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POSSIBLE ROLE OF LECITHIN IN THE DEVELOPMENT OF THE SLOW MUSCLE CONTRACTING ACTIVITY OF HUMAN PLASMA

BY

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In experiments on perfused lungs, Feldberg, Holden & Kellaway (1938) found that the venous perfusate, after the injection of snake venom, caused not only a rapid histamine-like contraction, but sometimes also a delayed and slow contraction of the isolated jejunum of the guinea-pig. They established that the " slow reacting substance " (SRS-C) was formed by the action of the venom. Cobra venom contains phospholipase A, which splits lecithin into lysolecithin and free fatty acid. As SRS-C had the properties of an unsaturated fatty acid, it was assumed that it consisted of the fatty acids which are split from lecithin by the venom. This was proved by showing that SRS-C was formed on incubation of pure lecithin with cobra venom in ether solution and that it was present in the unsaturated fatty acid fraction of the reaction mixture (Vogt, 1957). SRS-C is also formed by alkaline hydrolysis of lecithin (Vogt, 1957).

For many years the phospholipids of human serum were thought to be of three principal types: lecithins, cephalins and sphingomyelins. Phillips (1958) recognized another variety of phospholipid in human serum identified as lysolecithin. This fraction makes up as much as 7% of the total circulating phospholipid. Beta-palmitoyl lysolecithin has been isolated from plasma as well as liver, heart and the adrenal medulla. It is also interesting to note that Vogel & Zieve (1962) reported the conversion of lecithin to lysolecithin as a source of fatty acids in incubated plasma or serum.

The release of SRS-C from egg lecithin by snake venom, the conversion of lecithin to lysolecithin in incubated plasma and the similar chemical nature of SRS-C and of G acid (slow contracting substance isolated from human plasma (Gabr, 1956)) suggested the study of the effect of human plasma on egg lecithin *in vitro*.

METHODS

Preparation of citrated plasma

Human plasma was separated from fresh blood by centrifugation. Each blood donation was drawn in anticoagulant solution containing 2.2% trisodium citrate, 0.8% citric acid and 2.45% dextrose in pyrogen-free water (15 ml. solution for each 100 ml. blood). The plasma was generally pooled from 10 donors under sterile conditions.

Preparation of pure lecithin

Ovolecithin, containing a trace of lysolecithin and sphingomyelin, was prepared by the method of Rhodes & Lea (1957). It was further purified on a silicic acid column by the procedure of Phillips (1958). The lecithin thus obtained, free from other phospholipids, was stored at −10° C. as a 10% solution in chloroform. Plasma lecithin was also isolated according to Phillips (1958) and stored in a similar manner.

Effect of citrated plasma on egg lecithin

Purified egg lecithin was thoroughly dried *in vacuo* over paraffin wax and P_2O_5, and mixed with sterile citrated plasma in varying concentrations starting from 100 mg–500 mg for each 100 ml. of the plasma. The mixture was gently emulsified by means of a sterile syringe and incubated at 37° C for varying times up to 16 hr (under aseptic conditions) before assay on the isolated guinea-pig ileum. A sample of citrated plasma was taken from the same plasma pool and incubated under the same conditions. This sample acted as a control in the separation procedure, in the estimation of lecithin (Phillips, 1958) and free fatty acids (Dole, 1956), and in the assay on the isolated guinea-pig ileum.

Estimation of free fatty acids

Fatty acids were estimated by the method of Dole (1956) as modified by Gallai-Hatchard & Thompson (1965). The reaction was stopped by the addition of 5 ml. of isopropanol-heptane-1 N H_2SO_4 (40:10:1, v/v). After shaking and allowing to stand for at least 10 min, 3 ml. heptane and 2 ml. water were added, the tubes well shaken for 30 sec and then centrifuged lightly; 3 ml. upper phase were then transferred to another tube, 1 ml. indicator added (1 ml. saturated aqueous cresol red in 50 ml. ethanol), and then titrated with 0.005 N NaOH in 90% (v/v) methanol.

