Role of Val$^{509}$ in Time-dependent Inhibition of Human Prostaglandin H Synthase-2 Cyclooxygenase Activity by Isoform-selective Agents

Qiupeng Guo†, Lee-Ho Wang‡, Ke-He Ruan§, and Richard J. Kulmacz§

From the †Department of Biology, University of Houston, Houston, Texas 77204 and the ‡Division of Hematology, Department of Internal Medicine, University of Texas Health Science Center, Houston, Texas 77030

Prostaglandin H synthase (PGHS), a key enzyme in prostanooid biosynthesis, exists as two isoforms. PGHS-1 is considered a basal enzyme; PGHS-2 is associated with inflammation and cell proliferation. A number of highly selective inhibitors for PGHS-2 cyclooxygenase activity are known. Inhibition by these agents involves an initial reversible binding, followed by a time-dependent transition to a much higher affinity enzyme-inhibitor complex, making these agents potent and poorly reversible PGHS-2 inhibitors. To investigate the PGHS-2 structural features that influence the time-dependent action of the selective inhibitors, we have constructed a three-dimensional model of human PGHS-2 by homologous modeling. Examination of the PGHS-2 model identified Val$^{509}$ as a cyclooxygenase active site residue, that was not conserved in PGHS-1. Recombinant human PGHS-2 with Val$^{509}$ mutated to either Ile (the corresponding residue in PGHS-1), Ala, Glu, or Lys was expressed and assayed for cyclooxygenase activity and the effect of the mutations on cyclooxygenase activity and on inhibition by four agents reported to be selective for PGHS-2 (NS398, nimesulide, DuP697, and SC58125). All the recombinant proteins were of the expected mass. The mutants exhibited 45–210% of wild-type cyclooxygenase activity, with $K_m$ values for arachidonate of 2.1–7.6 $\mu$m (wild-type PGHS-2, 3.8 $\mu$m), indicating that changes in position 509 had modest effects on cyclooxygenase catalysis. Each of the agents inhibited wild-type PGHS-2 in a time-dependent fashion, and all but nimesulide did the same for the V509A mutant. In contrast, the V509E and V509I PGHS-2 mutants, like recombinant human PGHS-1, did not show time-dependent inhibition with any of the agents, and the V509K mutant responded in a time-dependent manner only to DuP697. Reversible inhibition was still observed with Val$^{509}$ mutants that did not show time-dependent inhibition. Thus, the side chain structure at position 509 markedly influenced the ability of PGHS-2 to undergo the time-dependent transition without removing inhibitor or substrate binding. These results indicate that Val$^{509}$ in PGHS-2 has a major role in the structural transition that underlies time-dependent inhibition by the isoform-selective agents.

Materials—Heme, dimethyl sulfoxide, and $\alpha$-tropophan were from Sigma; Tween 20 was from Pierce; arachidonate was from NuChek Prep, Inc.; and [1-14C]arachidonate (55 mCi/mmol) was from Amersham Corp. Nimesulide was from Cayman Chemical Co. DuP697, SC58125, and NS398 were generous gifts from Drs. Chakk Ramesha (Roche Pharmaceuticals) and Paul J. Marshall (CIBA Pharmaceuticals). Homologous Modeling—The structural model for human PGHS-2 was built from the crystallographic coordinates for ovine PGHS-1 (9). The cylooxygenase activity of prostaglandin H synthase (PGHS) catalyzes the first committed step in prostanooid biosynthesis, the bis-dioxygenation of arachidonic acid to form prostaglandin G2 (1). Two isoforms of PGHS are known, with PGHS-1 generally ascribed housekeeping roles, whereas the strong induction of PGHS-2 by cytokines is believed to be a key part in inflammatory processes (2). Many cyclooxygenase inhibitors have been discovered; the most potent include agents, such as indomethacin, which trigger a time-dependent change in the protein once bound in the cyclooxygenase active site, thus achieving essentially irreversible inhibition without covalent modification of protein or agent (3–5). More recently, a set of time-dependent cyclooxygenase inhibitors with very high selectivity for PGHS-2 has been identified (6–8). Little is known about the nature of the structural change(s) underlying noncovalent time-dependent cyclooxygenase inhibition of either isoform or about the protein structural features that lead to the remarkable specificity of the PGHS-2 inhibitors.

