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Inhibition of Highly Purified Mammalian Phospholipases A_2 by Non-Steroidal Anti-Inflammatory Agents

MODULATION BY CALCIUM IONS

Richard C. FRANSON, Drore EISEN, Robert JESSE and Carmine LANNI
Department of Biophysics, Medical College of Virginia, Richmond, VA 23298, U.S.A.

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Highly purified Ca^{2+}-dependent phospholipases A_2 that were isolated from human platelets, rabbit alveolar macrophages and peritoneal polymorphonuclear leucocytes and were active in the neutral-to-alkaline pH range were inhibited 50% by 75 μM-indomethacin in the presence of 5.0 mM added Ca^{2+}. Sodium meclofenamate and sodium flufenamate were also inhibitory; the sensitivity to inhibition was a function of Ca^{2+} concentration. The dose for 50% inhibition (ID_{50}) with meclofenamate was 0.4 mM in the presence of 2.5 mM added Ca^{2+}, but 50 nM in the presence of 0.5 mM added Ca^{2+}. Thus, inhibition of phospholipase A_2 activity by non-steroidal anti-inflammatory agents via Ca^{2+} antagonism may significantly contribute to the mechanism of drug action.

Arachidonate mobilization and prostaglandin production during inflammation is well-documented (Vane, 1971, 1972; Humes *et al.*, 1977) and until recently it was thought that the primary mechanism of action of non-steroidal anti-inflammatory agents was to block further metabolism of arachidonate by inhibition of the cyclo-oxygenase (Lands & Rome, 1976; Humes *et al.*, 1977). Kaplan *et al.* (1978) have shown that indomethacin also inhibits phospholipase A_2 activity of rabbit polymorphonuclear leucocytes in the presence of 10 mM added Ca^{2+}, but significant inhibition was apparent at concentrations of drug much greater than that required to block the cyclo-oxygenase (Humes *et al.*, 1977). We have recently isolated phospholipase A_2 from human platelets and rabbit alveolar macrophages as well as rabbit polymorphonuclear leucocytes (Franson & Waite, 1978; Jesse & Franson, 1979). These enzymes are toally Ca^{2+}-dependent (i.e. inactive in the absence of Ca^{2+}), but are maximally active with only 0.5–0.75 mM added Ca^{2+}. In view of extensive work by Northover (1977) that demonstrates that non-steroidal anti-inflammatory agents function in part as Ca^{2+} antagonists, we have examined the effect of Ca^{2+} on the sensitivity of these highly purified enzymes to inhibition by non-steroidal anti-inflammatory agents and provide evidence that inhibition of phospholipases A_2 at physiological Ca^{2+} concentration may contribute to the anti-inflammatory action of these drugs.

Materials and Methods

Membrane-associated phospholipases A_2 were isolated from intact cells (human platelets, BCG-induced alveolar macrophages and glycogen-induced peritoneal polymorphonuclear leucocytes from rabbits) by methods previously described, using solubilization by H_2SO_4 and ion-exchange chromatography. The platelet phospholipase A_2 was purified 3500-fold compared with the homogenate by this method and the leucocyte and macrophage enzymes were purified approx. 1500-fold (Weiss *et al.*, 1975; Franson & Waite, 1978; Jesse & Franson, 1979). Phospholipase A_2 activity was measured by established methods with either pure 1-acyl-2-[^{14}C]linoleoyl-*sn*-glycero-3-phosphoethanolamine or *Escherichia coli* grown on [1-^{14}C]oleate (to specifically label the 2-position of phospholipids) and then autoclaved to destroy microbial phospholipid-splitting activity. Reaction mixtures contained the indicated concentrations of $CaCl_2$ and/or drug, 75 nM buffer (Tris/maleate, pH 7.5, for leucocyte and macrophage; Tris/HCl, pH 9.5, for the platelet), 2.5×10^8 autoclaved *E. coli* cells (7000 c.p.m. corresponding to 5 nmol of phospholipid) or 40 nmol (10000 c.p.m.) of a sonicated aqueous dispersion of 1-acyl-2-[^{14}C]linoleoyl-*sn*-glycero-3-phosphoethanolamine. The total volume of the reaction mixtures was 0.5 ml with *E. coli* substrate and 1.0 ml with 1-acyl-2-[^{14}C]linoleoyl-*sn*-glycero-3-phosphoethanolamine. Incubation time at 37°C

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and enzyme content (0.5–4.0 μg of protein) were adjusted to ensure linear kinetics. The reactions were stopped by the addition of 3 vol. of chloroform/methanol (1:2, v/v) and the lipids were extracted and isolated as previously described (Franson & Waite, 1978). Phospholipase A_2 activity is calculated as nmol of non-esterified fatty acid formed/unit time and is expressed as a percent of the control (where the control value is the 100% uninhibited activity). Indomethacin (Sigma, St. Louis, MO, U.S.A.) was freshly prepared in ethanol for each experiment and appropriate controls were run; sodium meclofenamate and sodium flufenamate were supplied by Warner–Lambert/Parke–Davis (Ann Arbor, MI, U.S.A.) and were water-soluble and stored in solution at −10°C. Protein was measured by the method of Bradford (1976), with ovalbumin as a standard.