Sterility control, treatment with kaolin, and effect of calcium ions

Sterility control (Gabr, 1961) was carried out on all experiments before and after incubation. Aliquots of normal plasma and lecithin-plasma were treated with kaolin (Maizels, 1944). The effect of calcium ions on the slow-muscle-contracting (S.M.C.) effect produced by lecithin, on incubation with plasma, was studied as follows. To 19 ml. of citrated plasma, 1 ml. of M/2 solution of $CaCl_2$ was added. The plasma was stirred, allowed to stand in the deep-freeze overnight. The serum was separated the next day after thawing and centrifuging down the clot. Aliquots of the lecithin-plasma incubate were treated in a simliar manner.

Preparation of the esterase rich fraction of blood

This fraction is produced during the purification of serum albumin by the method of Kekwick, Mackay & Martin (1953). The method of preparation may be outlined as follows. Crude albumin, precipitated from the globulin supernatant at pH 5.0 by 25% ethanol and 9% ether, consists of 86% albumin and 14% globulin. The material was found to contain 60–70% of the total cholinesterase in plasma. Crude albumin, derived from 100 ml. of plasma, was dissolved in distilled water to a protein concentration of 2%, the pH adjusted to 5–5.1 with 0.2 M acetic acid and the ether concentration to 10 vol%. A precipitate was formed which contained the bulk of the globulin contaminants and 80–90% of the esterase present in the crude albumin fraction. The precipitate was suspended in 10 ml. of 0.85% saline and the pH was adjusted to 7.4 with 0.2 M NaOH.

Separation of the slow-muscle-contracting substance from human plasma and from the lecithin-plasma incubate by paper chromatography

A modification of the isolation procedure for G acid (Gabr, 1956) was applied to 100 ml. portion of sterile plasma incubated for 6 hr at 37° C and 100 ml. portion of lecithin-plasma (0.25% lecithin) which had been incubated for the same time period at 37° C under aseptic conditions. Both portions were separated from the same plasma pool. The details of this separation procedure have been previously described (Gabr, 1965).

In the case of lecithin-plasma, the S.M.C. activity eluted from paper was about 100% more than in the case of plasma to which no lecithin was added.

Biological assay

The determinations on the isolated guinea-pig ileum were made, as previously described (Gabr, 1961), using a chamber of 55 ml. capacity filled with oxygenated Tyrode solution, the standard of reference being a specimen of the same plasma pool which had been incubated for the same period of time as the lecithin-plasma incubate.

RESULTS

Incubation of human plasma with egg lecithin

Figure 1 shows the effect of incubated plasma and of the lecithin-plasma incubate on the isolated guinea-pig ileum. It is obvious that the S.M.C. effect of the plasma is nearly doubled after incubation with 0.25% egg lecithin for 6 hr. The same effect is

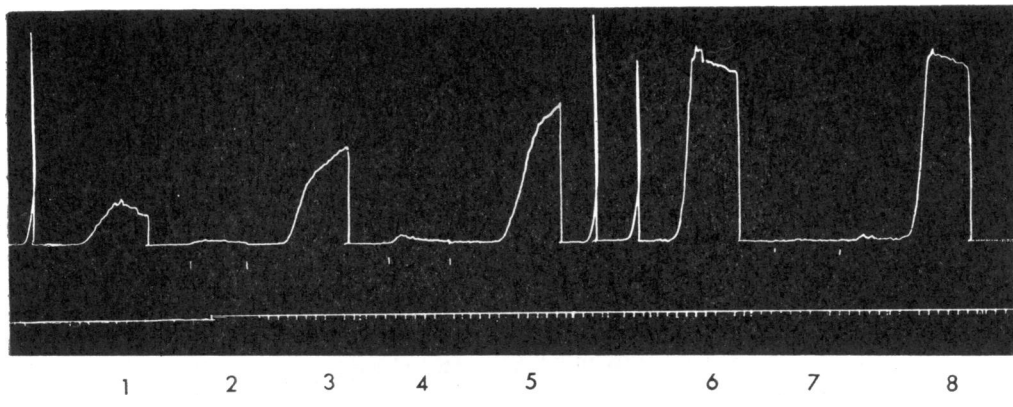

Fig. 1. Effect of human plasma on egg lecithin. Response of the isolated guinea-pig ileum to (1) 2 ml. plasma, (2) 2 ml. incubated lecithin (0.1% emulsion in saline), (3) 2 ml. incubated plasma, (4) 2 ml. incubated lecithin (0.25% emulsion in saline), (5) 2 ml. incubated lecithin-plasma (0.1% lecithin), (6) 2 ml. incubated lecithin-plasma (0.25% lecithin), (7) 2 ml. incubated lecithin (0.25% emulsion in saline), (8) 4 ml. incubated plasma and to 0.2 μg acetylcholine hydrochloride (not numbered). Time in 5 sec.

