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Structural requirements for time-dependent inhibition of prostaglandin biosynthesis by anti-inflammatory drugs

(fatty acid oxygenase/anti-inflammatory agents)

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ABSTRACT **Several anti-inflammatory drugs have been examined for their ability to cause a time-dependent destruction of the fatty acid oxygenase that produces prostaglandins. All of the time-dependent inhibitors contained carboxylic acid moieties, and in addition all but one of the drugs contained a halogen atom. Structural analogs (of the time-dependent inhibitors) lacking halogen atoms were unable to cause a time-dependent destruction of the enzyme.**

The time-dependent property of an inhibitor was totally eliminated after methylation of the carboxylate group. Methylation did not, however, alter the ability of the inhibitors to competitively inhibit the oxygenase.

Thus, the reversible binding of the agents at the active site was not appreciably dependent upon the free carboxyl group, whereas the subsequent irreversible process was.

Since the first report by Vane (1) on the inhibition of the biosynthesis of prostaglandins by aspirin, a wide variety of anti-inflammatory drugs has been shown to inhibit the synthesis of prostaglandins, including pyrazolones, arylacetic acids, fenamates, and salicylates (2). The concept that inflammation could be mediated by controlling prostaglandin synthesis has intensified the search for effective inhibitors of the formation of prostaglandins. Many inhibitors investigated contain hydrophobic portions likely to compete with substrate acid for binding to a hydrophobic site on the fatty acid oxygenase that produces prostaglandins. Data recently summarized (3, 4) support the concept of such a site.

Time-dependent inhibition of the fatty acid oxygenase by aspirin and indomethacin was first reported in 1971 (5). Awareness of this mode of inhibition has added another dimension to our understanding of the control of prostaglandin production, since the potency of an inhibitor may not depend solely on its affinity for binding the oxygenase, but also on the amount of time the drug remains in contact with the enzyme. The mechanism of the time-dependent loss of activity is not well understood, although recent evidence suggests that aspirin may exert its time-dependent effect by acylation of the fatty acid oxygenase (6). The current results make it unlikely that acylation is a general mechanism for all time-dependent inhibitions of anti-inflammatory agents since several time-dependent inhibitors lack an activated acyl group (see Fig. 2).

We initiated the present study to examine common structural features that might be associated with the time-dependent action of the irreversible agents.

MATERIALS AND METHODS

All fatty acids were high purity grade obtained from the Hormel Institute and NuChek Preps. The fatty acids were dissolved at 50–100 mM concentration in benzene containing 5 mM butylated hydroxy toluene (Calbiochem, L.A.) and stored at 0°. Aqueous solutions of 5–50 mM concentration were prepared for use by evaporating aliquots of stock benzene solutions under a stream of nitrogen and suspending, by vigorous shaking, in 0.1 M Tris·HCl pH 8.5. Flurbiprofen, ibuprofen, and an acetone powder preparation of sheep vesicular gland were donated by Upjohn Co. Indomethacin was a gift from the Merck Co. BL-2338 was donated by Dr. Peter Hebborn of Bristol Laboratories. BL-2365 (MJF 10875) was donated by Mead Johnson, and meclofenamic and mefenamic acids were gifts of Parke-Davis. All other chemicals, reagent grade, were obtained from common commercial sources. Deionized, distilled water was used for the preparation of all solutions. Buffer used for all incubations unless otherwise stated consisted of 0.1 M Tris·HCl (pH 8.5) containing 0.67 mM phenol.

The enzyme (see ref. 7 for preparation) was routinely homogenized in 0.1 M Tris·HCl (pH 8.5), containing 0.67 mM phenol, to give a concentration of 20–60 mg/ml in buffer. This was allowed to stand for 30 min at 25° to increase its activity (8).

Determination of Fatty Acid Oxygenase Activity. Oxygen consumption was measured at 30 ± 0.5° on a Yellow Springs Instrument Co. model 53 Oxygen Monitor. Continuous recordings were made on a dual channel recorder with one channel recording the oxygen electrode output and the other channel the same signal after it had been transformed to the first derivative (dO_2/dt). The total final volume in the sample chamber was usually 3.0 ml, and the maximum volume of all additions during an assay was 0.25 ml.