We have constructed a three-dimensional model for human PGHS-2 based on the crystal structure of ovine PGHS-1 (9) and identified Val$^{509}$ in PGHS-2 as one of the few residues in the cyclooxygenase active site that is not conserved in PGHS-1. Recombinant human PGHS-2 was expressed with four Val$^{509}$ mutations to assess their effects on cyclooxygenase activity and on inhibition by agents specific for PGHS-2. Several of the Val$^{509}$ mutations led to a loss of the characteristic time-dependent action of the agents without a large perturbation of substrate or inhibitor binding. The results point to a role for Val$^{509}$ in the time-dependent structural transition, which makes these agents such potent and selective inhibitors of human PGHS-2 cyclooxygenase activity.

EXPERIMENTAL PROCEDURES

*This work was supported in part by Grant GM 52170 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed: Division of Hematology, Dept. of Internal Medicine, MSB 5.284, University of Texas Health Science Center at Houston, 6431 Fannin St., Houston, TX 77030. Tel.: 713-792-5450; Fax: 713-794-4230; E-mail: kulmacz@heart.med.uth.tmc.edu.
the cDNA for human PGHS-1 was described previously (11). A plasmid DNA expression vector containing the human PGHS-2 cDNA was a kind gift from Dr. Timothy Hla (American Red Cross). The 3.1-kilobase EcoRI fragment including the PGHS-2 coding region was isolated from the plasmid and ligated into the parent pSG5 vector (Stratagene). Double digestion with BamHI and XhoI was used to verify the correct orientation and to remove extraneous DNA originating from the original pcDNAI/neo vector. The ends of the resulting 6.0-kilobase fragment were filled with Klenow enzyme and circularized with T4 DNA ligase. The final pSG5 construct containing the whole human PGHS-2 cDNA was excised, pCoxII, was chosen by several restriction digestion, by DNA sequencing, and for expression of cyclooxygenase activity in transfected COS-1 cells.

The pcDNAI plasmid served as the starting point for mutagenesis of Val509. The basic strategy (12) was as follows. Specific mutated primer pairs were designed for each Val509 mutation. Besides introducing the desired amino acid mutation, each specific pair of primers spanned the hA of the Nco site from that in the previous plasmid, allowing rapid verification of successful mutation. General upstream and downstream primers were designed to encompass the PstI restriction site upstream (1400 base pairs from the ATG) and the XhoI site downstream (1881 base pairs from the ATG) of the mutated region in the PCR products. To construct a particular mutant, the specific mutated primers were first amplified by PCR (13) with the corresponding upstream or downstream general primers, using pCoxII as the template. The products from this first round of PCR were purified by agarose gel electrophoresis and then combined for a second round of PCR with only the upstream and downstream primers. The second PCR product was then digested with PstI and XhoI and gel purified into a 464-base pair fragment containing the mutated Val509 codon. This mutated fragment was then inserted into the starting plasmid, which had been digested with the same restriction enzymes to remove the corresponding fragment containing the initial sequence. After ligation, the altered plasmid was transformed into XL1-Blue cells. Mutant colonies were selected, and the mutation was confirmed by restriction digestion and DNA sequencing.

The upstream primer was 5′-CGCTTTATGCTGAAGCCG-3′, and the downstream primer was 5′-GTTGACACTATAAGATTGGG-3′. The specific primers were (with mutated codons in bold and the removed and restored NcoI site underlined): V509I (NcoI site removed, pCoxII used as cloning vector), forward, 5′-GAAACCATGATAAGTTGG-3′; reverse, 5′-CTTCTTCAGTTTACCC-3′; V509K (NcoI site removed, pCoxII used as cloning vector), forward, 5′-GAAACCATGAAAGAACAGTTGG-3′; reverse, 5′-CTTCTTTCATGGTTCCACCC-3′; V509A (NcoI site restored, V509I plasmid used as cloning vector), forward, 5′-GAAACC- CATGGTTTCACC-3′; reverse, 5′-CTTCTTCGAGTTACCC-3′; and V509E (NcoI site restored, V509I plasmid used as cloning vector), forward, 5′-GAAACCAGGAAAGAACAGTTGG-3′; reverse, 5′-CTTCTTTCGAGGTACCC-3′.

