The phosphatidylinositol cycle and the regulation of arachidonic acid production

Eduardo G. Lapetina, M. M. Billah & P. Cuatrecasas

Department of Molecular Biology, The Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709, USA

An increase in the metabolism of phosphatidylinositol occurs in a wide variety of tissues by the action of specific ligands1,2. In platelets, the interaction of thrombin with its receptor initiates the degradation of phosphatidylinositol by the action of a specific phospholipase C (refs 4–9). In normal conditions of stimulation, the resultant 1,2-diacylglycerol is rapidly and completely phosphorylated to phosphatidic acid10,11. The formation of phosphatidic acid precedes the release of arachidonic acid from the phospholipids of stimulated platelets¹. This early appearance of phosphatidate might result in the initial production of arachidonic acid and lysophosphatic acid by the action of a phospholipase A₂ specific for phosphatidate12. Phosphatidate/lysophosphatidate could induce calcium-gating13-15 and subsequently stimulate phospholipases of the A₂-type6, that degrade phosphatidylcholine, phosphatidylethanolamine and a further fraction of phosphatidylinositol17. Alternatively, the lysophosphatidate produced may serve as a substrate for the transfer of arachidonate directly from other phospholipids16,17 to form new phosphatidate which in turn can release more arachidonate. Overall, such a sequence would be equivalent to phospholipase A₂ activation of other phospholipids. Our present data indicate that when the release of arachidonic acid is completely inhibited by cyclic AMP or quinacrine, phosphatidic acid is redirected entirely to phosphatidylinositol and there is no production of arachidonate. In these conditions, the availability of calcium might be profoundly restricted. The correlation in platelets of a phosphatidylinositol by a specific phospholipase A₂ might suggest that these phenomena are applicable to activations in other cell systems.

Phosphatidylinositol is not the only phospholipid that contributes to the production of arachidonic acid in stimulated platelets10-18. Phosphatidylcholine and phosphatidylethanolamine also release arachidonic acid by the action of phospholipase A₂ activities6. Both lysophosphatidylcholine and lysophosphatidylethanolamine have recently been found in stimulated platelets10,18. In a similar way, lysophosphatidic acid is also produced in intact platelets that have been prelabelled with ³²P and stimulated with thrombin (Fig. 1). Thrombin is very effective in producing phosphatidic acid6,19 and lysophosphatidic acid19, whereas ionophore A23187 forms virtually no phosphatidic acid6,5 or lysophosphatidic acid (Fig. 1). Calcium ions enhance the thrombin-induced formation of phosphatidic acid as well as of lysophosphatidic acid (Fig. 1). We have described elsewhere the existence of a specific phospholipase A₂ which is present in platelet membranes and which specifically degrades phosphatidic acid17. This enzyme activity (Kₐ = 20 μM) is most active at pH 7.0, requires Ca²⁺ (10 μM) for maximal activity and is inhibited by quinacrine12. The existence and specific properties of this enzyme suggest a possible important role in the production of arachidonic acid in stimulated platelets. This phosphatidate-specific phospholipase A₂ has distinctly different properties from those of the phospholipases A₂ that degrade phosphatidylethanolamine and phosphatidylcholine6, as its activity does not depend on the presence of detergents, alkaline pH or high concentration of Ca²⁺.

Phosphatidate is a key intermediate in the phosphatidylinositol cycle1-3. In this cycle, four consecutive enzyme activities are involved in the degradation and resynthesis of phosphatidylinositol (phosphatidylinositol-specific phospholipase C; 1,2-diacylglycerol kinase; CTP- phosphatidate: cytidyl transferase; CDP-1,2-diacylglycerol-inositol phosphatidyl transferase). To study the effects of calcium on the phosphatidic acid and phosphatidylinositol of stimulated platelets, platelets were labelled with ³²P-orthophosphate and resuspended in an EGTA-containing buffer. If those platelets are then incubated with quinacrine and stimulated with thrombin, the release of arachidonic acid is completely blocked but phosphatidic acid is formed as a consequence of the degradation of phosphatidylinositol6. After an initial period, the label in phosphatidate decreases while the labelling of phosphatidylinositol increases (Fig. 2). This increased conversion of phosphatidate to phosphatidylinositol is blocked by the addition of ionophore A23187 plus calcium ions (Fig. 2). In this case, there is a further accumulation of labelled phosphatidic acid while the increased labelling of phosphatidylinositol is completely blocked (Fig. 2). These results indicate that calcium inhibits the resynthesis of phosphatidylinositol from phosphatidic acid after thrombin stimulation (Fig. 2)¹⁰. In fact, Ca²⁺ has a direct inhibitory action on the enzymes involved in the resynthesis process (CDP-phosphatidate: cytidyl transferase and CDP-1,2-diacylglycerol-inositol phosphatidyl transferase¹⁰). The presence of quinacrine, which completely blocks the production of arachidonic acid from all platelet phospholipids, calcium