Preparation of Methyl Esters. Inhibitors (10–20 mg) were dissolved in 2 ml of anhydrous methanol in screw top test tubes, and 10 ml of a diethylether solution saturated with diazomethane was added. Tubes were capped tightly and allowed to stand for 15 min at room temperature. Samples were evaporated to dryness and an additional 10 ml of the diazomethane solution was added. After 15 min the samples were again evaporated, and the resulting methyl esters were dissolved in 4 ml of ethanol.

Preincubation Experiments. Inhibitors were examined for their ability to stimulate a time-dependent decay of enzyme activity by preincubating enzyme (60 mg/ml) with various levels of inhibitor in 5 × 75 mm test tubes. After various elapsed times, aliquots (usually 50 μl) were withdrawn and added to reaction chambers containing the substrate, arachidonic acid (50 to 100 μM). Alternately, enzyme and inhibitors were preincubated in the oxygen electrode chambers prior to the addition of the arachidonic acid.

RESULTS

Kinetics of time-dependent irreversible inhibition

Preincubation of anti-inflammatory drugs with the fatty acid oxygenase prior to assay of enzyme activity revealed that certain agents caused increasingly greater inhibition as the time of exposure to the enzyme was increased. This is il-

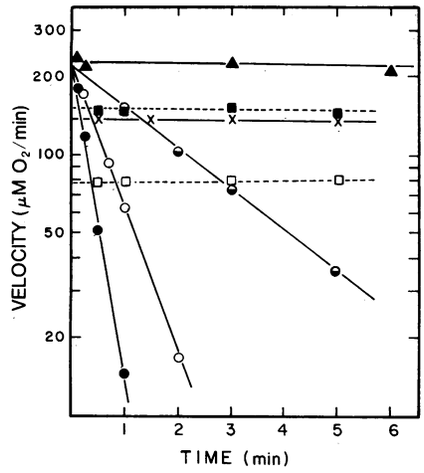

FIG. 1. Logarithm of initial velocity against preincubation time for the oxygenation of 5,8,11,14-eicosatetraenoic acid by vesicular gland preparations preincubated with various levels of flurbiprofen, methyl flurbiprofen, and ibuprofen. Assay procedure is described in *Materials and Methods;* inhibitor and enzyme were preincubated in the oxygen electrode reaction chambers. Levels of inhibitor: ●, 3.0 μM flurbiprofen; ○, 1.0 μM flurbiprofen; ⊖, 0.3 μM flurbiprofen; ■, 10 μM methyl flurbiprofen; □, 50 μM methyl flurbiprofen; ×, 50 μM ibuprofen; ▲, control, no inhibitor.

lustrated in Fig. 1, where the logarithm of remaining enzyme activity is plotted against the preincubation time with the anti-inflammatory agent, flurbiprofen. The kinetics of the inactivation of the enzyme had the properties of a first-order decay. Apparent decay constants (k_{app})* increased with increasing levels of inhibitor so that values of k_{app} divided by the concentration of inhibitor used gave an approximately constant value for each time-dependent inhibitor examined. This constancy is predicted from a simple kinetic model (9) for inhibition by time-dependent agents that act in two steps: a reversible binding followed by an irreversible decay.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k} E^{\dagger};\quad K_I = k_{-1}/k_1$$

This kinetic formulation has the following algebraic relationship in terms of the time-dependent loss of active enzyme:

$$\ln\left(\frac{E_{total} - E^{\dagger}}{E_{total}}\right) = \frac{-k[I]t}{K_I + [I]} = -k_{app}t$$

Thus, the properties described above for the observed apparent decay constants (k_{app}) are compatible with the situation in which the concentration of [I] is less than the value for K_I so that $k_{app}/[I] \simeq k/K_I$. Table 1 lists average k_{app}/I values for several time-dependent inhibitors. Flurbiprofen was the most potent time-dependent agent examined, having a k_{app}/I value approximately 5000 times that found for aspirin, which was the least potent of the time-dependent inhibitors tested.