not obtained when lecithin is incubated with 5% solution of human serum albumin for 6 hr (Fig. 2). This may be attributed to the separation of the enzymes which cause the release of the S.M.C. lipid-soluble acid(s) from egg lecithin, during the course of purification of human serum albumin. The S.M.C. effect produced by lecithin on incubation with human plasma is increased by increasing the amount of added lecithin (from 100 mg to 500 mg % of the plasma), by increasing the time of incubation (from 1 hr to 16 hr), and by adjustment of the lecithin-plasma emulsion to pH 8 before incubation (Gabr, 1964). In these experiments, the pH of citrated plasma was around 7. When the S.M.C. effect of the lecithin-plasma incubate becomes great as a result of one or more of the above-mentioned factors, the onset of muscle contraction becomes more rapid, the magnitude of contraction becomes more marked, and the S.M.C. effect persists

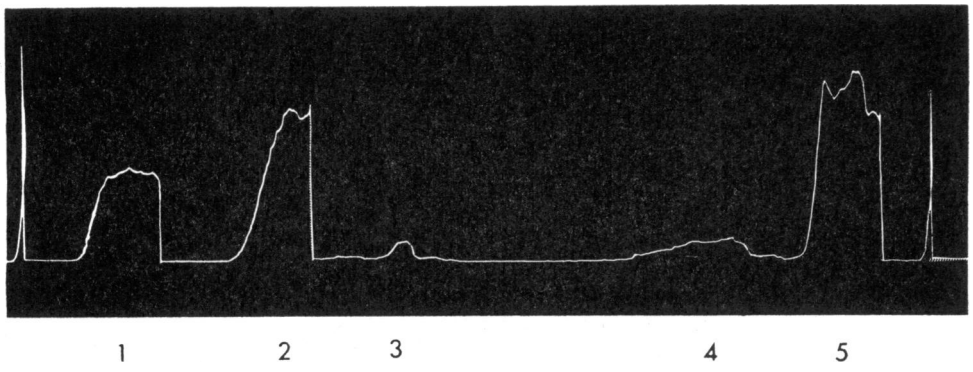

Fig. 2. Effect of human plasma and human serum albumin on egg lecithin. Response of the isolated guinea-pig ileum to (1) 2 ml. incubated plasma, 6 hr, (2) 2 ml. incubated lecithin-plasma, 1 hr, (3) 2 ml. incubated human serum albumin, 6 hr, (4) 2 ml. incubated lecithin-albumin, 6 hr, (5) 2 ml. incubated lecithin-plasma, 6 hr and to 0.2 μg acetylcholine hydrochloride (not numbered).

longer after washing out the organ bath with Tyrode solution. Extensive washings are sometimes necessary to effect complete recovery of the muscle preparation. This may take 2–3 min.

Human plasma derived from time-expired blood is equally active on the isolated guinea-pig ileum, yet its effect on lecithin is small when compared with the effect of fresh plasma. It is of interest to note that from time to time a muscle preparation is encountered which normally responds to histamine and acetylcholine but not to plasma or lecithin-plasma. The effect of the lecithin-plasma incubate on the isolated guinea-pig ileum is increased as the amount of added lecithin is increased up to 500 mg % of the plasma. Further addition of lecithin was not attempted owing to the difficulty of getting a satisfactory emulsion.

When human plasma is previously heated for 1 hr at 56° C, the S.M.C. effect on incubation with egg lecithin is not obtained, presumably because the enzyme responsible for the release of the S.M.C. lipid-soluble fatty acid(s) from lecithin is inactivated by this treatment. On incubation with human plasma, purified plasma lecithin was found to increase the same effect on the isolated guinea-pig ileum.

