Vol. 139, No. 1, 1986
August 29, 1986

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 115-121

ASPIRIN INHIBITS PHOSPHOLIPASE C

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Received July 11, 1986

SUMMARY: We have shown previously that aspirin (ASA) ingestion by normal human volunteers inhibits peripheral blood monocyte phospholipase C (PLC) activities ex vivo. In order to explore further the mechanism of action of ASA, normal human monocytes and differentiated human U937 cells were treated with ASA and other salicylates. Cells preincubated with ASA were found to have decreased PLC activities. Phospholipase A_2 activities were not affected by salicylates. Sodium salicylate and salicylic acid, nonacetylated relatives of ASA also inhibited PLC activity. This effect was dose and time dependent and addition of cycloheximide or actinomycin D to the preincubation mixture abrogated the inhibitory effect of salicylates on PLC. This PLC inhibitory protein induced by ASA appears distinct from lipocortin, a phospholipase A_2 inhibitory protein inducible by corticosteroids.

Aspirin (ASA) inhibits prostaglandin formation in a variety of experimental preparations by inhibiting cyclooxygenase, a key enzyme in the production of prostanoids (1-5). The mechanism of this inhibition is acetylation (6-7). Other nonacetylated nonsteroidal antiinflammatory drugs (NSAID) also inhibit prostanoid production by mechanisms other than acetylation (1-5). Thus, regulation of

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Materials: Phosphatidylcholine and phosphatidylinositol with [^{14}C]-arachidonic acid (AA), [^{14}C]-linoleic acid and [^{14}C]-oleic acid in the sn-2 position (PC-2A, PC-2O, PC-2L, PI-2A, PI-2L and PI-2O) and phosphatidylcholine and phosphatidylinositol with [^{3}H]- in the polar head group (PC-3 and PI-3).

0006-291X/86 $1.50

prostaglandin synthesis by acetylation of prostaglandin synthetase is a mechanism of action not shared by these compounds.

Other salicylates such as sodium salicylate, a nonacetylated salicylate, have less inhibitory effect on cyclooxygenase for a given dosage (IC 50) compared to other NSAIDs (1-5). However, patients with inflammatory disorders such as rheumatoid arthritis obtain clinical relief from sodium salicylate (8,9). This non-acetylated salicylate would thus appear to modulate eicosanoid formation by a mechanism other than direct inhibition of prostaglandin synthetase. We have shown that ASA ingestion in antiinflammatory doses by normal human volunteers inhibits monocyte PLC activities ex vivo (10). This paper extends these observations on the mechanism of salicylate inhibition of PLC activities using an in vitro system.

MATERIALS AND METHODS

Materials were obtained as we have described (10). Lactate dehydrogenase (LDH) Kit, actinomycin D, and cycloheximide were obtained from Sigma Chemical (St. Louis, MO). Human recombinant interferon was from Genentech (South San Francisco, CA) or AMGen (Thousand Oaks, CA).

Monocyte Isolation. Peripheral blood monocytes were isolated as we have previously described (11). Cells were pooled, centrifuged and resuspended at a concentration of 2 x 10^5 live cells/ml in RPMI 1640 and incubated in 2 ml polypropylene microfuge tubes at 37°C in a 5% CO_2/95% air incubator and incubated with or without compounds to be tested. 95% were usually positive for nonspecific esterase and cell viability as determined by trypan blue exclusion was greater than 95%.

U937 Cells. Cells were were maintained in RPMI 1640 with 10% heat inactivated fetal calf serum, penicillin (100 U/ml) and streptomycin (100 ug/ml) at 37°C in a 5% CO_2/95% air incubator and were induced to differentiate with 500 units of recombinant gamma interferon and were used 3-6 days following induction (12).

Phospholipase Assay: The assay was performed as we have described (10,13). PLA_2 activity was quantified by measuring lysophospholipid formation and PLC by measuring diacylglyceride. PLC was also quantified by measuring the radioactivity in the alcoholic phase with PI-3 and PC-3 to confirm our results obtained by measuring diacylglyceride formation.

Radioimmunoassay for Lipocortin. The radioimmunoassay was performed as described (14).

Protein Determination: Protein content of cell lysates was determined by the method of Bradford (15) using a dye reagent con-

centrate supplied by Bio-Rad Labs. with bovine serum albumin as standard.

LDH Determination: Production of lactate dehydrogenase was measured using a kit purchased from Sigma Chemical as described by the manufacturer.