Inhibition by analogs of time-dependent agents

Certain analogs of time-dependent inhibitors lacked the ability to stimulate enzyme decay. Methyl esters of all of the time-dependent drugs tested were totally devoid of time-dependent properties [even at concentrations 100 times that necessary to observe destruction of enzyme by the corresponding free acids (see Fig. 1)]. Compounds structurally related to the time-dependent inhibitors but lacking halogens were also unable to stimulate a time-dependent enzyme decay (aspirin was the only nonhalogen-containing time-dependent inhibitor examined).

K_I values for the inhibitors listed in Table 1 were measured to determine whether the loss of the time-dependent properties of an agent upon methylation or structural alteration was the result of significantly reduced ability of the drug to bind to oxygenase. To minimize enzyme destruction caused by the time-dependent inhibitors, K_I values were estimated from activity measurements obtained by introducing enzyme as the last addition to reaction chambers already containing various levels of inhibitor and substrate (therefore no preincubation of enzyme and inhibitor in the absence of substrate occurred). The velocity was obtained within 20 sec after enzyme was added. All inhibitors listed in Table 2 had less effect at higher substrate concentrations, and reversible competitive patterns were clearly observed for those inhibitors that were not time-dependent. Although the methyl ester of indomethacin had a K_I value two orders of magnitude lower than that for the free acid, the K_I values of the methyl esters were in most cases similar to those estimated for the corresponding free acids. Thus, the binding of these agents at the active site of the enzyme does not seem to be appreciably diminished by altering the free carboxylate group. Structural analogs that lacked halogens (i.e., BL-2365, ibuprofen, and mefenamic acid) also retained an undiminished ability to competitively inhibit the oxygenase (Table 2) when the carboxylate was altered.

Table 1. Time-dependent loss of oxygenase activity

Inhibitor	Estimated k_{app}/I value (μM^{-1} min^{-1})	
	Free acid	Methyl ester
Indomethacin	0.04 (20)	0 (8)
Aspirin	0.0003 (20)	0 (6)
Flurbiprofen	1.1 (16)	0 (8)
Ibuprofen	0 (8)	0 (8)
Meclofenamic	0.4 (12)	0 (4)
Mefenamic	0 (8)	0 (8)
BL-2338	0.08 (16)	0 (8)
BL-2365	0 (16)	0 (4)

Values in parentheses indicate the number of experiments that were averaged.

Table 2. Inhibition of prostaglandin synthesis by interference with substrate binding

Inhibitor	Estimated K_I values (μM)	
	Free acid	Methyl ester
Indomethacin	100 (3)	1 (3)
Aspirin	14,000 (5)	1,600 (5)
Flurbiprofen	1 (4)	0.5 (4)
Ibuprofen	3 (4)	6 (3)
Meclofenamic	4 (4)	1 (4)
Mefenamic	1 (4)	3 (3)
BL-2338	1 (2)	5 (4)
BL-2365	14 (5)	9 (3)

Values in parentheses indicate the number of experiments that were averaged.

* $k_{app} = 0.693/t$ 1/2, where t 1/2 is the half-life of the enzyme under the experimental condition.

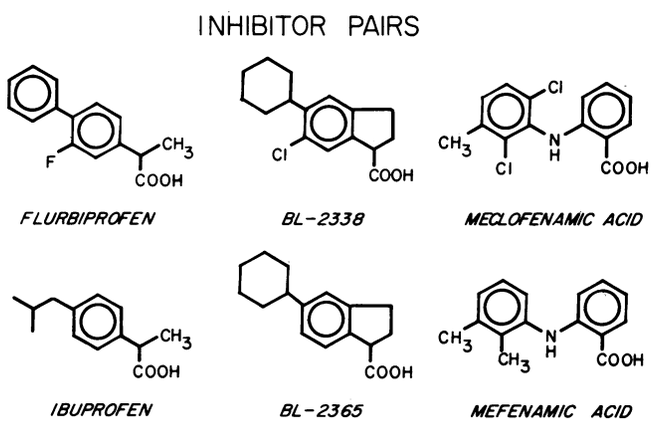

FIG. 2. Structures of anti-inflammatory agents. The upper three compounds cause irreversible loss of oxygenase whereas the lower analogs do not.

