile phase is composed of phosphoric acid.

A solid internal standard such as fluorescein in the present study is absolutely required for quantitative analysis. With our methods, as low as 10 ng of authentic phospholipid was required for the analysis of PA, PI, PS, and PE but about 150 ng was necessary for the determination of PC which has longer retention time. When biological materials such as platelets were used as samples, 10 times more amount was necessary because 90% of phospholipids were lost due to dilution and lipid extraction. Therefore at least 10^7 platelets per assay is required for the quantitative analysis of phospholipids in our HPLC system.

The phospholipids breakdown in thrombin stimulated platelets studied by the HPLC system was very similar with those by TLC in several literature (2-5,8). The simular study by HPLC with A_{23187} stimulated platelets also confirmed the reported data using TLC (3-5).

Several differences were observed in the amount of PI and PA by the simultaneous study of stimulated platelet both by HPLC and TLC. The amount of PA in thrombin stimulated platelets was much higher than that by TLC analysis, which was probably due to active incorporation of ^{32}P-ATP into PA. And the amount of PA formed in HPLC was much less than that of PI hydrolysed, which could be due to different UV absorption at 203 nm or to consumption in DG lipase (7,25) or phospholipase A_2 (3-6) mediated pathway. In A_{23187} stimulated platelets, the difference in the value of PA was also noticed, most likely due to de novo active phosphorylation of PA. These observations suggest that HPLC is superior to TLC in the quantitative analysis of phospholipid metabolism. However, further improvements are required to separate additional lipid such as DG and polyphosphoinositides for full application of HPLC in the study on cellular phospholipid metabolism.

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ANALYSIS OF PLATELET PHOSPHOLIPIDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY II—STUDIES ON HITHERTO UNKNOWN PEAK OF PHOSPHOLIPID OF HUMAN PLATELETS.

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ABSTRACT

With our proposed method to analyze platelet phospholipids, utilizing a normal phase high performance liquid chromatography (HPLC), it was able to quantify the amount of major platelet phospholipids (Thromb. Res. 36, 335-344, 1984). A prominent peak of unknown nature (PX) was identified close to the peak of phosphatidylcholine in the HPLC analysis of platelet phospholipids, and the attempts were made to elucidate the nature of PX. By applying several additional authentic phospholipids and those treated by acids on both the HPLC and thin layer chromatography, it was concluded that the major component of PX is 1-lyso phosphatidylethanolamine plasmalogen (PEP), artificially degraded from PEP by the acidic HPLC solvent system. No further degradation of 1-lyso PEP was observed and the absorbance of possibly co-migrated 2-lyso PE in activated platelets was negligible because it was devoid of C-2 double bonds sensitive to the absorbance used in the assay. Therefore, the relative amount of PEP may be detected based on the number of double bonds in platelet PEP. The amount of PEP thus measured was significantly decreased in thrombin-stimulated platelets, suggesting a possible participation of PEP in stimulus-linked platelet reaction.

INTRODUCTION

We have devised a simple and rapid method to quantify platelet phospholipids by a normal phase high performance liquid chromatography (HPLC) without use of radioisotopes (1). During the study, a prominent peak of unknown nature (PX) was identified, which was eluted just after the peak of phosphatidylcholine (PC). It was not possible, however, to elucidate the nature of PX, as none of authentic phospholipids tested yielded the corresponding peak to PX (1). Therefore, the attempts were made to characterize the nature of PX and the results were presented herein.

Key words: Human platelet, phospholipid, plasmalogen, TLC, HPLC

MATERIALS AND METHODS

Preparation and stimulation of human platelet suspension.....Fresh human blood anticoagulated with 0.38% trisodium citrate was obtained from healthy subjects. Preparation of washed platelets was performed as described previously, using PGI_2 for platelet cytoprotection (1). Washed human platelets (4×10^8/ml) were finally suspended in HEPES buffer (142 mM NaCl, 6.2 mM KCl, 2.4 mM $MgSO_4$, 6.5 mM HEPES, pH 7.4). For activation, they were incubated with 1 U/ml thrombin or saline (control) under stirring for designated times. Lipids were then extracted as described below.

Lipid extraction.....All lipid extraction procedures were performed according to the slightly modified method (1) of Bligh & Dyer (2). The extraction mixture was composed of sample (0.5 ml), [chloroform/methanol/fluorescein (100:200:1)] (2.0 ml), chloroform (0.6 ml) and distilled water (0.6 ml).

HPLC.....Analysis of phospholipids by HPLC was performed as described previously (1). Briefly, chromatography was run on a JASCO Model liquid chromatograph, consisting of DG-3310® degasser, VL-614® injector, BIP-I® solvent delivery system and UVIDEC-100-V® variable wave length UV detector (JASCO, JAPAN) and supplemented by a CR-1A CHROMATO PAC® integrating recorder (Shimazu, JAPAN). The column used was LS-320 packed with silica, 0.4×30 cm, 5-8 μm particle size (Toyo-Soda, JAPAN). Before analysis, the column was conditioned as follows; the column was eluted by 20 ml each of methanol, methanol/water (1:1) and mixture of acetonitrile-methanol-85% phosphoric acid (130:3:1.3 v/v) at a flow rate of 0.5 ml/min. The column condition was set up more than ten hours before running. The eluate used was a mixture of acetonitrile-methanol-85% phosphoric acid (130:3:1.3). The solvent was delivered to the column at a flow rate of 1.5 ml/min at room temperature. Monitoring of column effluent was done at 203 nm and absorbance range was 0.064. The samples were dissolved in 25 μl of methylene-chloride. After mixing vigorously for 15 sec, an aliquot (3 μl) was applied to HPLC.

TLC.....Separation of platelet phospholipids was performed by single dimension TLC. TLC plates (LK6D, Whatman, USA) were used. Chromatography was performed at room temperature using the solvent system [chloroform/methanol/water (70:35:7)]. The lipids separated were visualized with iodine vapor.

Miscellaneous.....The following authentic phospholipids were purchased from Serdary, CANADA; phosphatidylethanolamine (PE), PC, lyso-phosphatidylethanolamine (LPE) and lyso-phosphatidylcholine (LPC) from pig liver, phosphatidylethanolamine plasmalogen (PEP) from beef brain, phosphatidylcholine plasmalogen (PCP) from beef heart. All the chemicals for HPLC and TLC were of highest grade available and obtained from Nakarai, JAPAN. PGI_2 was kindly donated by Ono, JAPAN. Bovine thrombin was also donated by Mochida, JAPAN.

RESULTS

On the HPLC analysis of platelet phospholipids, a distinct peak of PX was always eluted with retention time of approximately 37 min right after the peak of PC as shown in FIG. 1. As a fixed amount of fluorescein (200-800 μg/ml) was always premixed with samples as an internal standard, an apparent decrease in the amount of PX as well as phosphatidylinositol (PI), PE and PC was observed in platelets stimulated by 1 U/ml thrombin for 5 min (FIG. 1-b).

In the previous study, none of the authentic phospholipids tested formed any peak corresponding to that of PX. Taking this fact into account, a

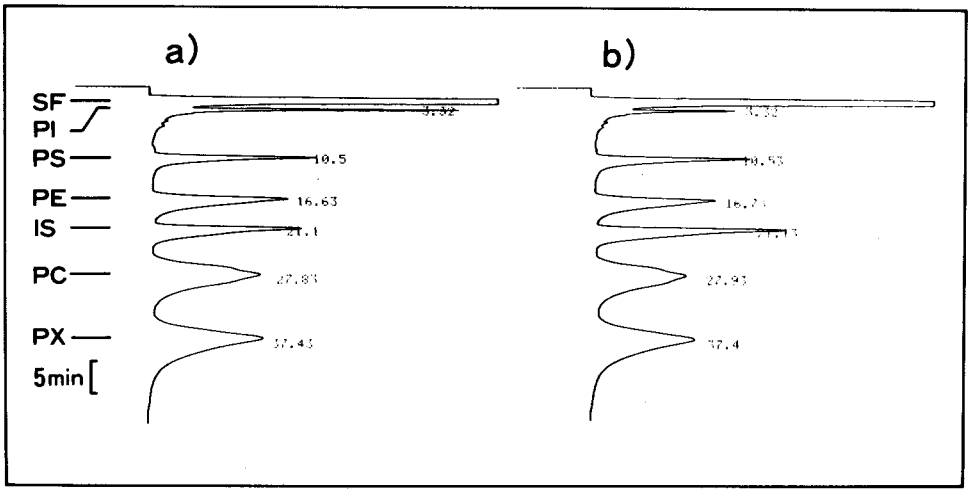

FIG. 1
A typical HPLC pattern of lipid extracts from human platelets. Lipid extraction and HPLC application were described in MATERIALS & METHODS. a) control; b) 5 min after 1 U/ml thrombin stimulation: chromatographic conditions; flow rate—1.5 ml/min, UV detection—203 nm, absorbance range—0.064, mobile phase—acetonitrile/methanol/85% phosphoric acid (130:3:1.3 v/v). SF—solvent front, PI—phosphatidylinositol, PS—phosphatidylserine, PE—phosphatidylethanolamine, IS—internal standard, PC—phosphatidylcholine.