Expression of Recombinant Enzymes—Transfection of COS-1 cells used a modification of the procedure described previously (11). Each 100-mm dish of cells received 1.6 mg of DEAE-dextran and 15 μg of plasmid DNA in 5 ml of Dulbecco’s modified Eagle’s medium; 450 μg of chloroquine was added 1 h later in 7 ml of Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum. Cells were cultured for 18 h after transfection and disrupted by sonication, and a membrane fraction was isolated by centrifugation at 40,000 rpm for 60 min in a Beckman Instruments 50Ti rotor. The membranes were resuspended in 0.1 M Tris, pH 7.5, and Tween 20 (10%) was added dropwise to give a final concentration of 1%. After 30 min of gentle shaking on ice, the residual membrane material was pelleted by centrifugation at 10,000 × g for 40 min. The membrane suspension was aliquoted on ice until use. Protein concentrations were determined by a modified Lowry method, which included a trichloroacetic acid precipitation step (14). Recombinant proteins were analyzed by gel electrophoresis and immunoblotting (11).

Cyclooxygenase activity was assayed with an oxygen electrode essentially as described previously for PGHS-1 (15). The standard reaction mixture contained 0.1 M potassium phosphate, pH 7.2, 100 μM arachidonate, 5 mM heme, and 5 mM d-tryptophan. This level of tryptophan was found to give maximal stimulation of the cyclooxygenase activity of PGHS-2. One unit of cyclooxygenase activity resulted in a maximal rate of oxygen consumption of 1 nmol of O2/min.

For determination of affinity for fatty acid substrate, fixed amounts of the solubilized recombinant enzyme were assayed in the standard reaction mixture containing 1-100 μM arachidonate. The arachidonate stock solutions were standardized by measurement of total oxygen consumption when aliquots were reacted with excess ovine PGHS-1, assuming 2 mol of O2/mol fatty acid. Velocity data were fitted to the Michaelis-Menten equation using a nonlinear least squares routine (Kaleidagraph software) to estimate the Km values.

The arachidonate metabolite profile was examined by incubating aliquots of the detergent-solubilized recombinant enzymes with [14C]-arachidonate (20 μM) in 0.1 M potassium phosphate, pH 7.2, 10 μM heme, and 5 mM d-tryptophan at room temperature for 2 min. Reactions were stopped by extraction with ice-cold diethyl ether-methanol-1 M citric acid (30:41). After drying over anhydrous sodium sulfate, the lipid products were analyzed by thin layer chromatography at 0 °C on Whatman LKE silica gel plates with diethyl ether-heptane-acetic acid (85: 15:0.1) as the solvent. The separated bands were visualized by autoradiography.

To assess susceptibility of recombinant PGHS to time-dependent inhibition, detergent-solubilized enzyme from transfected COS-1 cell membranes (100–200 μg of protein; 20–40 units) was added to a reaction cuvette containing 3 ml of 0.1 M potassium phosphate, pH 7.2, 5 mM d-tryptophan, and 10 μM heme. After incubation for 1 min to reconstitute the holoenzyme, inhibitor was added, and the incubation continued for the desired time before the surviving cyclooxygenase activity was assayed by injection of 100 μM arachidonate. Stock solutions of inhibitors were prepared in ethanol, dimethyl sulfoxide, or acetone; control incubations included the solvent without inhibitor. Time-dependent cyclooxygenase inhibition is characterized by integration of prolonged progression of inhibition (3). Recombinant PGHS-2 was first tested at several levels of each inhibitor to establish a concentration at which time-dependent inhibition clearly was observed. The inhibitor was then used at levels up to 10-fold higher to examine its effects on the PGHS-2 mutants and PGHS-1. In this manner, time-dependent inhibition of the PGHS-2 mutants could be detected even if the affinity for the inhibitor was considerably lower than that for wild-type PGHS-2.