© 1981 Macmillan Journals Ltd
Cyclic AMP inhibits the 'release reaction' of platelets as well as aggregation. The action of cyclic AMP on platelet enzymes has been variously ascribed to the inhibition of the conversion of arachidonic acid to cyclooxygenase metabolites, the production of arachidonic acid from phospholipids, and the formation of phosphatidic acid. All these actions ultimately reduce the production of arachidonate or its conversion to active cyclooxygenase products. We previously described the action of cyclic AMP in reducing phosphatidic acid to an inhibition of phospholipase C. Further studies now reveal that the phosphatidylinositol cycle is not inhibited by cyclic AMP despite the profound reduction in the quantity of phosphatidate produced. Figure 3 describes the action of cyclic AMP on the reactions related to the increased turnover of phosphatidylinositol in platelets prelabelled with 32P-orthophosphate. Cyclic AMP seems substantially to increase the rate of conversion of phosphatidylinositol to phosphatidate, thereby decreasing the steady state concentration of phosphatidate. As we are proposing that the production of arachidonic acid might be related to the formation of phosphatidate, this could serve as the basis for the cyclic AMP-induced inhibition of arachidonate production. In the presence of quinacrine, which completely blocks the formation of arachidonic acid from various phospholipids, thrombin greatly increases the breakdown and resynthesis (turnover) of phosphatidylinositol as shown by increased labelling of 32P-phosphatidylinositol (Fig. 3). These data indicate that the integrity of the phosphatidylinositol cycle is maintained in conditions (cyclic AMP or quinacrine) in which the specific release of arachidonic acid induced by thrombin is completely blocked.

The information presented here indicates that calcium interrupts the phosphatidylinositol cycle and leads to accumulation of the intermediate product, phosphatidic acid. In stimulated platelets a specific phospholipase A2 released arachidonic acid from the phosphatidate produced, with the consequent appearance of lyso phosphatidic acid. This phosphatidate-lyso phosphatidate interconversion might be important in the subsequent and specific mobilization of arachidonic acid from phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol. In thrombin-stimulated platelets cyclic AMP enhances the overall turnover of the phosphatidylinositol cycle by increasing the rate of conversion of phosphatidic acid to phosphatidylinositol, and thus inhibits the release of arachidonic acid from various phospholipids.

Received 16 February; accepted 22 May 1981.

1. Lapetina, E. O. & Michell, R. H. FEBS Lett. 31, 1-10 (1973).
2. Michell, R. H. Biochim. biophys. Acta 415, 81-147 (1975).
3. Michell, R. H. Trends biochem. Sci. 4, 128-131 (1979).
4. Lapetina, E. O., Chandrabose, K. & Castrenes, P. Proc. natn. Acad. Sci. U.S.A. 75, 818-822 (1978).
Derivatives of flavan have been synthesized as chemical intermediates, but the only reported biological action is the ability of certain alkyl and alkoxy derivatives to lower blood cholesterol concentrations. It was therefore surprising to discover that flavan itself (Table 1) is a highly effective inhibitor of the replication of certain serotypes of rhinovirus, and that a simple derivative, BW683C (4',6-dichloroflavan), is the most potent antiviral compound yet reported. The present work examines the antiviral activity of flavan derivatives with a view to selecting the compound most suitable for trial in volunteers infected with a common cold virus.

4',6-Dichloroflavan, which is new to the chemical literature, has been prepared by methods used for the synthesis of substituted flavans. It is a colourless crystalline solid, m.p. 101°C, soluble in water only to the extent of 1 mg l⁻¹ at room temperature. Antiviral activity was detected in vitro by means of plaque inhibition tests with monolayers of M-HeLa cells infected with rhinovirus 1B. Activity was measured by plaque reduction assays in which doubling concentrations of compound were incorporated into the overlay medium. Plaque counts, expressed as a percentage of the control value, were plotted against the logarithm of the compound concentration, to yield a dose-response line from which the IC₅₀ value could be determined. The IC₅₀ values for BW683C and several analogues are shown in Table 1. Flavan (R₆ = R₇ = H), with an IC₅₀ of 0.046 µM, is one of the least active of the compounds tested. The activity is generally increased by the presence of a single halogen substituent, and more so by the presence of two chlorine atoms, with the most active compound tested being BW683C, which, with an IC₅₀ of 0.007 µM, is some six times more potent than the parent compound. The IC₅₀ of BW683C was 0.02 µM.

For any agent to be effective in the prophylaxis or treatment of the common cold, it must be active against a high proportion of rhinovirus serotypes. There are at least 89 serotypes, the most prevalent being 1A, 1B, 2, 4, 15, 29, 30 and 31 (ref. 10). IC₅₀ values were obtained for BW683C against 19 serotypes (Table 2). Seven of the eight most prevalent serotypes were inhibited, although they varied considerably in sensitivity. The sensitivity of the other 11 serotypes was also variable, but was sufficient to suggest that the compound may be clinically useful.

In tissue culture tests 4',6-dichloroflavan did not inhibit the replication of other RNA viruses, including bunyavirus, coronavirus, equine rhinovirus, influenza virus (NWS strain), measles virus, poliovirus (Sabin 1), Semliki Forest virus, Sindbis virus and respiratory syncytial virus. It also failed to inhibit the DNA viruses adenovirus type 5 and herpesvirus type 1.

### Table 1 Structure–activity relationship of a selection of halogen-substituted flavans against rhinovirus type 1B

| R₆ | R₇ | IC₅₀(µM) |
|----|----|----------|
| H  | H  | 0.046    |
| F  | H  | 0.020    |
| Cl | H  | 0.050    |
| Br | F  | 0.018    |
| H  | Cl | 0.039    |
| H  | Br | 0.036    |
| F  | F  | 0.068    |
| Cl | Cl | 0.007    |
| Br | Br | 0.010    |

© 1981 Macmillan Journals Ltd