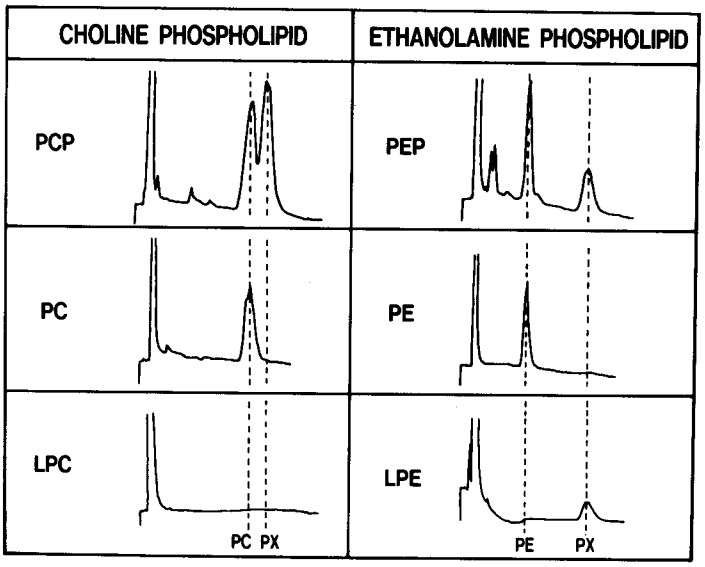

FIG. 2
HPLC patterns of different classes of choline- and ethanolamine-phospholipids. The amount of phospholipids applied to HPLC was as follows. PCP, LPC and LPE—10 μg, PEP—5 μg, PC—2 μg, PE—1 μg. See MATERIALS & METHODS for details of chromatographic conditions.

possibility was raised that PX might represent different types of well-known phospholipids such as alkyl- or alkenyl-type, if retention times are different between these phospholipids. At the same time, another possibility was brought up that PX may contain degraded phospholipids, as our HPLC eluate contains weak acid.

Thereby, additional authentic samples including alkenyl type and lyso phospholipids were analyzed on the HPLC. As shown in FIG. 2, it was found that alkenyl type choline- or ethanolamine-phospholipids (PC or PE plasmalogen (PCP or PEP)) contains two peaks, one of which is exactly corresponding to the peak of PX. Also, lyso-PE (LPE) was found to yield a small peak corresponding to that of PX, while no peak of lyso-PC (LPC) was appeared within 40 min after the application. Then, in order to study possible degradation of various phospholipids by phosphoric acid present in our HPLC eluate, various authentic phospholipids were incubated with the HPLC eluate for 20 min at room temperature and after neutralization with diluted NaOH in methanolic solution, the products were analyzed on TLC. As shown in FIG. 3, the product of PEP yielded an additional spot of the same Rf value as PX and LPE. Untreated PCP contained the same peak, which was not altered by the treatment. Furthermore, these alkenyl type of phospholipids were treated by strong acid (3N HCl in methanol) for 20 min and the products were analyzed by HPLC. As in FIG. 4, by such treatment, both peaks of PEP were slightly lowered and only PX peak of PCP was disappeared, suggesting that PX may contain LPEP and PCP.

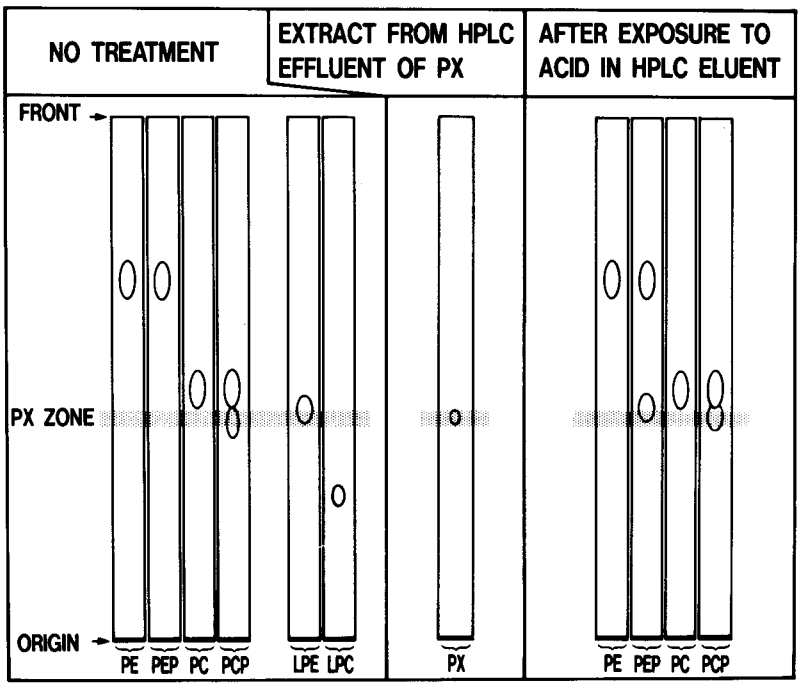

FIG. 3

Instability of alkenyl type of authentic phospholipids to weak acid such as phosphoric acid in the HPLC eluate was shown by TLC. The solvent used was a mixture of chloroform/methanol/water (70:35:7 v/v). The lipids separated were visualized with iodine vapor. Four samples on the right side were pretreated with HPLC eluate containing phosphoric acid for 20 min. See MATERIALS & METHODS for details of chromatographic conditions.

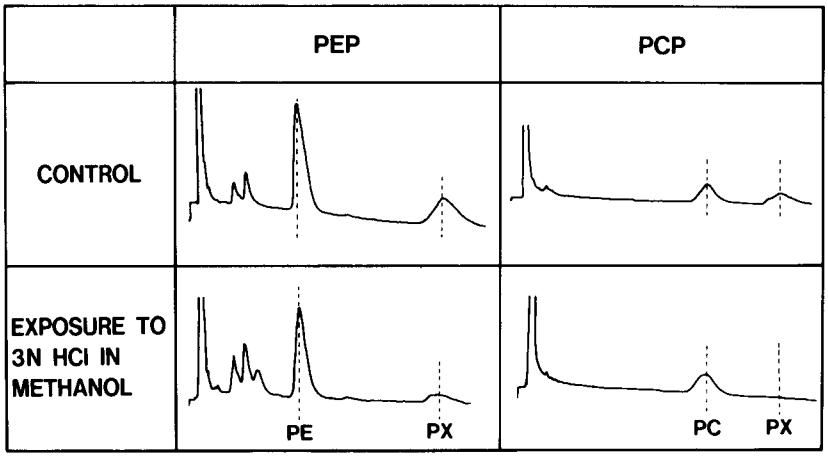

FIG. 4
Instability of alkenyl type of authentic phospholipids to strong acid (3N HCl in methanolic solution). After alkenyl phospholipids were treated by HCl for 20 min at 25°C, products were neutralized with NaOH. Lipids were extracted and analyzed by HPLC as described in MATERIALS & METHODS.

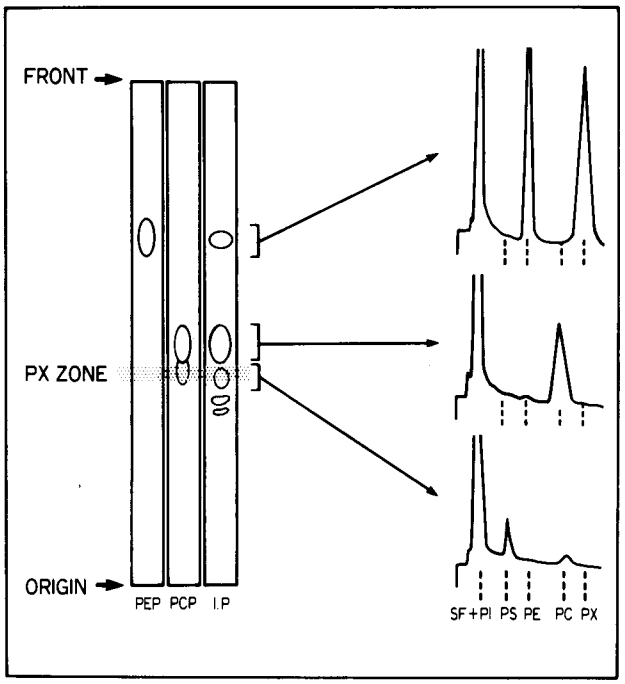

FIG. 5
HPLC analysis of platelet phospholipids fractionated on TLC. Platelet extracts and authentic samples (PCP or PEP) were separated on TLC. Those spots corresponding to PE, PC or PX were scraped off, extracted in chloroform/methanol (8:2), and dried under N_2 flush. An aliquot was applied to HPLC. Details of chromatographic conditions were described in MATERIALS & METHODS. I.P—phospholipid extracted from intact platelets.