RESULTS and DISCUSSION

Addition of ASA in varying concentrations to the cell sonicate mixture did not affect PLC nor PLA_2 activities (data not shown). Such results have been suggested by other workers examining the effects of ASA on human platelet phospholipase activities (16).

As addition of ASA to the cell sonicate did not affect phospholipase activities, human monocytes and gamma interferon differentiated U937 cells were therefore preincubated with varying concentrations of ASA (Fig. 1). Maximal inhibition was observed at 10^{-6} M, and greater concentrations (to 10^{-4} M) did not further inhibit PLC activity as measured using the cell sonicate. Irrespective of the dose used, the PLC activity was never completely abolished. However, we consistently observed approximately a 50% decrease in PLC activity in cells pretreated for 60 min with 10^{-6} M ASA. ASA inhibition of PLC activity was also time dependent (Fig. 2).

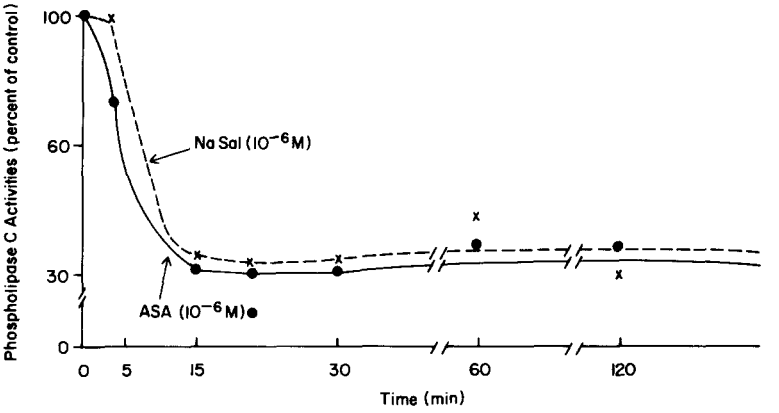

Figure 1: Inhibition of Phospholipase C Activities by Aspirin. Human monocytes and gamma interferon differentiated U937 cells were incubated in the presence or absence of ASA for 60 min, and then phospholipase assays performed as described in Materials and Methods. Data from a representative experiment of which 6 were performed using PC-2O, PC-2A, PI-2A or PI-2O. Experiments were performed in triplicate with standard deviation within 10% of mean.

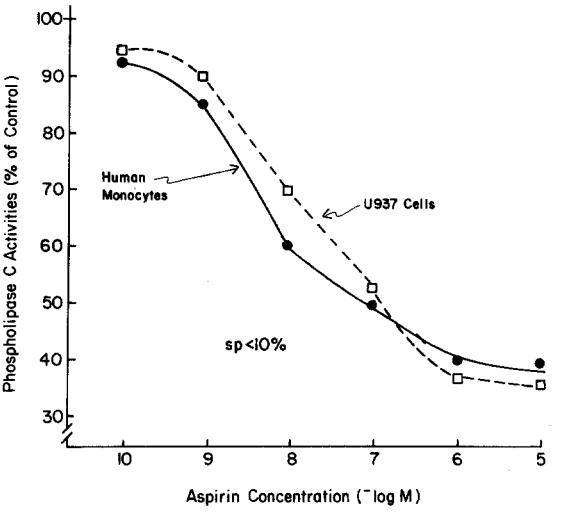

Figure 2: Time Course of Induction of Phospholipase C Inhibition by Aspirin and Sodium Salicylate. Data from a representative experiment using PC-20 as substrate with gamma interferon differentiated U937 cells performed in triplicate with standard deviation within 10% of the mean. Similar results were observed using substrates with arachidonic acid or linoleic acid in the sn-2 position, and with human monocytes. Cell viability as determined by trypan blue exclusion and LDH release was not affected.

The effects of preincubation with sodium salicylate and salicylic acid, nonacetylated salicylates, and indomethacin, on phospholipase activities were also examined (Table I). Preincubation of human monocytes with salicylates resulted in similar inhibition of PLC activity, whereas indomethacin (10^{-5} M), another cyclooxygenase inhibitor, had no effect. Indomethacin added to cell sonicate results in inhibition of PLA_2 activities (17). These data suggest that not all NSAIDs have PLC inhibitory activity. Inhibition of PLC activity by nonacetylated salicylates is also time dependent (Fig. 2) and dose dependent in a manner similar to the inhibitory action of ASA. Specificity of this inhibitory activity was also observed. When cells were pretreated with ASA, sodium salicylate, salicylic acid and indomethacin, no effect on PLA_2 activity was observed (Table I).