Results

The inhibition of phospholipases A_2 purified from human platelets, and rabbit polymorphonuclear leucocytes and macrophages is shown in Fig. 1. In the presence of 5.0 mM-Ca^{2+} and [1-^{14}C]oleate-labelled *E. coli* as substrate, activity was inhibited in a dose-dependent fashion by indomethacin; the dose for 50% inhibition (ID_{50}) was approx. 75 μM in each case. In subsequent studies, we used the phospholipase A_2 isolated from the polymorphonuclear leucocyte and pure 1-acyl-2-[^{14}C]linoleoyl-*sn*-glycero-3-phosphoethanolamine as substrate.

The non-steroidal anti-inflammatory agents sodium meclofenamate and sodium flufenamate, analogues of indomethacin, also inhibited phospholipase A_2 activity (Fig. 2). The ID_{50} with 2.5 mM added Ca^{2+} was comparable with that for indomethacin (Fig. 1) (approx. 100 μM). Thus the inhibition of phospholipid-splitting activity by non-steroidal anti-inflammatory agents under these conditions is considerably less sensitive, by several orders of magnitude, than the well-established inhibition of the cyclo-oxygenase by these agents (Lands & Rome, 1976; Humes *et al.*, 1977), and it is currently thought that the primary site of drug action with respect to prostaglandin production is at the level of the cyclo-oxygenase and further metabolism of arachidonate rather than the mobilization of arachidonate from complex lipids such as membrane phospholipid.

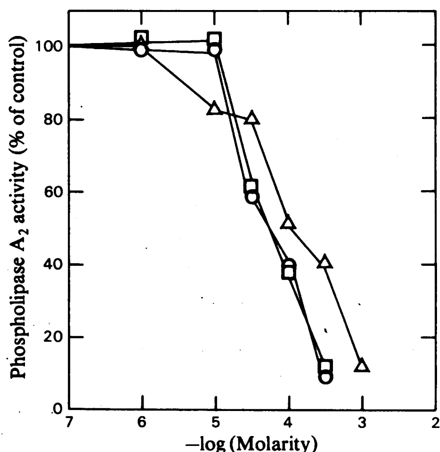

Fig. 1. *Inhibition of phospholipases A_2 by indomethacin*
Phospholipase A_2 activity was measured with autoclaved *E. coli* as substrate in the presence of 5.0 mM added Ca^{2+} and 0.5–4.0 μg of protein as described in the Materials and Methods section. Each point is the mean for duplicate or triplicate determinations. All results are corrected for non-enzymic release, which was constant and less than 0.045 nmol of non-esterified fatty acid (1.2% of total label). The enzyme controls (i.e. uninhibited activity) ranged in activity from 0.6 to 1.0 nmol of non-esterified fatty acids/10 min incubation and were unaffected by ethanol concentrations (less than 1%) used to dissolve indomethacin. The source of the phospholipase A_2 was: △, polymorphonuclear leucocytes; O, platelets; □, macrophages.

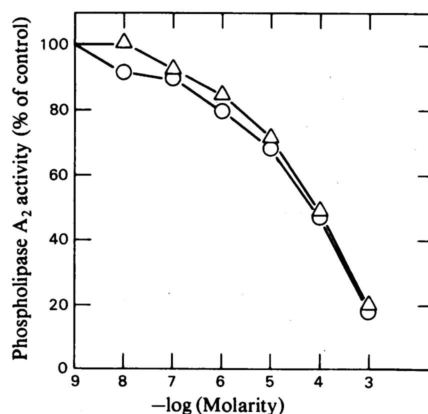

Fig. 2. *Inhibition of polymorphonuclear-leucocyte phospholipase A_2 by sodium meclofenamate and sodium flufenamate*
Activity was measured in the presence of 2.5 mM added Ca^{2+} and pure 1-acyl-2-[^{14}C]linoleoyl-*sn*-glycero-3-phosphoethanolamine as substrate. Reaction mixtures contained 1.0 μg of protein and were incubated at 37°C for 15 min. Each point in Figs. 2 and 3 is the mean for triplicate determinations corrected for non-enzymic release, which was 0.8 nmol of non-esterified fatty acids (2% of total label). The activity of the enzyme control (i.e. 100%) was 8.0 nmol of non-esterified fatty acids/15 min. Symbols: △, flufenamate; O, meclofenamate.