RESULTS AND DISCUSSION

Structural Model of the PGHS-2 Active Site—A high resolution crystallographic structure for human PGHS-2 is not yet available. However, a three-dimensional structure has been determined for ovine PGHS-1 complexed with flurbiprofen, a cyclooxygenase inhibitor (9). Human PGHS-2 shares about 60% overall amino acid identity with human and ovine PGHS-1 and much higher conservation in residues identified as being required for activity in PGHS-1 (16). The two purified isoforms have comparable cyclooxygenase and peroxidase activities (6, 17, 18). This high level of sequence conservation and functional similarity indicates that the positions of the main chain atoms in PGHS-2 are likely to be relatively close to those in PGHS-1 (19). Also, flurbiprofen has comparable inhibitory potency toward the two human PGHS isoforms (20). Thus, the PGHS-1 crystal structure is a reasonable template for construction of a structural model of human PGHS-2 using the homologous modeling approach (10).

The ovine PGHS-1 and human PGHS-2 sequences align very well, with disjunctions only at the amino and carboxyl termini (21). These matched segments were not resolved in the PGHS-1 crystal structure (9), so there were no gaps to be bridged when the PGHS-2 main chain was superimposed on that of PGHS-1 in the modeling process. The cyclooxygenase active site regions of the resulting PGHS-2 structural model, as well as the PGHS-1 template, are shown in Fig. 1. Twelve residues were found within 4.5 angstroms of flurbiprofen bound in the PGHS-1 cyclooxygenase active site. Val116, Arg230, Val349, Leu352, Ser353, Tyr355, Tyr359, Ile362, Gly366, Ala372, Ser390, and Leu391. It was remarkable that all but one of these residues in the immediate vicinity of the inhibitor are conserved in PGHS-2 and are predicted to adopt comparable positions (Fig. 1). This is consistent with the roughly similar action of flurbiprofen on the two PGHS isoforms (20).

The one major difference in the two active site models is Val505 of PGHS-2, which corresponds to Ile362 in PGHS-1 (Fig. 1). This residue sits on the opposite wall of the cyclooxygenase channel from Ser356 (Ser353 in PGHS-1), the residue acetylated by aspirin (22–24). The shorter side chain of Val505 in PGHS-2
provides a potential basis for discrimination between the isoforms in interactions with selective inhibitors such as NS398 (25) and identified Val509 as a mutagenic target. Accordingly, recombinant human PGHS-2 proteins with isoleucine, alanine, glutamate, or lysine at position 509 were expressed by transient transfection of COS-1 cells, and the properties of the mutant proteins compared with those of wild-type human PGHS-2 and PGHS-1 (see "Experimental Procedures").

Expression of the Val509 PGHS-2 Mutants—Immunoblot analysis of the recombinant PGHS-2 proteins (Fig. 2) revealed major bands with $M_r$ values of about 75,000, demonstrating that each of the mutants had the mass expected for the full length monomer (16). Analysis of COS-1 cells expressing recombinant human PGHS-1 with antibody raised against ovine PGHS-1 also detected a single major band near $M_r$ 75,000 (data not shown). The V509I and V509E mutants were expressed at somewhat higher levels, and the V509A and V509K mutants at somewhat lower levels, than PGHS-2 itself (Fig. 2).

Each of the PGHS-2 mutants was found to have considerable cyclooxygenase activity (Table I). The specific activities were highest for the V509I and V509E mutants and lowest for the V509K and V509A mutants. The similar relative ranking of protein expression (Fig. 2) and specific activity (Table I) indicates that the mutations at position 509 did not grossly alter the cyclooxygenase catalytic competence of PGHS-2. The metabolite profiles of the recombinant enzymes were analyzed using $[^{14}C]$arachidonate, as described under "Experimental Procedures." Wild-type PGHS-1 and PGHS-2 and the PGHS-2 Val509 mutants each displayed comparable metabolite patterns (data not shown), much like that observed with purified recombinant PGHS-2 (26). This indicates that the Val509 mutations did not result in marked changes of product specificity.

