Biochemical Pharmacology, 1963, Vol. 12, pp. 415–420. Pergamon Press Ltd., Printed in Great Britain.

PHARMACOLOGICALLY ACTIVE ACIDIC PHOSPHOLIPIDS AND GLYCOLIPIDS

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(*Received* 21 *December* 1962)

Abstract—The occurrence and smooth muscle stimulating action of "darmstoff" is described and the identification of a phosphatidic acid and other acidic phospholipids as active principles of darmstoff preparations. From investigations of several purified phospholipids and glycolipids it is concluded that the smooth muscle stimulating activity of such complex lipids depends on their acid nature which enables them to form lipid-soluble salts with inorganic cations. Of particular interest is a possible relationship between pharmacological activity and Ca-binding of these compounds.

THE discovery that some phospholipids have a pronounced stimulating effect on smooth muscle was the result of investigations into the chemical nature of "darmstoff". This name had been given to an unknown substance appearing in the bath fluid of isolated frog intestine preparations.[1] The substance was able to evoke or to intensify rhythmic contractions of the isolated frog rectum and to contract other smooth muscle preparations.[2] The contractions produced in the rabbit gut were not inhibited by atropine, they were, however, reduced, in the guinea pig ileum.[3] The blood pressure of rabbits was hardly affected.[4] Isolated strips of rabbit duodenum were suitable for assay, since they contracted in response to darmstoff in concentrations of 10^{-7} or even 10^{-8}. By countercurrent distribution experiments darmstoff was shown to be an acid, soluble as free acid in most organic solvents and forms water soluble alkali salts.[5]

Since further investigations of the chemical nature were not possible with the minute amounts of darmstoff obtainable from frog intestinal dialysates, attempts were made to extract darmstoff by boiling minced horse intestine in water. Again, a strong smooth muscle stimulating effect was obtained in a fraction consisting of lipid-soluble acid material. Most of the activity was resistant to treatment with hot dilute alkali. In later experiments, therefore, the intestines were boiled in 0·2 N NaOH, for 10 min, a procedure which facilitated extraction.[6]

At that time, no other smooth muscle stimulating acid was known to occur in intestine besides darmstoff. Consequently the material obtained by hot alkaline extraction of intestinal tissue was thought to be identical with that from dialysates of surviving intestine. As it is now known that the extracts contain several active lipid-soluble acids it may well be that darmstoff from dialysates is different in its composition from that of boiled intestine. Hot extraction of minced tissue may bring into solution compounds which do not diffuse out of intact living cells. Nevertheless such material may be locally active under physiological conditions.

In a sample of darmstoff extracted with 0·2 N NaOH and purified by countercurrent distribution one active principle was identified as acetalphosphatidic acid. Other

acidic phospholipids were, however, also present in the purified sample and contributed to its pharmacological activity.[6]

In order to identify these compounds extracts from horse intestine were prepared either by boiling in alkali or by extraction with chloroform–methanol. Fractionation of these extracts on silicic acid columns with chloroform and methanol led to the separation of further active principles and to their identification as cephalins and phosphoinositides.[7] In brain extracts obtained by extraction with chloroform–methanol the main active complex lipids were found to be phosphatidic acid and gangliosides.[8] In addition, both tissue extracts contain other lipid acid fractions which cause a contraction of intestinal muscle but these do not contain phosphorus. According to their behaviour in silicic acid chromatography and other purification methods they consist of certain fatty acids. Most likely these are hydroxy-acids. The reason for this assumption is given later in this paper. Moreover, Ambache and Reynolds found a hydroxy-acid in brain extracts.[9]

Naturally, not all phospholipids produce a contraction in smooth muscle. Various purified and some synthetic compounds have been investigated in order to find a relation between chemical structure and biological activity.[10] In Table 1 the activity of these compounds, measured in the isolated rabbit duodenum, is given in units of a

TABLE 1. SMOOTH MUSCLE STIMULATING ACTIVITY OF PHOSPHOLIPIDS AND GLYCOLIPIDS, GIVEN IN UNITS/μG OF PREPARATION AND ASSAYED ON ISOLATED STRIPS OF RABBIT DUODENUM