Then we proceeded to study the content of these alkenyl type of phospholipid in human platelets. Platelet phospholipid extracts were separated on TLC with neutral solvents along with PEP and PCP and then lipids extracted from these zones corresponding to PE, PC and PX were analyzed on HPLC. As in FIG. 5, PE fraction from TLC yielded two peaks on HPLC corresponding to that of PE and PX, respectively. Neither PC or PX fraction formed any peak on HPLC corresponding to PX. These results indicated that major constituent of PX is LPEP in human platelets.

Since we found that the amount of PEP in human platelets may be quantified as the amount of PX by the HPLC, the time dependent changes in the amount of PEP as well as PI, PS, PE and PC were determined in platelets stimulated by 1 U/ml thrombin. As shown in FIG. 6, the amount of PX was decreased within 5 min as PI, PE and PC (statistically significant; $P<0.05$ in Student's t test), suggesting the possible participation of PEP in stimulus-linked metabolism of phospholipids.

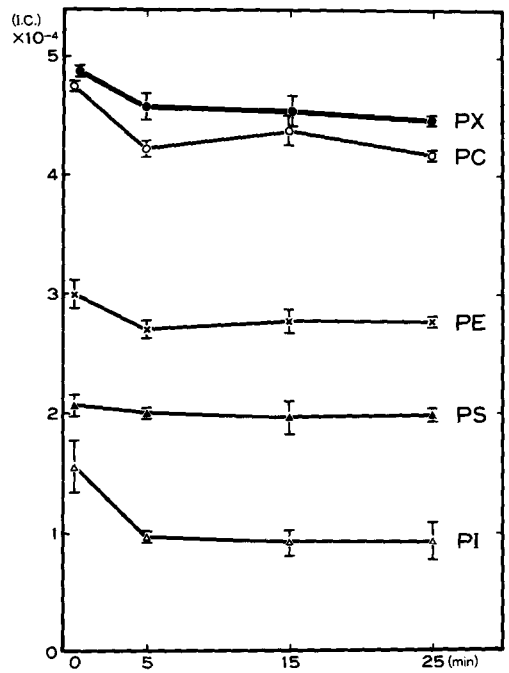

FIG. 6
Changes in phospholipids of thrombin-stimulated platelets. Washed human platelets (5×10^8/ml in calcium free HEPES buffer) were stimulated by 1 U/ml thrombin. Lipid extraction and HPLC analysis were done as described in MATERIALS & METHODS. I.C. = integrated count. Values shown are means ± S.D. from three experiments.

DISCUSSION

In the foregoing paper, it was not able to elucidate the nature of PX just by running major authentic phospholipids on the same HPLC (1), though it was suspected that PX is a class of phospholipid and is not a natural metabolite as it was extracted by lipid extraction and was rich in resting platelets. In our present study, it was found that authentic LPE yields small but exactly the same peak as that of PX on the HPLC. As detection of peaks in our HPLC is based on the absorbance at 203 nm, arising mainly from double bonds of unsaturated fatty acids linked to C-2 position of glycerol structure in phospholipids (3,4), lyso-phospholipids lacking unsaturated fatty acid in C-2 position should yield much smaller peak. Furthermore, there should not be a considerable amount of lyso-phospholipids in resting platelets (5). Taking these facts together into account, it was further speculated that PX may be

1-lyso PE formed during the HPLC, which is rich in double bonds. The eluate in our HPLC contains phosphoric acid (1%), which could degrade certain species of phospholipids. Among them, the plasmalogens, alkenyl type of phospholipids, have been known to be labile upon acid treatment (6). And as demonstrated in the present study, both authentic PEP and PCP yielded two peaks, respectively, one of which was most likely due to contamination as it was hardly possible to separate alkenyl- or alkyl-type of single class of phospholipid by conventional methods (7-9). Actually, as shown in the present study, not PCP but PEP was degraded by phosphoric acid present in the HPLC eluate. And in the separation of platelet phospholipids the degradation is likely to be processed immediately after the injection of sample, as the peak of PX was always clearly separated without interfearing other peaks. The additional fact that both authentic PEP and PCP were degraded by 3N HCl in methanol may be explained by a possible contamination in commercially available authentic plasmalogens of cyclic acetal phospholipids, which are resistant to weak acid (10).

Natarajan et al. (11) reported that PEP represents 45.3 mol % of ethanolamine phospholipids and PCP does only 1.4 mol % of choline phospholipids in human platelets. It was also reported by Mueller et al. (12) that PEP and PCP represent 60.4 and 8.8 mol % of ethanolamine and choline phospholipids, respectively. Therefore, we concluded that the predominant component of PX is 1-lyso PEP formed by the action of phosphoric acid during the HPLC and that the total area of the peak is proportional to the amount of PEP in platelets, as co-eluted 2-lyso PE (LPE) possibly formed in activated platelets may be negligible because of the lack of C-2 double bonds sensitive to absorbance at 203 nm (3,4). In the foregoing paper (1), we reported the phospholipid constituents of human platelets analyzed by the HPLC, where the amount of PE was smaller than that reported by other investigators (5,11-13). This might be well explained by the fact that we were separating PEP from PE, while others were not. It was not possible, however, to quantify the absolute content of PEP in platelets, unless the pure authentic PEP is available.

The plasmalogen was first discovered by Feulgen and Voit in 1924 (14) and it was roughly quantified in human platelets in 1960 (15). Until present, the plasmalogens have been measured by the following method; i) assay of fatty aldehydes (16,17) or phosphate ester (18) of $HgCl_2$ or acid treated samples. ii) assay of iodination to alkenyl ether bond (19,20). iii) TLC (21-23), GLC (24) or HPLC (25) assay on enzyme treated, acetylated, HCl-gas treated or vitride treated samples. Comparing our method with above listed procedures, it might be concluded that our method is advantageous over them, especially in simplicity and rapidity. Using the HPLC assay method, we have also demonstrated that the amount of PEP is decreased upon stimulation of platelets by thrombin, suggesting possible participation of PEP in stimulus linked intermediary reaction of platelets, details of which have yet to be elucidated, though Rittenhouse suggested the active metabolism of PEP in human platelets (26).

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INTERRELATIONSHIP BETWEEN SECRETION, PROTEIN PHOSPHORYLATION AND INTRACELLULAR Ca^{2+} CONCENTRATION IN PLATELETS STIMULATED BY THROMBIN OR THROMBOXANE A_2 ANALOGUE

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ABSTRACT

The interrelationship between ATP-secretion, protein phosphorylation and intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) was studied in both ^{32}P and quin 2 loaded human platelets stimulated by thrombin or thromboxane A_2 analogue (STA_2). In platelets stimulated by thrombin, the degree of 47,000 dalton polypeptides (P47) phosphorylation was observed in completely dose-related manner, regardless of the amount of $[Ca^{2+}]i$. In the same condition, the degree of myosin light chain (P20) phosphorylation, however, was well correlated with ATP secretion and $[Ca^{2+}]i$, when platelets were stimulated by lower dose of thrombin. The similar results were obtained in platelets stimulated by STA_2. These findings suggested that P20, but not P47, phosphorylation in activated platelets is mediated by a rise of $[Ca^{2+}]i$ and is well correlated with the secretory reaction. It was unlikely that P47 phosphorylation plays any role in promoting platelet activation.

