Incorporation of synthetic 1,2-diacylglycerol into platelet phosphatidylinositol is increased by cyclic AMP

Eduardo G. Lapetina

Department of Molecular Biology, The Wellcome Research Laboratories, Burroughs Wellcome Co., Research Triangle Park, NC 27709, USA

Received 23 October 1985

1,2-Didecanoylglycerol (diC_{10}) is taken up by human platelets and sequentially converted to 1,2-didecanoylphosphatidic acid (PA_{10}) and 1,2-didecanoylphosphatidylinositol (PI_{10}). Agents that increase cyclic AMP in platelets, such as prostacyclin and forskolin, sequentially convert diC_{10} to PA_{10} and PI_{10}. They decrease formation of PA_{10} with a parallel accumulation of PI_{10}. This might reflect an inhibition of phosphatidylinositol kinase.

Inositol phospholipid *Phosphatidylinositol kinase* *Phosphatidic acid*

1. INTRODUCTION

It has been observed that an increase of cyclic AMP in platelets decreases thrombin-induced formation of phosphatidic acid (this arises from phosphorylation of 1,2-diacylglycerol, which is produced by phosphodiesteratic cleavage of the inositol phospholipids) and increases accumulation of phosphatidylinositol [1,2]. It was indicated that cyclic AMP does not inhibit phospholipase C (phosphodiesteratic cleavage of the inositol phospholipids) despite a profound reduction in the quantity of phosphatidate produced [1]. It was hypothesized that cyclic AMP increases the conversion rate of phosphatidate to phosphatidylinositol, thereby decreasing the steady-state concentration of phosphatidate [1,2].

More recently it became apparent that phosphatidylinositol bisphosphate is the preferential phospholipase C substrate for the formation of 1,2-diacylglycerol and inositol trisphosphate, which are now considered as second messengers [3–6]. During agonist-induced stimulation, inositide kinases replenish phosphatidylinositol bisphosphate by sequential phosphorylations of phosphatidylinositol and phosphatidylinositol monophosphate [3,5]. Therefore, inhibition of phosphatidylinositol kinase by cyclic AMP decreases formation of phosphatidic acid and accumulation of phosphatidylinositol [1,5]. In agreement with this, platelet-activating factor in the presence of cyclic AMP induces degradation of phosphatidylinositol bisphosphate and inhibits its subsequent resynthesis [7].

We have now used a synthetic 1,2-diacylglycerol, 1,2-didecanoylglycerol (diC_{10}), that is metabolized by platelets to 1,2-didecanoylphosphatidic acid (PA_{10}) and activates protein kinase C [8,9]. diC_{10} is further converted to 1,2-didecanoylphosphatidylinositol (PI_{10}). Cyclic AMP decreases PA_{10} and increases PI_{10} similar to previous results with the endogenously produced phosphatidate and phosphatidylinositol [1,2].

2. EXPERIMENTAL

2.1. *Materials*

Carrier-free [^{32}P]orthophosphate was obtained from New England Nuclear. *myo*-[2-^{3}H]Inositol was from Amersham. diC_{10} was from Serdary, Ontario. Precoated silica gel G-25 plates without gyp-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

sum (layer thickness 0.25 mm) were from Macherey Nagel, Duren, FRG. Prostacyclin was from The Wellcome Research Laboratories, Beckenham, England. Forskolin was from Calbiochem-Behring.

2.2. *Preparation and labeling of human platelets with ^{32}P or [^{3}H]inositol*

Blood (200 ml) was obtained from healthy human voluneers who had not received any medication in the previous 3 weeks. Blood was anticoagulated with 0.2 vol. ACD buffer (85 mM trisodium citrate, 111 mM dextrose and 71 mM citric acid, pH 5.5). Platelet-rich plasma was obtained by centrifugation at 200 × *g* for 20 min and was further centrifuged at 250 × *g* for 15 min. The platelet pellet obtained was resuspended in 10 ml of a modified Tyrode-Hepes buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.36 mM NaH_2PO_4, 1 mM $MgCl_2$, 5 mM glucose, 10 mM Hepes; pH 7.4) containing 5 ng/ml prostacyclin, and the platelets were incubated at 37°C for 90 min with 2.5 mCi ^{32}P. Platelets were washed twice with 30 ml of the modified Tyrode-Hepes buffer containing 5 ng/ml prostacyclin and resuspended in the same buffer without prostacyclin. Platelet concentration was adjusted to 8.0 × 10^8/ml using a Coulter Counter 2F (Coulter Electronic, USA). Samples (0.5 ml) were incubated at 37°C in a shaking incubator bath with 10 μM diC_{10} for various times as indicated in each experiment. Forskolin (0.1 mg/ml) or prostacyclin (0.1 μg/ml) were added to the platelets 2 min before diC_{10} addition.