Acidic phospholipids	Units/μg
Lyso-phosphatidic acid* (1)	49
Lyso-phosphatidic acid* (2)	30
Phosphatidic acid* (3) (saturated)	1
Phosphatidic acid (from lecithin)	4
Cardiolipin	0·2
Phosphatidylserine	0·8
Monophosphoinositide	<0·1
Lyso-monophosphoinositide	0·4
Diphosphoinositide	0·4
Neutral phospholipids	**Units/μg**
Lecithin	<0·1
Lyso-lecithin	0
Sphingomyelin	0
Sphingosyl-phosphorylcholine	0 (inhibits stimulation by acetyl-choline)
Glycolipids	**Units/μg**
Ganglioside	0·4
Cerebroside	<0·1

* (1), (2) = two different preparations of lyso-phosphatidic acid, obtained from lysolecithin by brominolysis; (1) was free of bromine, (2) contained 15% Br. (3) = dimyristoylphosphatidic acid synthetized by Dr. E. Baer, Toronto.

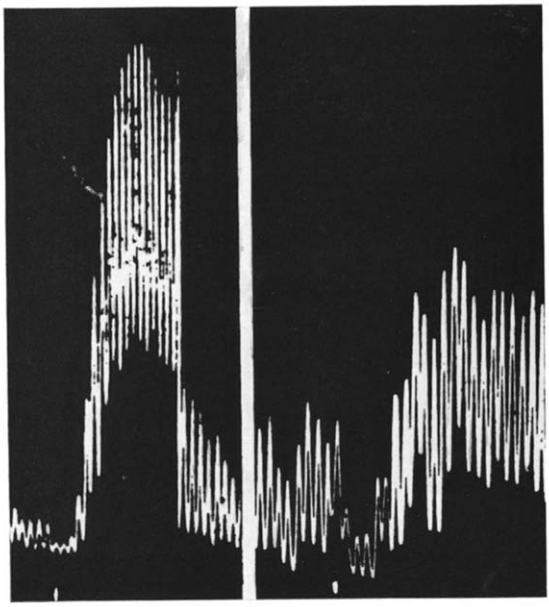

FIG. 1. Rabbit duodenum preparation, in 10 ml Tyrode solution. *Left:* contraction produced by 0·5 μg lysophosphatidic acid, Na-salt. *Right:* 500 μg lecithin. (From *Arch. exp. Path. Pharmak.* **240**, 134, 1960.)

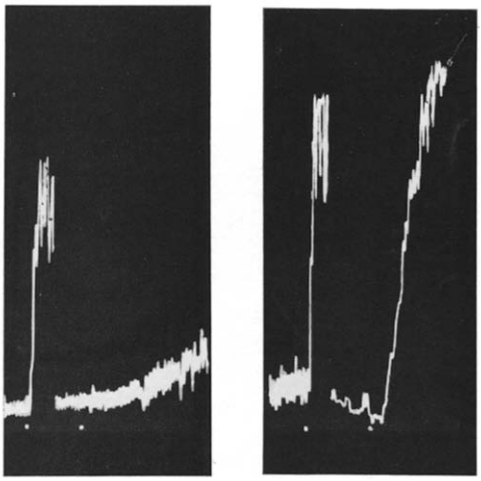

FIG. 2. Two different rabbit duodenum preparations, 10 ml Tyrode solution. *Left:* at the first dot 0·1 μg acetylcholine, at the second dot 500 μg cerebroside. *Right:* at the first dot 0·05 μg acetylcholine, at the second dot 200 μg ganglioside.

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standard preparation[11] of darmstoff. It is evident that all acidic phospholipids do contract the intestine with the exception of monophosphoinositide.

The more pronounced the acidity the greater is the activity of the compound. On the other hand, the neutral choline phosphatides cause a smaller or no contraction of smooth muscle. Fig. 1 shows the strongest effect ever obtained in a rabbit duodenum with 500 μg of lecithin. The effect is much smaller than that of 0·5 μg of lysophosphatidic acid. The basic phosphatide sphingosyl-phosphoryl-choline not only has no contracting activity itself but it even inhibits the contractions elicited by other drugs such as acetylcholine.[12] Table 1 shows further that the activity of acidic phospholipids is dependent on their solubility or dispersibility, respectively, in water. Thus, the activity increases from the wholly saturated dimyristoyl-phosphatidic acid to the partly unsaturated phosphatidic acid prepared from egg lecithin and, further, to lysophosphatidic acid. The solubility increases in the same order. Lysophosphatidic acid is the most active phospholipid so far known.