INTRODUCTION

Stimulus linked phosphorylation of several polypeptides in platelets has been implicated as the important regulatory mechanism of the platelet reaction (1)(2)(3), though the physiological significance of these phosphorylation has not been fully elucidated. Among several polypeptides phosphorylated in stimulated platelets, it has been well known that P47 (4)(5) and P20 (6)(7) (identified as myosin light chain) are phosphorylated by C-kinase (8) and myosin light chain kinase (MLCK) (6), respectively. C-kinase can be activated by Ca^{2+} or diacylglycerol (DG) (8) and MLCK by calmodulin and Ca^{2+} (9). As the measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) in smaller cells such as platelets and lymphocytes became possible in our hands, using ingenious device of quin 2 system (10), the interrelationship

Key words: platelets, intracellular $[Ca^{2+}]$, quin 2, P20, P47, phosphorylation

between protein phosphorylation, $[Ca^{2+}]i$ and secretion was studied in stimulated platelets to obtain some clues to elucidate physiological roles of protein phosphorylation in platelet reaction.

MATERIALS AND METHODS

Preparation of washed platelets (WP) and the study of platelet reaction..... Human WP in HEPES buffered saline (145 mM NaCl, 5mM KCl, 1 mM $MgSO_4$, 10 mM HEPES, 5 mM glucose, pH 7.4, at 37°C) were prepared from fresh human blood anticoagulated with 0.38% trisodium citrate, as described elsewhere (10). The final platelet count was adjusted to be 1×10^8/ml. To simulate the condition in which $[Ca^{2+}]i$ was measured using quin 2 system described hereafter, WP were incubated with 2.5 μM quin 2 AM for 25 min. at 37°C and unloaded quin 2 AM was removed by washing with the same buffer. Then, quin 2-loaded platelets were stimulated in the Lumi-aggregometer® in the presence of varied extracellular Ca^{2+} concentration ($[Ca^{2+}]e$) and aggregation and ATP-secretion were monitored simultaneously as described previously (11).

A desired $[Ca^{2+}]e$ in suspending buffer was prepared using strictly adjusted Ca^{2+}-EGTA buffer, basically as described previously (12). As a certain amount of $[Ca^{2+}]i$ had been chelated by quin 2-loading to platelets (10)(13), $[Ca^{2+}]i$ was elevated to about 90 nM by a passive influx of Ca^{2+} upon addition of 1 mM $CaCl_2$ to the suspending medium and after 5 min. $[Ca^{2+}]e$ was then adjusted by adding a fixed amount of EGTA in order to adjust $[Ca^{2+}]e$ strictly.

Determination of $[Ca^{2+}]i$.....WP were incubated with quin 2 AM dissolved in 0.25% dimethylsulfoxide in the condition described above. The intracellular concentration of quin 2 was estimated to be 1.2 mM in our method (10). The final platelet count was adjusted to be 7×10^7/ml. Fluorescence of quin 2 was recorded at 37°C in HITACHI fluorescence spectrophotometer (model 650-40) with excitation at 336 nm and emission at 500 nm, as described elsewhere (10). $[Ca^{2+}]i$ was calculated from fluorescent signals as described by Rink et al. (14).

Measurement of P20, P47 phosphorylation.....WP were incubated with inorganic ^{32}P (Amersham Japan, 200 μCi/ml) for 60 min. at 37°C and at the same time with 2.5 μM quin 2 AM for 25 min. Then, unincorporated ^{32}P and quin 2 AM were removed by washing. Before stimulation, the loaded platelets were incubated with 1 mM $CaCl_2$ for 5 min. and with a fixed amount of EGTA to adjust $[Ca^{2+}]e$. In preliminary experiments, time dependency of P20 and P47 phosphorylation in stimulated platelets was studied and the maximal phosphorylation of both polypeptides was obtained at 30 sec. and 15 sec. for thrombin and STA_2, respectively. After an addition of thrombin for 30 sec. or STA_2 (thromboxane A_2 analogue) for 15 sec., the reaction was terminated by an addition of 1.3 vol of 4% sodium dodecyl sulfate containing 10% mercaptoethanol. The mixture was heated at 100°C for 1 min. and subsequently glycerol (10% in final concentration) and bromphenol blue (1%) were added. Then solubilized proteins along with calibrations proteins (Mr. 340,000, 170,000, 97,400, 55,400, 36,500, 20,100, Boehlinger Japan) were applied to polyacrylamide gel electrophoresis according to the method of Laemmli (15). Then gels were stained by 0.25% Coomassie brilliant blue. After destaining, the gels were dried by vacuum and were autoradiographed on Sakura® X-ray film for overnight. Then, the degree of phosphorylation was measured densitometrically. In preliminary experiments, the gels were sliced into several pieces to determine the radioactivity by scintillation counting and the result was found to be well correlated with the densitometric assay.

Miscellaneous.....Quin 2 AM was obtained from Dojindo Laboratories (Kumamoto, JAPAN). STA_2 was kindly donated by Ono Pharmaceutical Company (Osaka, JAPAN). Bovine thrombin was supplied by Mochida Pharmaceutical Company (Tokyo, JAPAN). All other chemicals were of highest grade available and obtained from Nakarai Chemicals (Kyoto, JAPAN).

RESULTS

$[Ca^{2+}]i$ monitoring in platelets stimulated by thrombin or STA_2 are shown in FIG. 1. In the presence of 1 mM $[Ca^{2+}]e$, a rapid increase in $[Ca^{2+}]i$ to over 1 μM was always observed, followed by gradual decrease in fluorescent intensity when platelets were challenged by 0.5 U/ml thrombin. In the same condition, stimulation by STA_2 resulted in sharp but transient increase in fluorescent intensity as shown in FIG. 1. This rapid change was also confirmed by the image of fluorescence by memoryscope.

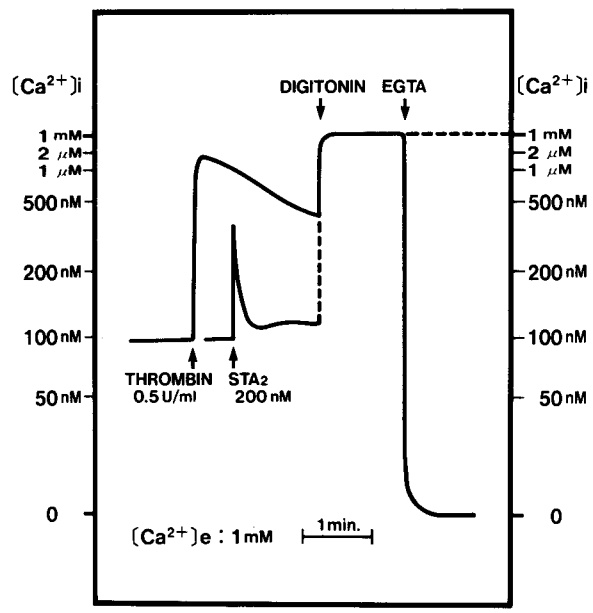

FIG. 1 Typical patterns of fluorescence monitoring of quin 2 loaded platelets stimulated by 0.5 U/ml thrombin or 200 nM STA_2. Calculated $[Ca^{2+}]i$ was also shown. See MATERIALS AND METHODS for details of the procedure.

First, we investigated the interrelationship of agonist concentration, $[Ca^{2+}]i$ and $[Ca^{2+}]e$. When quin 2 loaded WP were challenged with thrombin in the presence of 1 mM $[Ca^{2+}]e$, maximal $[Ca^{2+}]i$ was increased in a dose related manner, reaching up to 1 μM with a high dose of thrombin as in FIG. 2. On the contrary, in the presence of 20 nM $[Ca^{2+}]e$, maximal $[Ca^{2+}]i$ of about 250 nM was obtained even with more than 0.1 U/ml thrombin. Obviously, the increment of maximal $[Ca^{2+}]i$ in the presence of these different amounts of $[Ca^{2+}]e$ may be considered to be Ca^{2+} influx.

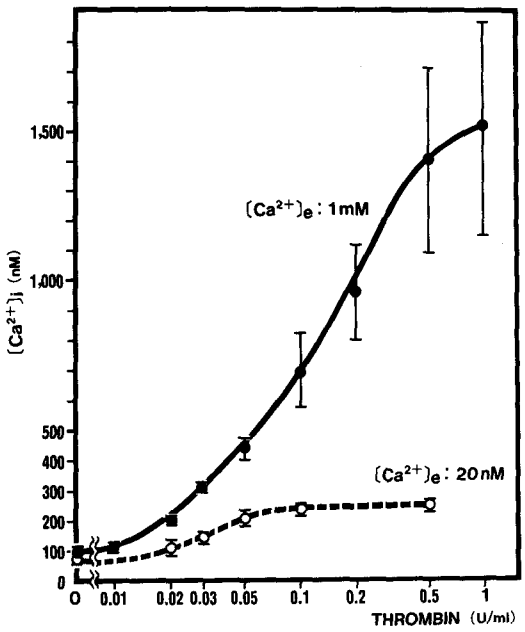

FIG. 2 Determination of maximal $[Ca^{2+}]i$ of quin 2 loaded platelets stimulated by varied concentrations of thrombin in the presence of 20 nm (○) or 1 mM (●) $[Ca^{2+}]e$. Values were shown as mean ± SD of three separate experiments.