DISCUSSION

The difference between reversible inhibitors and irreversible, time-dependent inhibitors has an important consequence in interpreting drug effectiveness. For example, a reversible inhibitor can influence the oxygenase only to the degree that the binding site is occupied by the agent. Dilution of the inhibitor or competitive reversal of its binding by increased substrate allows formation of prostaglandin to proceed. A time-dependent inhibitor, however, may be present at low concentrations that do not occupy more than 10% of the enzyme sites at a given moment, but lead to complete loss of activity with time. In this situation the inhibited system cannot be restored by further dilution of the inhibitor or by adding more substrate. As the results in Fig. 1 indicate, low concentrations of flurbiprofen (0.3 μM) can rapidly become more inhibitory than 50 μM of ibuprofen, which has a similar apparent reversible binding constant.

There are a number of structural similarities between several of the time-dependent agents. Flurbiprofen, BL-2338, and meclofenamic acid all contain aromatic halogens that may be in similar proximities to carboxyl moieties (Fig. 2). In each inhibitor the halogen is α to another ring substituent (phenyl, cyclohexyl, or methyl). Gryglewski (4) has compared a series of monosubstituted *N*-phenylanthranilates as inhibitors of prostaglandin synthesis and indicated that substitution with an apolar radical in position 3 of the phenyl ring produced the most potent inhibitor. The position of the substituent was regarded as important, as was the type of substituent.

The drug, BL-2365, is identical in structure to BL-2338 except a hydrogen is substituted for a chlorine atom, but BL-2365 is totally devoid of time-dependent properties. Similar comparisons can be made between ibuprofen and flurbiprofen, and between mefenamic and meclofenamic acids. In each case, the nonhalogenated derivative lacks the ability to cause a time-dependent destruction of the fatty acid oxygenase. The drug pirprofen has a structure similar to flurbiprofen (with a chlorine α to a pyrroline ring), and as a result of the present study we would predict it to be a time-dependent inhibitor. However, Ku and Wasvary (10) have recently reported that the drug is not a time-dependent inhibitor of the fatty acid oxygenase but instead is a competitive reversible inhibitor. These studies did not include preincubations of the inhibitor with enzyme, which would have more conclusively decided the question of time dependency.

The time-dependent inhibitory effect appears to depend on the combined presence of a halogen and a free carboxylic acid group. Its progressively greater effect with time may indicate a chemical modification of a component at the active site (i.e., through a halogenation or an arylation). Alternately, the destruction could be due to an allosteric structural alteration in the enzyme that follows the binding of the inhibitor. The observation that the acetylcholine receptor affinity for cobra neurotoxin increased by a time-dependent process prompted the suggestion (11) of the possibility of an intramolecular rearrangement. Similarly, a kinetic study (12) on the binding of glucocorticoids to mouse glucocorticoid receptor fit a mechanism involving a two-step binding process; (*i*) a rapidly-formed weak association, followed by (*ii*) a slow conversion to a tight complex. Careful distinction between chemical or allosteric alteration can best be done when the oxygenase is available in a pure form.

The results of the present study clearly indicate that the destructive event can be eliminated by masking the carboxylic acid moiety of the agent. Nevertheless, even though all time-dependent decay activity is abolished, the methyl esters retain their ability to reversibly inhibit the oxygenase. This finding is compatible with the earlier report of Whitehouse and Famaey (13) that conversion of the carboxylate groups to a primary alcohol did not diminish some activities of a number of nonsteroidal anti-inflammatory agents. Those authors, in acknowledging a role for the carboxylate anion of anti-inflammatory drugs, suggested that it might affect biodistribution. The present study indicates that greater inhibitory action may be observed for compounds with the unblocked carboxylate function due to a greater cumulative effect of the irreversible process in contrast to that for the reversible event.

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