2.3. *Determination of phospholipids*

Reactions were stopped by addition of 1.88 ml chloroform/methanol/concentrated HCl (100:200:2, by vol.). Phases were separated by adding 0.6 ml chloroform and 0.6 ml of 2 M KCl. The organic phase was dried under a flow of N_2 and the inositides were separated on thin-layer plates (impregnated with 1% potassium oxalate containing 2 mM EDTA) using chloroform, methanol and 4 N NH_4OH (45:35:10, by vol.). The lipids were visualized by autoradiography [10]. Experiments were run at least 5 times; each gave similar qualitative results. A representative experiment in each case is presented.

3. RESULTS

3.1. *Formation of 1,2-didecanoyl[^{32}P]-phosphatidate and 1,2-didecanoyl[^{32}P]-phosphatidylinositol in human platelets prelabeled with ^{32}P and exposed to exogenously added 1,2-didecanoylglycerol*

It has been shown that *sn*-1-oleoyl-2-acetylglycerol [11] and 1,2-diC_{10} [8] are converted to the corresponding [^{32}P]phosphatidates in platelets prelabeled with ^{32}P. These species of phosphatidate are readily separated from [^{32}P]phosphatidate, which is produced in thrombin-stimulated platelets because of their fatty acid compositions.

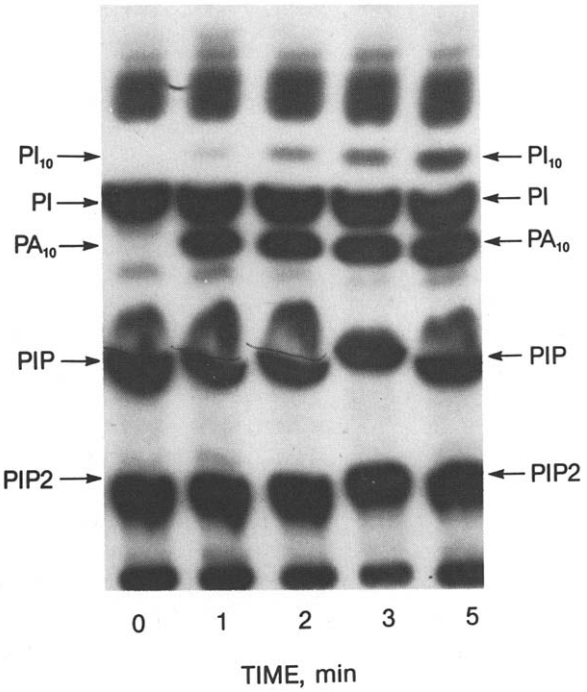

Fig.1. Autoradiography of the formation of 1,2-didecanoyl[^{32}P]phosphatidic acid and 1,2-didecanoyl[^{32}P]-phosphatidylinositol. Platelets prelabeled with ^{32}P were exposed to 10 μM 1,2-didecanoylglycerol for the indicated times. PIP2, phosphatidylinositol bisphosphate; PIP, phosphatidylinositol monophosphate; PA_{10}, 1,2-didecanoyl[^{32}P]phosphatidic acid; PI, phosphatidylinositol; PI_{10}, 1,2-didecanoyl[^{32}P]phosphatidylinositol. Unmarked radioactivity at the bottom indicates the origin and unmarked radioactivity near the solvent front corresponds to other platelet ^{32}P-labeled phospholipids that were not individually resolved.

The endogenous species contain predominantly stearic acid in position 1 and arachidonic acid in position 2 [12]. We used oxalate-impregnated thin-layer chromatographic plates [10] to separate the endogenous [^{32}P]inositol phospholipids from the 1,2-didecanoyl[^{32}P]phosphatidate and 1,2-didecanoyl[^{32}P]phosphatidylinositol (fig.1). These 1,2-didecanoyl[^{32}P]phospholipids were eluted and the phosphatidate further identified by separation in a previously reported chromatographic system [8]. The 1,2-didecanoyl[^{32}P]phosphatidylinositol is cleaved with phospholipase C (*Clostridium welchii*), and the [^{32}P]inositol monophosphate is separated on Dowex 1 columns [5]. Maximal formation of [^{32}P]PA_{10} occurs at 5 min (figs 1 and 2). Thereafter, there is a gradual disappearance of [^{32}P]PA_{10}, with a concomitant increase in the formation of [^{32}P]PI_{10} (fig.2).