Using quin 2 loaded WP prepared by the same way, ATP secretion and aggregation by varied concentrations of thrombin were studied. The amount of ATP secreted by 3 min. stimulation with thrombin in the presence of 1 mM $[Ca^{2+}]e$ was increased in a dose related manner up to 0.2 U/ml thrombin as shown in FIG. 3. The degree of aggregation was of similar pattern as ATP-secretion but values were not consistent because of relatively low platelet counts.

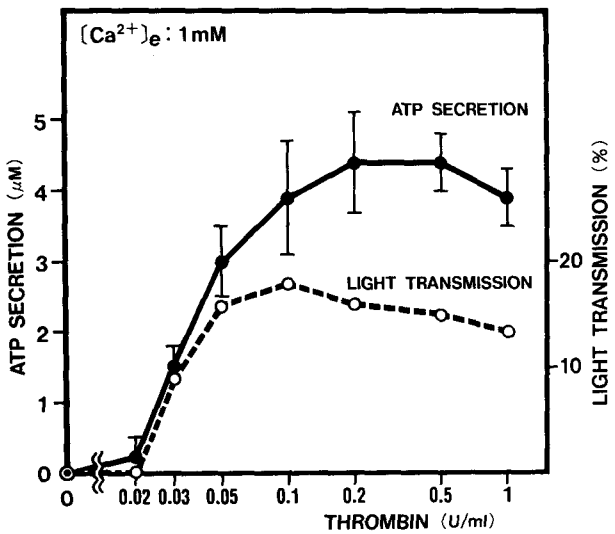

FIG. 3 ATP-secretion (●) and aggregation (○) of quin 2 loaded platelets stimulated by varied amount of thrombin in the presence of 1 mM $[Ca^{2+}]e$. Vertical bars represents ± SD (n=3).

In the similar condition as above, phosphorylation of P20 and P47 in thrombin stimulated platelets was then studied. As shown in FIG. 4, the phosphorylation of both polypeptides was increased in a dose related manner up to 1 U/ml thrombin, though the degree of phosphorylation of P47 was more than twofold of that of P20 by stimulation with thrombin above 0.1 U/ml.

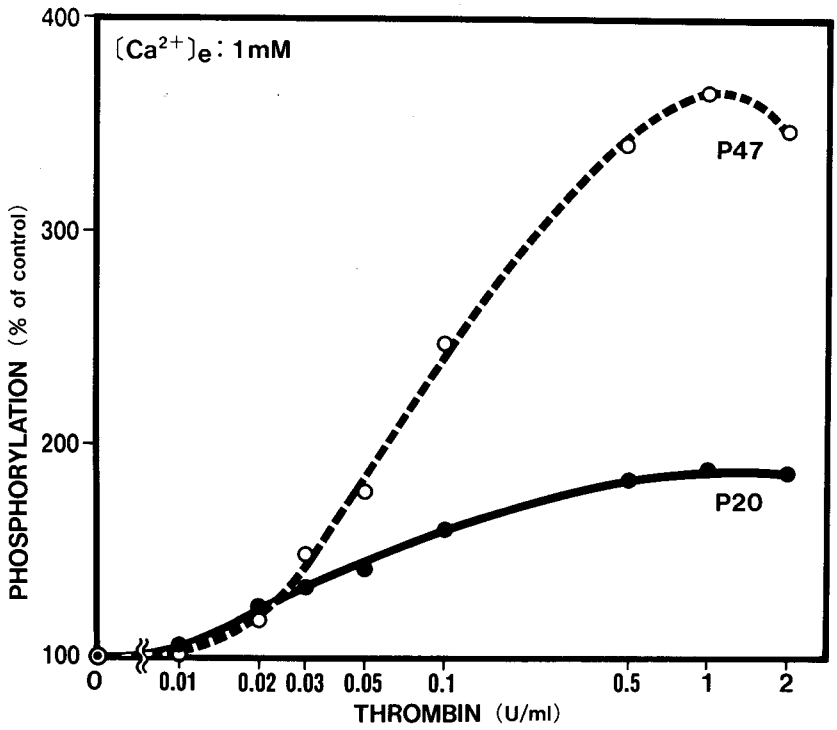

FIG. 4 Phosphorylation of P20 (●) and P47 (○) polypeptides in quin 2 loaded platelets stimulated by varied amount of thrombin. The degree of phosphorylation was expressed as relative density, 100% being the density in non-stimulated platelets.

Then, protein phosphorylation, ATP secretion and $[Ca^{2+}]i$ in quin 2 loaded platelets stimulated by thrombin (0.03 U or 0.5 U/ml) were studied in the presence of varied concentration of $[Ca^{2+}]e$. As illustrated in FIG. 5, when platelets were stimulated by 0.5 U/ml thrombin, the degree of ATP secretion and protein phosphorylation was not influenced by varied concentration of $[Ca^{2+}]e$, though maximal $[Ca^{2+}]i$ was increased in proportion to $[Ca^{2+}]e$. On the contrary, when they were stimulated by 0.03 U/ml thrombin, ATP secretion and P20 phosphorylation were proportionally increased as $[Ca^{2+}]i$, while P47 phosphorylation was not influenced by $[Ca^{2+}]e$ at all.

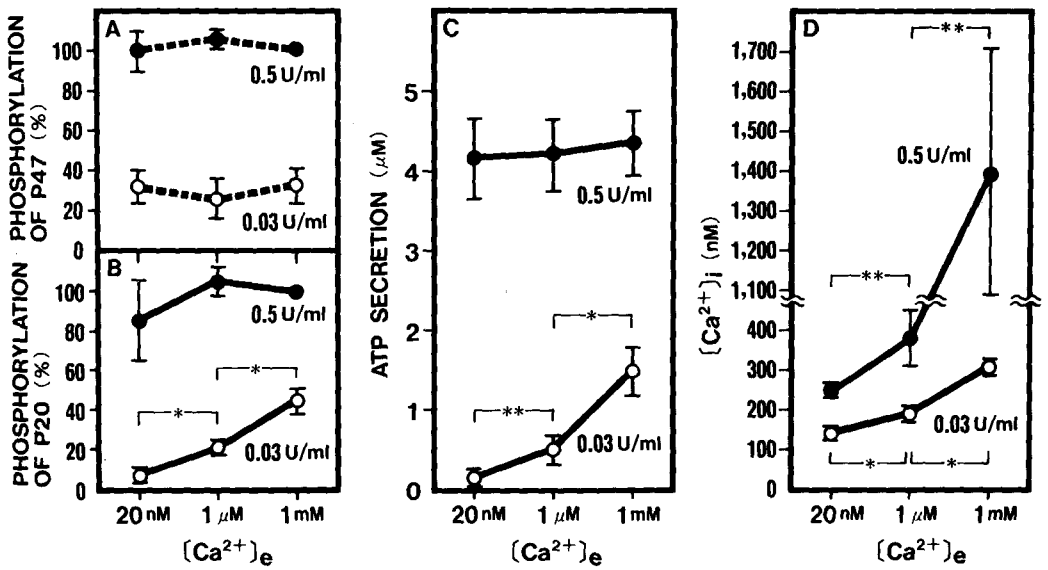

FIG. 5 Phosphorylation of P47 (A) and P20 (B), ATP-secretion (C) and $[Ca^{2+}]i$ (D) in quin 2 loaded platelets stimulated by 0.03 U/ml (○) or 0.5 U/ml (●) thrombin in the presence of varied $[Ca^{2+}]e$. In (A) and (B), the maximal densitometric value in the presence of 1 mM $[Ca^{2+}]e$ was designated as 100% phosphorylation. Values are expressed as mean ± SD from 3 to 5 experiments. Statistical analysis was performed by Student's t test. (* — $P<0.01$, ** — $P<0.05$)

Similar experiments were conducted using an optimal concentration of STA_2 as an agonist. In preliminary experiments, the concentration of STA_2 for maximal ATP-secretion and phosphorylation was 200 nM (data not shown). As shown in FIG. 6, P20 phosphorylation, ATP secretion were increased in proportion to the amount of $[Ca^{2+}]e$, while P47 phosphorylation was constant regardless of varied $[Ca^{2+}]e$.