Estimation of free fatty acids

The results of estimation of lecithin and free fatty acids in normal plasma and lecithin-plasma (containing 0.25% egg lecithin) are shown in Table 1. The values listed show the decrease in lecithin at the times stated, and also the amounts of free fatty acids accumulating at these times. The results also show that the extractable acidity increased at a rate of 3.1 μ-equiv %/hr upon incubation of plasma at 37° C; in the case of lecithin-plasma, the extractable acidity increased at a rate of 5.3 μ-equiv %/hr. Dole (1956) found that the extractable acidity increased at a rate of 3.5 μ-equiv %/hr during incubation of plasma at 37° C —" presumably by enzymatic hydrolysis of esterified fatty acids." Vogel & Zieve (1962) observed that the rate of release of fatty acids in incubated plasma is adequately accounted for by the conversion of lecithin to lysolecithin.

TABLE 1

EFFECT OF HUMAN PLASMA ON EGG LECITHIN

Concentration of lecithin and free fatty acids in normal and lecithin-plasma before and after incubation
Results given are the mean of three experiments

Incubation time (hr)	0	1	4	6
Lecithin concentration (μ-mole/100 ml. plasma)				
(a) Normal plasma	193	186	171	159
(b) Lecithin-plasma (0·25% lecithin)	505	496	458	432
Free fatty acids (μ-equiv/100 ml. plasma)				
(a) Normal plasma	53	57	66	72
(b) Lecithin-plasma (0·25% lecithin)	54	60	76	86

The increase in S.M.C. activity of human plasma on incubation with egg lecithin may be attributed to the release of S.M.C. lipid-soluble acids which arise from the conversion of lecithin to lysolecithin. Glomset, Parker, Tjaden & Williams (1962) showed that when (^{14}C) linoleic acid-labelled lecithin is incubated *in vitro* with rat or human plasma, radioactive cholesterol linoleate is formed as well as ^{14}C free fatty acid, triglycerides, mono- and diglycerides, and probably also cephalin. If G acid were the only causal factor, then the increase in the S.M.C. activity of the lecithin-plasma over the control in 6 hr may be attributed to the release of 1.7 μ-equiv of G acid/100 ml. plasma, or about 12% of the total fatty acids released from egg lecithin (20 μg of G acid—that is, 0.07 μ-equiv of G acid gives a slow contraction on the isolated guinea-pig ileum equal in magnitude to that obtained with 4 ml. of dialysed citrated human plasma).

It may be interesting to note that the increase in S.M.C. activity of normal plasma and lecithin-plasma on incubation is not exactly proportional to the amount of free fatty acids released in each case. This controversy may be due to the formation of other S.M.C. substances and probably to different specificities of the acting enzymes for plasma lecithin and egg lecithin, or for different fatty-acid residues of the lecithin molecule. The latter explanation was given by Moore & Williams (1964) in their study of the hydrolysis of egg lecithin by phospholipase A.

Effect of dialysis and calcium ions on the S.M.C. effect produced by lecithin on incubation with human plasma

It has been shown by Schachter (1956) that the S.M.C. effect produced by human plasma is best observed after dialysis, since dialysis removes the quick contracting agents. Gabr & Aly (1959) and Gabr (1961) observed that the S.M.C. effect produced by human plasma becomes less potent after storage for six months at room temperature. Analysis of the deposit which is formed in liquid plasma during this period of storage leads to the conclusion that the deposit retains 40% of the original S.M.C. activity of the plasma before storage. G acid is probably bound to calcium which is present in the deposit. An equivalent amount of calcium ions has been shown to precipitate G acid from pure systems (Gabr, 1956).

After the addition of calcium ions to plasma up to M/40 it was found that about 50% of the S.M.C. effect produced by the plasma disappears. The same amount of

calcium ions was without effect on the isolated guinea-pig ileum, at the same level of dosage. Figure 3 shows what happens to the S.M.C. effect produced by the lecithin-plasma incubate after dialysis against Tyrode solution, and after addition of calcium ions up to M/40 following the dialysis procedure. The relative changes in the slow contraction of smooth muscle produced by the lecithin-plasma incubate as a result of these treatments are indistinguishable from the relative changes that are produced by incubated plasma which has been subjected to the same treatments. Dialysis removes the quick contracting agents from the lecithin-plasma incubate at first, then the calcium ions reduce the S.M.C. effect of the incubate to less than 50% of the effect produced by the incubate after the dialysis procedure.