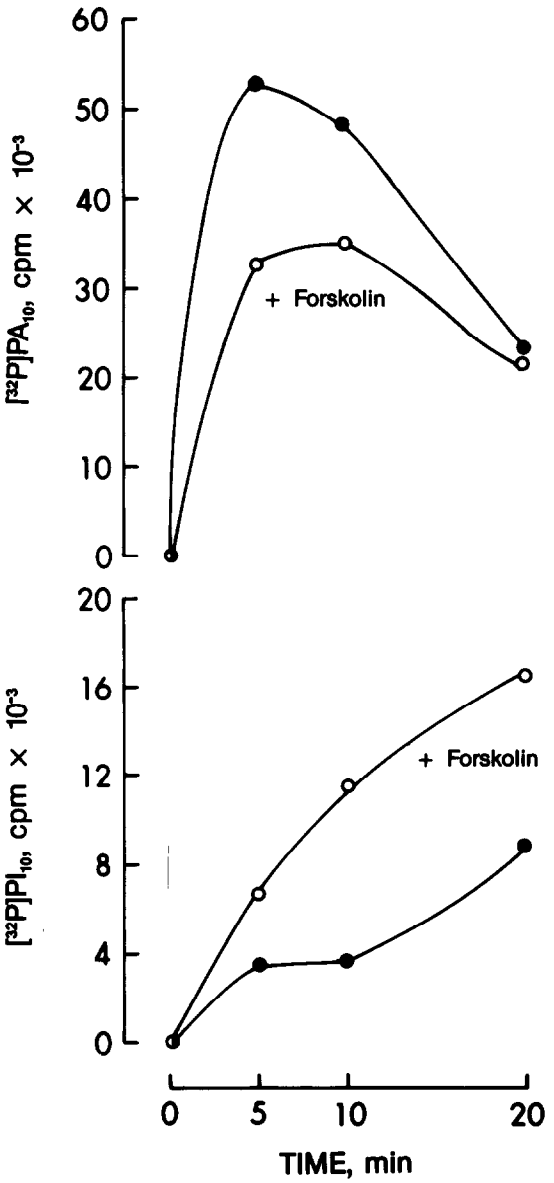

Fig.2. Effect of forskolin on the formation of 1,2-didecanoyl[^{32}P]phosphatidylinositol (PI_{10}) and 1,2-didecanoyl[^{32}P]phosphatidic acid (PA_{10}). ^{32}P-labeled phospholipids were separated as in fig.1. Other details as in fig.1.

3.2. *Effect of prostacyclin and forskolin on 1,2-didecanoyl[^{32}P]phosphatidate and 1,2-didecanoyl[^{32}P]phosphatidylinositol*

Prostacyclin and forskolin stimulate adenylate cyclase and increase the levels of cyclic AMP in platelets [13–15]. This activates cyclic AMP-dependent protein kinases and phosphorylates specific proteins; this is most prominent with a 50 kDa protein [16,17]. Cyclic AMP decreases the formation of the endogenous 1-stearoyl-2-arachidonylphosphatidate and increases the endogenous 1-stearoyl-2-arachidonylphosphatidylinositol in platelets that have been stimulated with thrombin [1,2]. Similarly, 0.1 μg/ml prostacyclin (not shown) or 0.1 mg/ml forskolin (fig.2) decrease the formation of PA_{10} and increase accumulation of PI_{10}. These results are consistent with the hypothesis that an increase in platelet cyclic AMP might inhibit phosphatidylinositol kinase.

4. DISCUSSION

sn-1-Oleoyl-2-acetylglycerol – as well as several synthetic 1,2-diacylglycerols with saturated acyl chains of 4, 6, 8 or 10 (diC_{10}) carbons – can be delivered into platelets and activate protein kinase C [8,9,11]. Under these conditions, 1,2-diacylglycerols are phosphorylated to the corresponding phosphatidic acid species by 1,2-diacylglycerol kinase [8,11]. Chromatographic separations for 1-oleoyl-2-acetylphosphatidate, 1,2-didecanoylphosphatidate and 1-decanoyllysophosphatidic acid have been reported [8,11,18]. In experiments that tested the effects of 1,2-diacylglycerols on the metabolism of platelet inositides labeled with ^{32}P [19], we recorded the formation of 2 new ^{32}P-labeled products: [^{32}P]PA_{10} and [^{32}P]PI_{10} (fig.1).