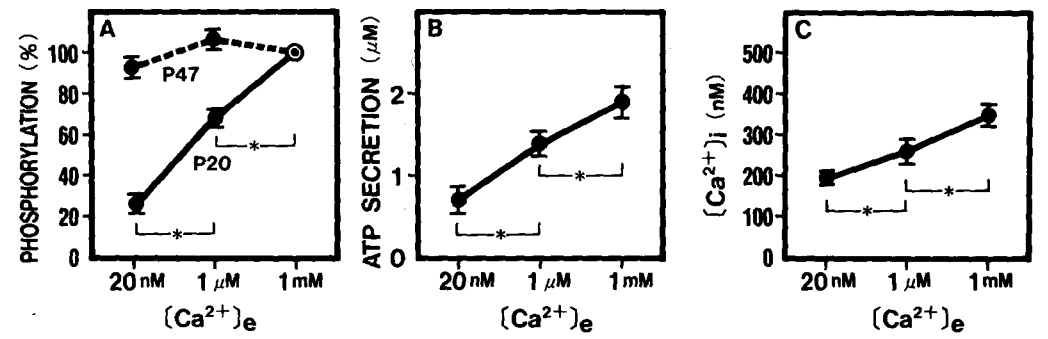

FIG. 6 Phosphorylation of P47 and P20 (A), ATP-secretion (B) and $[Ca^{2+}]i$ (C) in quin 2 loaded platelets stimulated by 200 nM STA_2 in the presence of varied $[Ca^{2+}]e$. Values are expressed as mean ± SD from 3 to 5 experiments. Statistical analysis was performed as in FIG. 5.

FIG. 7 shows a typical autoradiogram of SDS-solubilized polypeptides of ^{32}P-, quin 2-loaded platelets. A marked phosphorylation of P20 and P47 was seen in platelets stimulated by 0.5 U/ml thrombin. When platelets were stimulated by 0.03 U/ml thrombin, the degree of P20 but not P47 phosphorylation was proportional to $[Ca^{2+}]e$.

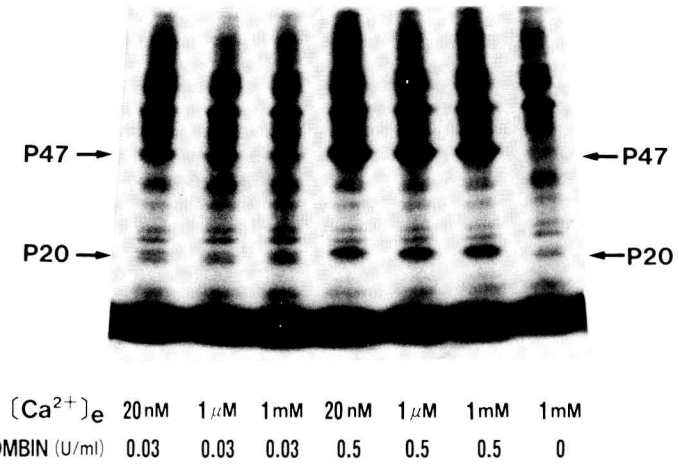

FIG. 7 A typical autoradiogram of SDS-solubilized polypeptides of ^{32}P and quin 2 loaded platelets stimulated by varied concentration of thrombin in the presence of varied $[Ca^{2+}]e$.

DISCUSSION

As one of the important intermediary reactions of platelet activation, stimulus-linked phosphorylation of polypeptides have been extensively studied in recent years (16)(17)(18). It has gained unanimous agreement that P20 phosphorylation is leading to generation of contractile force by actin, activated myosin ATPase (6)(7). A possible involvement of actin-binding protein (P260) phosphorylation in cytoskeletal motility has been proposed (19), but it was not possible to detect any significant changes in P260 phosphorylation in our present study (data not shown). The significance of P47 phosphorylation in promoting platelet reaction has been controversial partly because the nature of P47 has not been fully elucidated yet (5). The phosphorylation of P20 and P47 has been shown to be mediated by Ca^{2+} at least in vitro. Little information on the relationship between the phosphorylation and $[Ca^{2+}]i$ in platelets is available, though such relationship has been studied recently in permeabilized platelets (20)(21). It is possible to adjust $[Ca^{2+}]i$ exactly in permeabilized platelets but it may be impossible to simulate a sharp rise in $[Ca^{2+}]i$ seen in intact platelets stimulated by physiological agonists such as thrombin. Thereby, the present study was conducted to elucidate the relationship between $[Ca^{2+}]i$, protein phosphorylation and platelet reaction, utilizing quin 2 system. Though $[Ca^{2+}]i$ determination by quin 2 system is fairly accurate and reproducible, this may exert an inhibitory effect on

platelet reaction by chelating an excessive amount of intracellular Ca^{2+} (10). Thus, much attention was paid not to load quin 2 excessively and platelet reaction and protein phosphorylation were studied in platelets, to which the same amount of quin 2 was loaded as that used for $[Ca^{2+}]i$ determination. In platelets stimulated by thrombin, the degree of P47 phosphorylation was observed in totally dose-related manner, regardless of the amount of $[Ca^{2+}]e$ or $[Ca^{2+}]i$. P20 was also phosphorylated in the agonist-dose related manner in the presence of 1 mM $[Ca^{2+}]e$. However, in platelets stimulated by a lower dose of thrombin (0.03 U/ml), which is considered to be more physiological, the degree of P20 phosphorylation was well correlated with ATP-secretion and $[Ca^{2+}]i$, while no such correlation with P47 phosphorylation was observed. The very similar results were obtained in platelets stimulated by STA_2. These findings suggested that P20, but not P47, phosphorylation in activated platelets is mediated by a rise of $[Ca^{2+}]i$ and is well correlated with the secretory reaction. P47 phosphorylation in stimulated platelets may not be mediated by a rise in $[Ca^{2+}]i$ but it seems to be agonist-dose dependent phenomena such as polyphosphoinositides breakdown (13). Though the degree of P47 phosphorylation is impressively marked among polypeptides of platelets phosphorylated by activation, it is unlikely that P47 phosphorylation is promoting platelet activation. In supporting to the aforementioned idea, platelet activation without accompanying P47 phosphorylation has been reported (16)(22), and other roles of C-kinase dependent P47 phosphorylation have been proposed such as negative feed back mechanism in phosphoinositide metabolism (23)(24) and in P20 dependent expression of myosin ATPase (17) and as terminating the transduction process (24).

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参考論文

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索引用語：先天性総胆管嚢腫(拡張症)，胆汁酸，胆道内圧

緒　言

妊娠期発症する先天性総胆管嚢腫（以後C.C.C.と略す）が存在することは，良く知られている．妊娠合併率をAlonso-Lej[1]は7％，田所[2]らは成人発症例466例中12例（2.6％）と，それぞれ報告している．妊娠期発症の成因について，妊娠子宮の圧迫，分娩後の内臓下垂に伴う総胆管の屈曲[1]によると想定されてきたが，それを実証した報告はみられない．今回われわれは，妊娠期発症C.C.C. 3例を報告しうち1例に対し，分娩前後における嚢腫内圧，胆汁酸組成，胆汁粘度，および胆汁排出量を比較測定し，妊娠期発症の成因について考察を行なった．また自験3例を含む過去10年間の本邦妊娠期発症28例を集計し，文献的考察をおこなった．

症　例

症例1　24歳女性，第2子妊娠10カ月にて右上腹部痛，嘔吐出現し入院した．眼球結膜の黄染，腹部膨隆，右上腹部圧痛を認めたが，腫瘤を触知しなかった．第6病日黄疸増強のため帝王切開術施行し，術中上腹部に腫瘤触知した．第8病日全身状態の悪化，高熱のため化膿性胆汁管炎の合併を疑い，外胆汁瘻術を施行しC.C.C.と診断した．第74病日嚢腫十二指腸吻合術を行った（図1）．

症例2　24歳女性　初回妊娠9カ月に右上腹部痛，呕吐出現し，妊娠10カ月に黄疸増強のため入院した．眼球結膜の黄染，腹部膨隆，右上腹部圧痛を認めたが，腫瘤を触知しなかった．第13病日黄疸遷延のため帝王切開術施行し，術中C.C.C.と診断し外胆汁瘻を造設した．第40病日嚢腫切除Roux-en-Y肝管空腸吻合術を行った（図2）．

症例3　26歳女性　初回妊娠6カ月にて嘔吐出現し，7カ月に至り右悸肋部痛出現のため入院した．小学生の頃から，自家中毒症状を繰り返していた．眼球結膜の黄染を認めなかったが，上腹部圧痛および腫瘤を触知した．第3病日起音波検査にてC.C.C.と診断し，第6病日外胆汁瘻造設した．第83病日満期自然分娩し，第110病日嚢腫切除Roux-en-Y肝管空腸吻合術をおこなった（図3）．