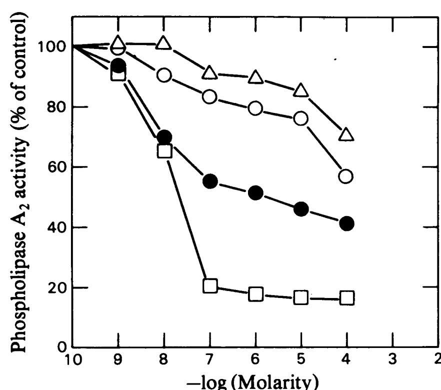

Fig. 3. *Inhibition of polymorphonuclear-leucocyte phospholipase A_2 by sodium meclofenamate as a function of Ca^{2+} concentration*

Reaction mixtures containing various amounts of $CaCl_2$ were as described in Fig. 2. The concentration (mM) of added Ca^{2+} was: △, 5.0; ○, 2.5; ●, 1.0; □, 0.5.

Because the isolated phospholipases A_2 are extremely sensitive to, and dependent on, Ca^{2+} for reactivity (Franson & Waite 1978; Jesse & Franson, 1979) and because of previous reports that suggest non-steroidal anti-inflammatory agents function as Ca^{2+} antagonists (Northover, 1977), we examined the possibility that the sensitivity of phospholipases A_2 to inhibition by non-steroidal anti-inflammatory agents may be modulated by Ca^{2+}. Fig. 3 demonstrates clearly that the extent of inhibition by a given concentration of meclofenamate is, indeed, dependent on the concentration of added Ca^{2+}. Thus with 2.5mM-Ca^{2+} the ID_{50} was 0.4mM, whereas with 0.5mM-Ca^{2+} the ID_{50} was 50nM, or 10^4 times more sensitive. In similar experiments with the membranous substrate, autoclaved *E. coli*, and 0.5mM added Ca^{2+}, inhibition of the polymorphonuclear-leucocyte phospholipase A_2 was reproducibly measured at 10pM-meclofenamate (26%) and was 50% inhibited with 50pM concentrations of the drug (results not shown). Although somewhat less sensitive, similar results were obtained with sodium flufenamate; dexamethasone and aspirin had no effect on phospholipase A_2 activity at all concentrations tested (10^{-10}–10^{-4}M).

Discussion

These results demonstrate that isolated phospholipases A_2 are inhibited by low concentrations of non-steroidal anti-inflammatory agents. The sensitivity to inhibition is Ca^{2+}-dependent and readily detectable at 10nM–10pM concentrations of non-steroidal anti-inflammatory agents with submillimolar concentrations of Ca^{2+}. By comparison, indomethacin inhibits the macrophage cyclo-oxygenase with an ID_{50} of 20nM (Humes *et al.*, 1977). Thus the anti-inflammatory activity of these drugs may be attributed not only to inhibition of further metabolism of arachidonate by the cyclo-oxygenase, but also to direct regulation of the availability of non-esterified fatty acids in those cells and tissues that mobilize arachidonate by deacylation of unsaturated fatty acids from the 2-position of membrane phospholipids.

Kaplan *et al.* (1978) previously described the inhibition of the polymorphonuclear-leucocyte phospholipase A_2 by indomethacin in the presence of 10mM added Ca^{2+} and obtained results comparable with those shown in Fig. 1. In their study indomethacin was equally effective on the membrane-associated enzyme in disrupted leucocytes, and kinetic data suggested non-competitive inhibition presumably due to interaction of drug with enzyme. Our results with the polymorphonuclear-leucocyte, macrophage and platelet phospholipases A_2 stress the importance of low 'free' Ca^{2+} concentrations in both enzyme activation and sensitivity to inhibition by non-steroidal anti-inflammatory agents. Although the mechanism(s) by which Ca^{2+} affects enzymic activity is poorly understood, it has been shown with phospholipases A_2 isolated from the pancreas and polymorphonuclear leucocyte that Ca^{2+} may bind to either enzyme (de Haas *et al.*, 1978) and/or substrate (Franson & Waite, 1978) to induce alterations in surface charge of the substrate or conformation of the enzyme to markedly influence catalysis.

It is evident from the study of a variety of intact cells and tissues, such as the platelet or adrenal gland, that intracellular translocation of Ca^{2+} is critical to biological events, i.e. platelet aggregation and release (Rittenhouse-Simmons *et al.*, 1977; Rittenhouse-Simmons & Deykin, 1978) and adrenal secretion (Jaanus & Rubin, 1971). Modulation of membrane-associated phospholipase A_2 activity by non-steroidal anti-inflammatory or other agents that affect intracellular 'free' Ca^{2+} concentrations may be important to the maintenance of membrane integrity and cell function in both normal and inflammatory processes.

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