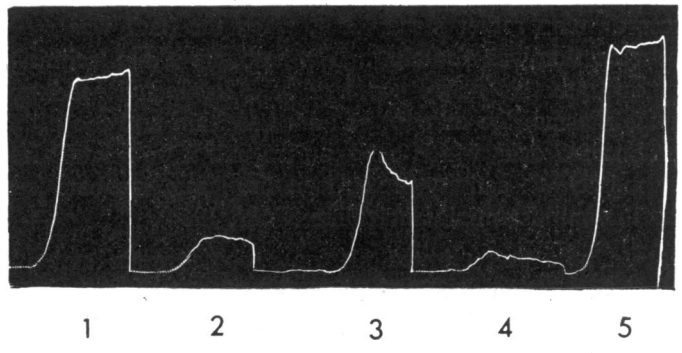

Fig. 3. Effect of dialysis and calcium ions on incubated lecithin-plasma. Response of the isolated guinea-pig ileum to (1) 2 ml. lecithin-plasma, (2) 2 ml. calcified lecithin-plasma, (3) 2 ml. dialysed lecithin-plasma, (4) 2 ml. calcified lecithin-plasma after dialysis and (5) 2 ml. lecithin-plasma. The writing point was detected from the lever afterwards. Concentration of egg lecithin, 0.25%, incubation time, 6 hr.

Effect of kaolin treatment and blood esterase rich fraction on the S.M.C. effect produced by lecithin on incubation with human plasma

The slow contraction of smooth muscle produced by human plasma becomes less potent after treatment with kaolin according to the method devised by Maizels (1944) for the clarification of the plasma. The recovery of G acid from such kaolin treated plasma is about 50% of the yield which is obtained from the same plasma before kaolin treatment (Gabr, 1961). Parallel experiments showed that the S.M.C. effect, which is produced by lecithin on incubation with human plasma, is abolished by the same treatment. The S.M.C. effect of the kaolin treated incubate is indistinguishable from the S.M.C. effect of incubated plasma which has been treated with kaolin, under the same conditions.

On incubation with the esterase rich fraction (1:4, v/v) for 6 hr, it has been found that the S.M.C. effect of normal plasma and the S.M.C. effect of lecithin-plasma were not destroyed under the same conditions which destroy the effect of acetylcholine. It may be surprising to note that this blood-esterase rich fraction increases the S.M.C.

effect produced by egg lecithin on incubation with the plasma. This may be explained by assuming that the enzyme which releases the S.M.C. lipid-soluble acid(s) from egg lecithin is present in the esterase rich fraction in a concentration which is relatively higher than its concentration in the plasma. The S.M.C. effect produced by the lipid-soluble acids, which have been separated from the incubated plasma and from the lecithin-plasma incubate by paper chromatography, is not influenced by the esterase rich fraction of human blood.

DISCUSSION

The present experiments demonstrate the release of a substance or substances with a slow contracting activity (S.M.C. activity) on plain muscles, through the action of human plasma or purified egg lecithin. Not only did the contraction produced by the S.M.C. substances develop rather slowly, but, when the bath was replaced with fresh Tyrode solution, relaxation was also slow. The amounts of the lecithin-plasma incubate necessary to demonstrate this newly acquired S.M.C. effect depend upon the time of incubation with the plasma, the amount of added lecithin and the pH of the incubate. Similar to the action of G acid, which has been isolated from the G.2 fraction of human plasma, the acquired S.M.C. activity of the lecithin-plasma incubate is not antagonized by atropine or by the antihistaminic antazoline (Gabr, 1964).

It is important to note that citrated plasma becomes more active on the isolated guinea-pig ileum on incubation at 37° C. This may be attributed to the formation, by glass activation, of kinins (Margolis, 1958) and other smooth muscle stimulating substances, in small amounts. Therefore, the plasma used as a standard of reference was incubated for the same period of time as the lecithin-plasma, before assay on the isolated guinea-pig ileum.