症例1，2，3，ともAlonso-Lej I型C.C.C.であり，症例2，3には膵胆管合流異常を伴っていた．

嚢腫内圧

症例3に対し分娩前後における嚢腫内圧を測定した（図4）．5ml/5secの割合で生食を注入する定流潅流法を用いた．嚢腫内容を可及的に吸引し測定を開始したため，必然的に基礎圧は0となっている．分娩前後での内圧曲線は類似した変化を示しながら上昇し，各変

図1　症例1　24歳♀

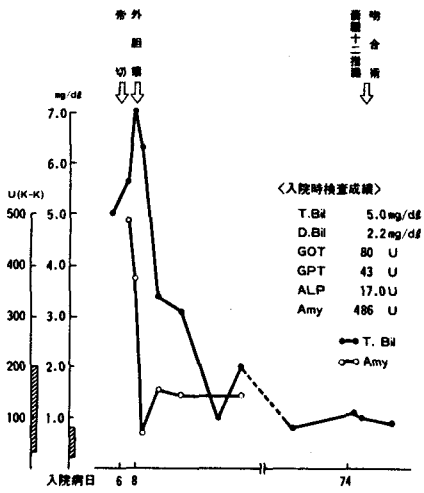
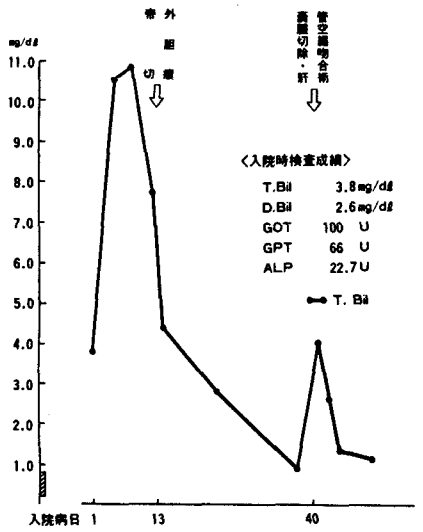


図2　症例2　24歳♀



化点はそれぞれ十二指腸への排出開始，持続排出の時期と一致し，分娩前後での差はみられない．その後の直線的上昇は，注入量と十二指腸への排出量との差が一定し嚢腫の受動的な拡張のもたらされる時期を表わしている．この直続部での⊿V/P(コンプライアンス)を比較すると，分娩前3，分娩後2.2ml/cmH_2Oと分娩前のコンプライアンスが高く，嚢腫壁の伸展性が高まっている．総注入量は分娩前315，分娩後195mlであり，その時の最高内圧は分娩前後ともほぼ30cmH_2Oと近似している．200sec.後の残圧は分娩前4，分娩後3 cmH_2Oと差を認めない．圧変化を比較する限りでは，妊娠子宮の影響は認められない．

図3　症例3　26歳♀

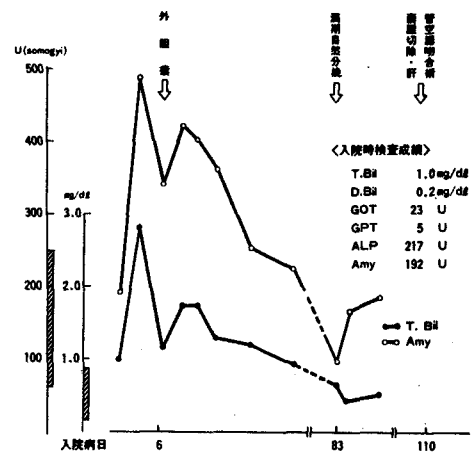
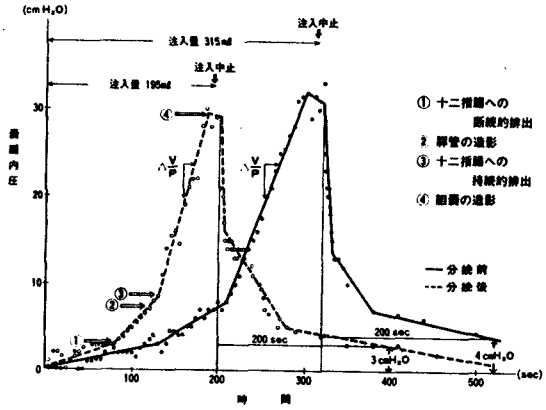


図4　分娩前後の嚢腫内圧変化



胆汁酸組成

遠藤[3]らの方法に従い分娩前後の胆汁酸測定を行った．分娩前169mg/dl，CDC13%，CA87%，分娩後114mg/dl, CDC24%, CA64%，その他12%と分娩前後とも2次胆汁酸欠乏および腸肝循環のブロックを示すものの，外胆汁瘻造設時の胆汁酸パターンと同様のもので有意差を認めず，また異常胆汁酸も検出されなかった．

胆汁粘度

妊娠時に胆汁粘度に変化をきたし嚢腫発症の一因になるかも知れないと考え，分娩前後の胆汁粘度を測定した（図5）．

対照として総胆管結石術後，Tチューブを留置した4例を用いた．化膿性胆管炎を合併していた対照4を除き，分娩前後および対照1，2，3，に有意差を認

めなかった．

胆汁排出量

症例3について，外胆汁瘻造設後から根治手術までの期間の1日平均胆汁排出量を測定した(図6)．妊娠月数とともに，外胆汁瘻からの胆汁排出量は増加し，分娩後も持続した．対照として総胆管切開術後の10例を用いたが，10例の胆汁排出量と比較し有意に($p<0.005$)，妊娠期および分娩後の胆汁排出量は増加していた．

本邦妊娠期発症先天性総胆管嚢腫集計

過去10年間本邦妊娠期発症したC.C.C. 28例を集計した(表1)．集計例よりその詳細を検討すると(表2)，腹痛(79%)，黄疸(75%)の発現率が高く，異常分娩様式(帝切28%，早産24%)をとるものが多い．結石合併率は約20%であり，成人報告例[2)4)]の割合と同率である．分娩前診断率は17%と低く，それゆえ手術時期も分娩後手術例が19例と多く，うち12例が内瘻術式をうけている．症状発現時期と分娩経過との関係を比較すると(表3)，妊娠7カ月以降に症状発現のピークがみられ，殆んどの例が異常分娩様式をとっている．分娩後にも黄疸増強例，腫瘤触知例が増加し，症状発現

図5　分娩前後における胆汁の粘度変化

	粘度		$\bar{X}$	
先天性総胆管嚢腫(分娩前)	1.00	0.98	0.990	
〃 (分娩後)	0.97	0.93	0.950	
対照1	0.99	0.94	0.965	
対照2	0.91	0.90	0.905	
対照3	0.94	0.94	0.940	
対照4 (胆管炎例)	1.44	1.37	1.405	$p<0.05$

測定方法
(機　種)
粘度計：ELD型粘度計(東京計器)
ローター半径：2.4cm
ローター円錐角：1°34′
スプリング：フルスケール・トルク 674dyne·cm
恒温循環装置：クールニクスCTE 310型(ヤマト科学)
(測定条件)
試料量：1.2～1.5ml
測定温度：36.5℃ ±0.5℃
せん断速度：384 sec^{-1} (100 r.p.m)
サンプルカップ注入後放置時間：7分
粘度値：測定開始2分後の値(約1分で一定の値を示す)