Solubility is, however, only of secondary importance and it cannot replace the acidic nature to make a phospholipid pharmacologically active. Lysolecithin, though more soluble in water than phosphatidic acid does not cause a contraction of the intestine. In the guinea pig ileum it is even inhibitory.[13]

The importance of the acidic nature for biological activity is also seen in glycolipids. Neutral cerebroside does not stimulate the rabbit gut whereas the acidic ganglioside does (Table 1 and Fig. 2). On the other hand, acidic nature and lipid-solubility are not sufficient by themselves to ensure smooth muscle stimulating activity. This is shown by the following facts. Cerebroside sulphate is inactive, according to a personal communication of Dr. Ambache, and so are fatty acids of the straight chain series, regardless whether saturated or unsaturated. When the "slow reacting substance" (SRS-C) liberated by cobra venom from egg yolk or perfused tissues had been recognized as consisting of unsaturated fatty acids originating from phosphatides,[13] it was thought that certain simple unsaturated fatty acids would contract smooth muscle. Jaques[14] found arachidonic acid to be particularly active, in this respect. Dakhil and Vogt[15], however, demonstrated that simple unsaturated fatty acids including arachidonic acid did not stimulate guinea pig or rabbit intestine unless they were oxidized to hydroperoxides. Pickles has suggested in a personal communication that uterine muscle may behave differently in that it reacts to pure arachidonic acid. Some preliminary experiments in the author's laboratory, however, do not support this assumption. From the work on prostaglandin and irin and from other findings it would appear that non-peroxidized fatty acids are only active if they contain hydroxyl groups. This has probably some relevance concerning the mode of action of lipidsoluble acids in general (see below).

The physiological role of the active lipid acids was at first believed to be that of a stimulant for smooth muscle organs. The increased release of darmstoff-like material from frog stomach during vagus stimulation suggested a transmitter function of darmstoff[1] which could explain the atropine-resistance of the intestinal muscle response to vagal stimulation in certain species. Ambache,[16] however, showed that darmstoff does not act peripherally but at neuronal sites. This view is in agreement with my own experiments performed later.[3, 17] Further, the occurrence of acidic phospholipids in non-muscle-containing organs such as brain precludes the assumption of a mere motor function.

The dependence of smooth muscle stimulating activity on acid nature and lipid-solubility gave rise to the idea that acidic phospholipids—and phosphatidic acid in particular—could act as a carrier in the sodium pump system.[18, 19] Phosphatidic acid forms lipid-soluble salts with Na^+-ions which in this salt combination could penetrate cell membranes more easily than as free ions. If phosphatidic acid were formed at the inner side of the cell membrane, e.g. by cleavage from lecithin, it might diffuse out as sodium salt. At the outside the lecithin could be resynthetized and would move back to the inside leaving the Na^+ behind and thereby completing the pumping cycle. Such a mechanism would require a phosphatidic acid forming enzyme system at the inside and a phosphatidic acid destroying system at the outside of the cell membrane. Hokin and Hokin[20] found, indeed, such enzyme systems and supported the carrier idea by experiments on the salt extrusion in the salt gland of sea birds.[21] Kirschner attributed a similar role to phosphatidyl serine.[22] Other phospholipids have also been taken into consideration, based on the fact that they can take inorganic ions into organic solvents.[23] Unless phospholipids are acidic they cannot, however, combine with cations alone but only with complete salts. This makes a cation carrier function of neutral phospholipids unlikely.

Recent calculations have cast some doubt on the validity of the hypothetical carrier role of phosphatidic acid. The activity of the enzymes found by Hokin and Hokin which would keep the carrier cycle going does not seem to be sufficiently high to account for the actual Na^+-transport, at least if the figures for calculation are taken from brain.[24]

Another approach to the problem of Na^+-transport by phosphatidic acid has been tried by Passow[25] and in the author's laboratory. Red blood cells were incubated with