The S.M.C. activity of the lecithin-plasma incubate is not assumed to be identical with that of G acid, but the term simply serves to characterize the liberated substances by their pharmacological action. Similar to G acid, the S.M.C. substance of the lecithin-plasma incubate can be extracted with ethyl alcohol, and is soluble in ether and absolute methanol. The small amounts of active material, separated by paper chromatography, made it difficult to proceed further to characterize the active substance which is formed in the lecithin-plasma incubate. It is interesting to note that a similar S.M.C. effect is obtained by chemical hydrolysis of egg lecithin (Vogt, 1957).

The S.M.C. effect of the lecithin-plasma incubate may be attributed to a mixture of lipid-soluble acids, and it is not certain whether the slow contraction produced by the active material separated by paper chromatography was the effect of one or several such acids. The S.M.C. lipid-soluble acid(s), separated by paper chromatography seemed to be unsaturated, since it discharged the colour of iodine solution. It is certain, however, that not all unsaturated fatty acids have this property (Gabr, 1956 ; Vogt, 1957). The exact chemical structure of the acid or acids which are released from lecithin by different treatments and which produce a slow contraction is yet unknown. These treatments are alkaline hydrolysis of lecithin and incubation of lecithin with snake venom or with fresh human plasma. The conversion of lecithin to lysolecithin in incubated plasma suggests the release of the S.M.C. lipid-soluble acid(s) in human plasma through a

mechanism which may be similar to the mechanism of formation of SRS-C in egg yolk by cobra venom (Vogt, 1957).

The S.M.C. substance separated from incubated plasma and the S.M.C. substance separated from the lecithin-plasma incubate showed the same behaviour on paper chromatograms. In both cases, the R_F value was 0.8, which is the same R_F value found for G acid, using the following solvent system: 96% ethanol, ammonia solution (density, 0.88) and water (80:4:16, v/v). Moreover, the lipid-soluble acid(s), separated from incubated plasma and from the lecithin-plasma incubate, could not be resolved into more than one component by paper chromatography using other solvent systems such as: (a) ethanol, ammonia, water (80:4:16, v/v); (b) n. butanol, ethanol, ammonia, water (10:70:4:16, v/v); (c) n. butanol, ethanol, ammonia, water (40:40:4:16, v/v).

Olive oil, cottonseed oil and butter fat do not produce similar effect on smooth muscle when emulsified with human plasma and incubated for 6 hr at pH 8. It is of interest that the amount of egg lecithin which doubles the S.M.C. effect of human plasma is in the range of 200–250 mg/100 ml. of the plasma, a concentration which nearly corresponds to the concentration of the naturally occurring lecithin in human plasma. Parallel results were not obtained after incubation of egg lecithin with 5% solution of human serum albumin in saline.

The S.M.C. effect produced by the lipid-soluble acids, which are formed in incubated plasma and in the lecithin-plasma incubate, is similar in many respects to the effect of G acid which has been isolated from the G.2 fraction of human plasma. In neither case was the effect antagonized by atropine or by the antihistaminic antazoline. Like G acid, the lipid-soluble acids separated from incubated plasma and from the lecithin-plasma incubate stimulate the isolated guinea-pig ileum at the same level of dosage (20 μg in 55 ml. organ bath), and have no effect on the isolated rat colon. The factors which cause this effect are not dialysable at the physiological pH and appear to be precipitated from plasma in the presence of a relative excess of calcium ions. In both cases, the S.M.C. factors are probably lipid-soluble unsaturated acids and can be removed from the plasma by extracting with alcohol, or by adsorption to kaolin. These similar properties, however, do not prove identity of chemical structure.

SUMMARY

An incubate of egg lecithin with human plasma under sterile conditions produces on the guinea-pig ileum a slow contraction which is, in many respects, indistinguishable from the slow contraction produced by the original plasma. The substance responsible for the slow contraction which is formed in the plasma on incubation with lecithin, can be extracted with alcohol, can be adsorbed on kaolin, and can be precipitated by calcium. It can be separated by paper partition chromatography and seems to be an unsaturated lipid-soluble acid. On incubation with human plasma, purified lecithin which was separated from the same plasma was found to increase the same effect on the isolated guinea-pig ileum.

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