表1　妊娠，分娩を契機に発症した先天性総胆管嚢腫　過去10年間集計

症例	報告者(年度)	年令	主要症状 嘔吐	腹痛	黄疸	腫瘤	時期	分娩 様式	時期	診断 分娩前	分娩時	分娩後	手術 分娩前	分娩時	分娩後	その他
1	中山 ('68)	23	−	+	+	+	6 M	正常		Probe			CCD			
	松永 ('74)															
2	越智 ('69)	27	−	+	−	+	分娩後	正常				RI			CCD+G	
	三好 ('70)															
3	長瀬 ('70)	26	−	−	+	−	7 M	早産	9M			Probe			ED ·CCD	新生児死亡．
4	野口 ('71)	33	−	+	−	−	10 M	帝切	10M			Probe			CCD	経産．新生児死亡．
5	浅井 ('71)	28	−	+	+	+	9 M	正常				○			○	
6	杉山 ('72)	25	−	+	+	−	7 M	早産	8M			Ba.Ang			CCJ	
7	小坂 ('72)	25	−	+	+	−	9 M	早産	9M			Ba			CCJ	経産．
8	木村 ('73)	26	+	+	+	−	8 M	帝切	8M		Probe			ED	CCJ +HJ	胎児死亡．壊死性胆管炎．
9	吉田 ('73)	21	−	+	+	−	10 M							〈ED+HE〉		
10	亀山 ('74)	23	+	+	−	−	9 M	帝切	10M			Echo			CCJ	母体死亡．巨大骨腫(8000ml)．
	山村 ('76)	-														
11	清水 ('75)	25	−	+	+	−	8 M	早産	8M			Ba.RI			ED→CCJ	巨大骨腫 (5300ml)．
12	酒井 ('75)	33	-	+	+	−	8 M	中絶	8M			Probe			CD	結石．
13	斉藤 ('76)	24	−	−	+	−	7 M	正常				Ba			CCJ	
14	寺尾 ('76)						分娩後									
15	〈2例〉						分娩後									
16	佐藤 ('76)	24	−	-	+	−	分娩後	正常				PTCD			CD	経産．結石．
17	小泉 ('76)	22	−	+	+	−	7 M	正常				PTCD			CCD	
18	伊原 ('77)	25	−	+	+	+	6 M	中絶	7M			PTCD			HJ	結石．
	谷村 ('78)															
19	永田 ('77)	27	−	+	−	−	分娩後	正常				DIC			HJ	結石．
20	武藤 ('78)	23	−	−	+	+	7 M							〈 CJ		
21	〈2例〉	23	−	−	+	+	7 M							〈ED+HJ〉		
22	石田 ('78)		+	+	−	−	8 M	帝切			Probe			ED	CCJ	
23	河井 ('78)	23	−	+	+	+	7 M	早産	8M	Echo					CCJ	巨大骨腫 (6500ml)．
24	白川 ('79)													〈 CCD 〉		結石．
25	〈2例〉													〈 CCD 〉		
26		24	+	+	+	−	10 M	帝切	10M			Probe			ED ·CCD	化膿性胆管炎．経産
27	自験例	24	+	+	+	−	10 M	帝切	10M		Probe			ED	HJ	
28		26	+	+	−	+	7 M	正常		Echo			ED		HJ	

Operation　CCD=Choledochocystoduodenostomy ; CCJ=Choledochocystojejunostomy ; ED=External drainage
HJ=Hepaticojejunostomy ; HD=Hepaticoduodenostomy ; CD=Choledochoduodenostomy
HE=Hepaticoenterostomy ; CJ=Choledochojejunostomy ; G=Gastrectomy

Diagnosis　Probe=Probe laparotomy ; RI=Radio isotope ; Ba=Barium study ; Ang=Angiography

〈　〉: 時期不明例　　○: 術式不明例

図6　胆汁排出量の変化

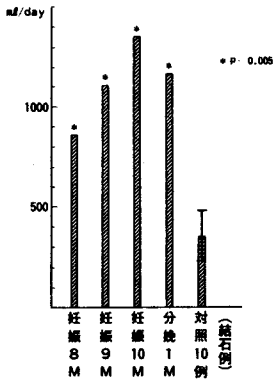


表2

主要症状発現率

腹　痛	79%（19／24例）
黄　疸	75%（18／24例）
腫　瘤	33%（8／24例）
嘔　吐	25%（6／24例）

分娩様式

正常満期	38%（8／21例）
帝王切開	28%（6／21例）
早期分娩	24%（5／21例）
人工流産	10%（2／21例）

結石合併率

有結石例	20%（5／25例）
無結石例	80%（20／25例）

診断時期

分娩前	17%（3／18例）	
	Probe Lapa.	1
	Echo	2
分娩時	17%（3／18例）	
	Probe Lapa.	3
分娩後	66%（12／18例）	

＊分娩後症状出現例を除く

手術方法

分娩時期	例数	外瘻術	内瘻術	胆道再建術
分娩前	2	1	1（CCD 1）	
分娩時	3	3		
分娩後	19		12（CCD 4，CCD+G 1，CCJ 7）	7（HJ 5，CD 2）

CCD：Choledochoduodenostomy
CCJ：Choledochojejunostomy
G：Gastrectomy
HJ：Hepaticojejunostomy
CD：Choledochoduodenostomy

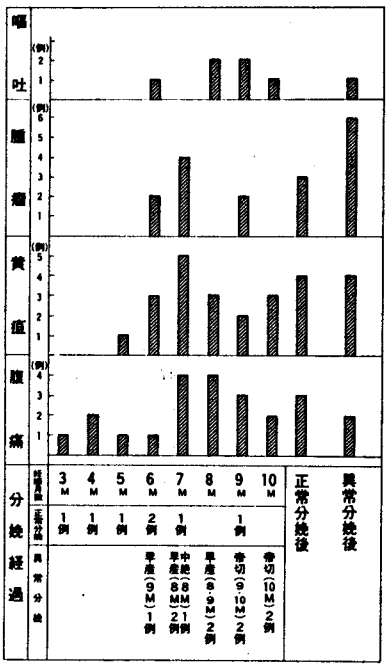
のもう1つのピークがある．

考　察

1．妊娠期発症の成因

従来妊娠子宮の増大による物理的圧迫が，総胆管末端部の通過障害を増加させ胆道内圧の上昇をもたらし，発症に至ると考えられてきた．今回われわれは，妊娠期発症の成因を検討するため，分娩前後における嚢腫内圧，胆汁酸組成，胆汁粘度，および外胆汁瘻よりの胆汁排出量を比較測定した．その結果分娩前後の嚢腫内圧には変化が認められず，また胆汁酸組成，胆汁粘度にも変化を認めなかったが，分娩前に嚢腫壁の伸展性が高まっていた．一方外胆汁瘻よりの胆汁排出量は，妊娠月数とともに増加しており，妊娠期に胆汁分泌が増加すると考えられた．従って今回の測定結果からは，妊娠期発症の成因が妊娠子宮の物理的圧迫によるもののみとは考えられず，妊娠期に嚢腫壁の伸展性が高まり，また胆汁分泌が増加することも成因の1つであると考えられた．胆汁分泌が増加し，総胆管末端部狭窄部位の通過障害を増強させた時，胆道内圧の著しい上昇を伴わなくとも容易に嚢腫壁が伸展拡張し，症状発現に至るのではないかと，推測された．われわれの集計例中，3例（10%）の巨大嚢腫の合併をみるため，妊娠期に嚢腫がより拡張しやすいと考えられた．

表3　症例発現時期と分娩経過



2．妊娠時診断と治療

妊娠期C.C.C.の診断には，DIC, ERCP, PTC，シンチグラムなどの補助診断法を用い得ないため，分娩前診断率は低い．また腫瘤を触知される例が少なく特異症状に乏しいため，妊娠悪阻，妊娠時膵炎，妊娠時肝炎，および妊娠期胆石との鑑別診断が，必要である．妊娠時膵炎の合併率は，Walker 0.007%[6]，Wilkinson 0.034%[6]と低く，Berk[7]は妊娠期膵炎のうち胆石症を基礎疾患として有する例が，53.1%と高率であり，妊娠後期および産褥期に好発すると述べている．Haemmerli[8]によれば，妊娠時黄疸の合併率も0.067%と低く，ウイルス肝炎によるものが最も多いと述べている．妊娠期に膵炎および黄疸の出現する頻度は非常に少ないが，鑑別診断時に常にC.C.C.を念頭におく必要がある．

妊娠期診断には，超音波検査が最も有効である．Russel[5]は妊娠期C.C.C. 2例に，超音波検査を行いその有効性を示唆し，胆石症および肝内胆汁うっ滞症との鑑別が必要であると述べている．松永[9]らは超音波診断上，腫大した胆嚢，肝嚢腫，右腎嚢腫，水腎症，膵嚢胞との鑑別を要すると述べ，その鑑別点を記載しているが，多くは臨床症状からその鑑別は困難ではない．

C.C.C. の根治術式は，嚢腫切除胆道再建術[10][11]であると考えられている．妊娠期発症例についても，可能なかぎり根治術式を選択すべきであるが，内瘻術式が多く用いられてきた．分娩後症状増悪し初めて診断され，外科治療をほどこされている例が多いため，手術侵襲の比較的少ない内瘻術式が適応されてきたためだと考えられる．全身状態の改善を得てのち，根治手術を施行するため，われわれは外胆汁瘻造設後根治手術を行う2期手術方式を選択してきた．自験例では，外胆汁瘻の造設および長期間の外胆汁瘻の存在は，妊娠分娩経過に対しても，根治手術時においても何らの悪影響をおよぼすことはなかった．この2期手術方式は，妊娠期発症したC.C.C. の治療方式として推奨される．

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