The effects of salicylates on PLC activities requires preincubation. In order to determine the mechanism of time depen-

Table I: Effect of Preincubation with Salicylates or Indomethacin on Phospholipase Activities*

Compound	Phospholipase Activities (Percent of control)			
	PLC		PLA_2	
	Monocyte	U937	Monocyte	U937
Control	100 ± 2	100 ± 1	100 ± 2	100 ± 4
Aspirin (10^{-6} M)	54 ± 3**	49 ± 4**	94 ± 15	98 ± 3
Sodium salicylate (10^{-6} M)	61 ± 5**	45 ± 8**	88 ± 11	90 ± 10
Salicylic acid (10^{-6} M)	52 ± 6**	50 ± 7**	98 ± 9	94 ± 8
Indomethacin (10^{-5} M)	94 ± 7	93 ± 8	104 ± 6	97 ± 5

* Monocytes and gamma interferon differentiated U937 cells were incubated for 1 hr at 37°C in a 5% CO_2/95% air incubator. At the end of the incubation period, the cells were processed as described in Materials and Methods with PC-20 as substrate for PLC and PC-3 as substrate for PLA_2. Similar results were observed using PC-2A or PC-2L as substrate for PLC, with PI-2 substrates, and when PC-3 and PI-3 were used as substrate and polar head group metabolites measured. Phospholipase activities from a representative experiment performed in triplicate expressed as pmol substrate hydrolyzed/mg protein/min ± S.D., and expressed as percent of control.

** $p < 0.05$

dent salicylate induced inhibition of PLC activity, we preincubated cells with actinomycin D (AD) or cycloheximide (CX) before adding salicylates (Table II). The inhibitory effects of salicylates on

Table II: Effects of Actinomycin D and Cycloheximide on Salicylate Induced Inhibition of Phospholipase Activities*

Compound	Phospholipase C Activities		
	Control	+AD	+CX
Control	12.3 ± 1.2	12.4 ± 0.9	11.9 ± 1.4
Aspirin (10^{6} M)	6.2 ± 1.4**	11.4 ± 1.0	10.2 ± 1.3
Sodium Salicylate (10^{-6} M)	7.6 ± 0.2**	10.4 ± 1.1	11.1 ± 0.1
Salicylic Acid (10^{-6} M)	7.4 ± 1.0**	12.0 ± 0.4	10.9 ± 0.2

* Monocytes were incubated for 1 hour at 37°C in a 5% CO_2/95% air incubator. At the end of the incubation period, the cells were processed as described in Materials and Methods. The final concentration of CX was 100 ug/ml and 10 ug/ml for AD. Phospholipase activities from a representative experiment performed in triplicate using PC-2A as substrate and expressed as pmol substrate hydrolyzed/mg protein/min ± S.D. Similar results were obtained using PC-20 or PI-20 as substrate, and with gamma interferon differentiated U937 cells.

** $p < 0.05$

monocyte PLC activities were abrogated by preincubation with AD or CX. Thus, translational and transcriptional control mechanisms to induce a new protein(s) are likely to be involved in salicylate inhibition of PLC activities.

Endogenous phospholipase inhibitory proteins have been described in various cells (14,18,19). To examine the possibility that the protein induced by ASA may be lipocortin, a PLA_2 inhibitory protein, lipocortin activity was assayed by radioimmunoassay (RIA) (14). ASA treated monocytes and U937 cells did not contain elevated lipocortin activities compared to control cells (18.1 $\pm$ 2.9 ng/ml of cell sonicate for ASA vs 17.5 $\pm$ 2.9 ng/ml for control, N=6). Thus, ASA may induce a unique PLC regulatory protein; alternatively, salicylates may inhibit protease degradation of this endogenous PLC inhibitory protein or modulate structure-activity relationships.

From these data we conclude that salicylates inhibit PLC activity and that this inhibition may be relevant to explain the therapeutic effects of these drugs. The mechanism of action of ASA appears to extend beyond inactivation of cyclooxygenase (20-22). In addition, our data are consistent with the hypothesis that there are at least two different types of PLC activities; one is sensitive to ASA treatment and the other(s) are not. Multiple types of PLC have been isolated and characterized from sheep seminal vesicles and bovine myocardium (23,24). Thus, there is precedence for the existence of multiple forms of the PLC enzyme, one subset of which may be affected by ASA.

ACKNOWLEDGEMENTS: We thank Dr. Brian Mandell for performing the LDH assays, Dr. Robert B. Zurier for helpful suggestions, and E. Lobb for assistance in preparation of the manuscript.

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