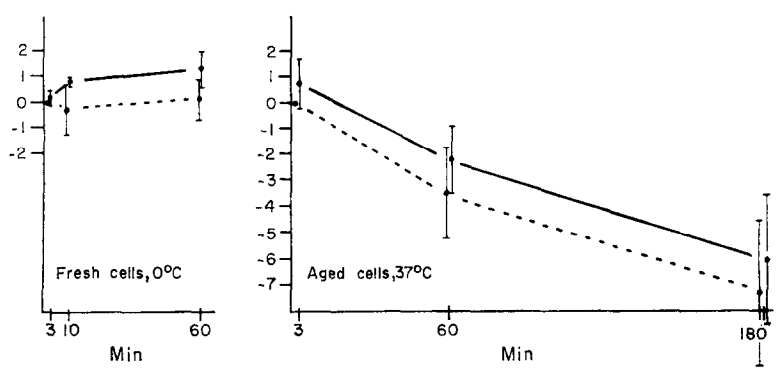

FIG. 3. Sodium content of human red cells in whole plasma. Ordinate, changes in μeq Na^+/l. red cells as compared to the original Na^+ content.
Abscissa, time of incubation. *Left:* fresh blood incubated in ice-cold water bath. *Right:* blood after storage for 4 days at 4°C, incubated at 37°C. ——— Blood incubated with 0·5 mg/ml phosphatidic acid. - - - - - Control blood. ⊥ Mean values of from four to five experiments and standard deviation.

phosphatidic acid and the effect on Na^+- and K^+-movements was measured. Passow, working with red cells in saline found an increased permeability to both Na^+ and K^+ to the effect that the cells lost K^+ and gained Na^+. In my own experiments whole blood was used. There was no effect on K^+ movement but the cells incubated with phosphatidic acid took up a little more Na^+ than the controls (Fig. 3). The effect did

not increase with prolonged incubation and was too small to be statistically significant, though it was present in all experiments. Further investigations showed that large amounts of phosphatidic acid were bound to Ca^{2+} of the plasma and, possibly, of the red cell membranes. Passow, too, found that Ca^{2+} inhibited the action of phosphatidic acid on permeability.

These experiments indicate that phosphatidic acid does indeed increase the permeability of a cell membrane to Na^+ and K^+, but this effect would become striking only in regions of low Ca^{2+} content. The results give no support for a specific effect of phosphatidic acid towards Na^+-permeability.

The observed effects of phosphatidic acid on red cells may have some relevance for the mechanism of smooth muscle stimulation. As mentioned above the correlation between acid nature and biological activity of phospholipids suggested a physiological role in the outward movement of Na^+ from cells. At the same time, it suggested a pharmacological influence on the inward movement of Na^+ which might explain the stimulating action when the acidic phospholipids are applied to the outside.

It has been shown by Bülbring and her collaborators[26] that the permeability of smooth muscle cells to Na^+ is extremely high, and that it is strongly influenced by changes in the external Ca^{2+} concentration. The experiments with red cells show that in the presence of physiological concentrations of Ca^{2+} the effect of phosphatidic acid on Na^+ permeability is minimal. It may well be that the effect of phosphatidic acid on the Ca^{2+} will turn out to explain its biological activity in smooth muscle. Calcium has a much higher affinity to phosphatidic acid than Na^+[27] and calcium-phosphatidate is also soluble in non-polar organic solvents. Furthermore, Ca^{2+} seems to be of similar or even greater importance for mechanical and electrical activity of smooth muscle than Na^+.[26, 28] The penetration of ionized Ca^{2+} into cells leads to stimulation. If phosphatidic acid were able to carry Ca^{2+} as a lipid-soluble salt through the cell membrane this might explain its stimulating effect better than a direct effect on Na^+ movement. In this connexion it seems worthwhile to mention the possibility that not only phospholipids but also the smooth muscle contracting hydroxy-fatty acids combine with Ca^{2+} to form lipid-soluble Ca^{2+} salts due to their hydroxyl groups which could provide chelating sites. For instance, prostaglandin forms soluble salts with alkaline earth ions.[29] Acidic lipids which lack such groups and do not form lipidsoluble Ca^{2+} salts, e.g. simple unsaturated fatty acids and cerebroside sulphates do not have smooth muscle stimulating activity. A study of the interaction of acidic lipids with Ca^{2+} will probably be a fruitful line in our attempt to uncover the mechanism of the pharmacological action and a possible physiological role of these lipids.

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