Exogenous 1,2-diacylglycerols, with a fatty acid pattern different from the endogenously produced 1,2-diacylglycerol, follow a similar metabolic pathway and can be differentiated chromatographically; this provides an opportunity to study the regulation of the related enzymatic reactions. We have used the endogenous [^{32}P]ATP pools to label the added, unlabeled 1,2-diacylglycerol. The ^{32}P-labeled products of this 1,2-diacylglycerol are chromatographically separated from the endogenous ^{32}P radioactive phospholipids. In our present experiments, [^{32}P]PI_{10} is separated from [^{32}P]PI; we have not been able to separate and identify the resultant products of further phosphorylations of PI_{10} by inositide kinases.

Our present information indicates that increases of platelet cyclic AMP affect the sequential conversion of diC_{10} to PA_{10} and PI_{10} in a similar manner to the 1,2-diacylglycerol that is endogenously produced in platelets stimulated with thrombin [1,2]. The similarity of the cyclic AMP effect in both cases suggests that phospholipase C is not affected, because the exogenously added 1,2-diacylglycerol bypasses the activation of phospholipase C. The accumulation of PI_{10} induced by cyclic AMP may reflect an inhibition of phosphatidylinositol kinase [5,7]. The inhibition of this kinase drastically reduces formation of polyphosphoinositides and the phospholipase C-induced formation of inositol trisphosphate and 1,2-diacylglycerol [7] with concomitant accumulation of phosphatidylinositol [5]. The development of methods to separate diC_{10}-derived polyphosphoinositides from the endogenous species will allow the study of this possibility and will provide a technique to test the effect of platelet agonists on the inositol phospholipids produced from synthetic 1,2-diacylglycerol added to platelets.

ACKNOWLEDGEMENTS

I thank Bryan Reep for his excellent technical help, Allen Jones for his manuscript revision and Tonya Beasley for her careful preparation of this manuscript.

REFERENCES

[1] Lapetina, E.G., Billah, M.M. and Cuatrecasas, P. (1981) Nature 292, 367–369.
[2] Lapetina, E.G. (1982) Trends Pharmacol. Sci. 3, 115–118.
[3] Berridge, M.J. (1983) Biochem. J. 212, 849–858.
[4] Rittenhouse, S.E. (1983) Proc. Natl. Acad. Sci. USA 80, 5417–5420.
[5] Watson, S.P., McConnell, R.T. and Lapetina, E.G. (1984) J. Biol. Chem. 259, 13199–13203.
[6] Chung, S.M., Proia, A.D., Klintworth, G.K., Watson, S.P. and Lapetina, E.G. (1985) Biochem. Biophys. Res. Commun. 129, 411–416.
[7] Billah, M.M. and Lapetina, E.G. (1983) Proc. Natl. Acad. Sci. USA 80, 965–968.
[8] Watson, S.P., Ganong, B.R., Bell, R.M. and Lapetina, E.G. (1984) Biochem. Biophys. Res. Commun. 121, 386–391.
[9] Lapetina, E.G., Reep, B., Ganong, B.R. and Bell, R.M. (1985) J. Biol. Chem. 260, 1358–1361.
[10] Billah, M.M. and Lapetina, E.G. (1982) J. Biol. Chem. 257, 12705–12708.
[11] Kaibuchi, K., Takai, Y., Sawamura, M., Hoshijima, M., Fujikura, T. and Nishizuka, Y. (1983) J. Biol. Chem. 258, 6701–6704.
[12] Broekman, M.J., Ward, J.W. and Marcus, A.J. (1981) J. Biol. Chem. 256, 8271–8274.
[13] Lapetina, E.G., Schmitges, C.J., Chandrabose, K. and Cuatrecasas, P. (1977) Biochem. Biophys. Res. Commun. 76, 828–835.
[14] Gorman, R.R., Bunting, S. and Miller, O.V. (1977) Prostaglandins 13, 377–388.
[15] Tateson, J.E., Moncada, S. and Vanl, J.R. (1977) Prostaglandins 13, 389–397.
[16] Nishizuka, Y. (1983) Phil. Trans. R. Soc. Lond. Ser. B 302, 101–112.
[17] Lapetina, E.G., Watson, S.P. and Cuatrecasas, P. (1984) Proc. Natl. Acad. Sci. USA 81, 7431–7435.
[18] Watson, S.P., McConnell, R.T. and Lapetina, E.G. (1985) Biochem. J., in press.
[19] Watson, S.P. and Lapetina, E.G. (1985) Proc. Natl. Acad. Sci. USA 82, 2623–2626.