The $K_m$ values of the mutants for arachidonate were measured to probe for changes in substrate binding (Table I). The $K_m$ value for recombinant human PGHS-1 was 2.5 $\mu$m (Table I), similar to reported values of 4.5–5.6 $\mu$m (6, 20). Recombinant PGHS-2 had a $K_m$ near 4 $\mu$m, comparable with the values of 0.9–5.4 $\mu$m reported previously (6, 17, 20). The $K_m$ value was

**Fig. 1.** Comparison of cyclooxygenase active sites in ovine PGHS-1 and human PGHS-2. A, stereogram of residues in the vicinity of bound flurbiprofen in the ovine PGHS-1 A subunit. The side chains of the 12 residues within 4.5 angstroms of are shown. For clarity, only Val116, Val349, Tyr355, Tyr385, Ile523, and Ser530 have been labeled. Coordinates are those determined for the inhibitor-enzyme complex (9). In this orientation, heme is above Tyr385, and the cyclooxygenase channel opening lies below Tyr355. B, stereogram of active site residues in structural model of human PGHS-2. The PGHS-2 residues shown correspond to those near flurbiprofen in the PGHS-1 crystal structure (A). The PGHS-2 coordinates were determined by homology modeling based on the PGHS-1 crystal structure, as described under "Experimental Procedures."
little changed in the V509A, V509E, and V509I mutants. Only with the positively charged V509K mutation, with a $K_m$ value near 8 $\mu$m, was there any indication of altered fatty acid binding. Thus, residue 509 in PGHS-2 does not appear to be a major determinant of cyclooxygenase substrate binding. It may be that the predicted proximity of Val509 to the position of bound flurbiprofen in the crystal structure does not accurately reflect its relationship to bound arachidonate in the native enzyme.

Inhibitor Characteristics with the Val

509 Mutants—The effects of the mutations of Val509 on inhibitor interactions were examined with NS398, nimesulide, DuP697, and SC58125 (see Fig. 3 for structures), agents expected to be selective time-dependent inhibitors of PGHS-2 (6, 7, 25, 27); the results with NS398 are shown in Fig. 4. With wild-type PGHS-2, the cyclooxygenase inhibition was proportional to the NS398 concentration and the length of the preincubation period (Fig. 4A). This is the expected behavior of an inhibitor that first binds the cyclooxygenase rapidly and reversibly, with the enzyme-inhibitor complex then undergoing some slower transition to form a tighter complex (3, 28). This progression from a weaker (EI) to a tighter (EI') complex is indicated in Equation 1, with the initial, readily reversible binding governed by $K_{eq1}$, and the poorly reversible transition to the tighter complex governed by $K_{eq2}$.

$$K_{eq1} = E + I \rightleftharpoons EI \rightleftharpoons EI' \quad \text{(Eq. 1)}$$

The inhibitory pattern for the V509A mutant and NS398 was similar to that seen for wild-type PGHS-2 (Fig. 4, A and B), indicating that this PGHS-2 mutant also was susceptible to time-dependent inhibition by NS398. In contrast, cyclooxygenase inhibition in the V509E, V509I, and V509K PGHS-2 mutants and in PGHS-1 was independent of the length of preincubation, even at NS398 levels 10-fold higher than that producing time-dependent inhibition in PGHS-2 (Fig. 4). Inhibition of the V509E, V509I, and V509K mutants did increase...
with the NS398 concentration (Fig. 4, C–E), confirming that NS398 was able to reversibly inhibit these PGHS-2 mutants. Thus, the time-dependent action of NS398 was blocked in these mutants, although the agent was still able to bind.

The three other isoform-specific agents were also characterized for their inhibitory effects on the recombinant enzymes, using the approach described for NS398; the results are summarized in Table II. With recombinant PGHS-2, preincubation with nimesulide, DuP697, or SC58125 produced a progressive inhibition of the cyclooxygenase, following the pattern seen with NS398 in Fig. 4. Thus, all four agents can be classed as time-dependent inhibitors for wild-type PGHS-2 (Table II). For recombinant PGHS-1, on the other hand, preincubation did not increase the potency of any of the agents, even at levels at least 6-fold higher than those that showed progressive inhibition of PGHS-2. The present results thus confirm previous reports characterizing these agents as selective time-dependent inhibitors for PGHS-2 (6–8). The Val509 mutations in PGHS-2 produced varied responses to the inhibitors (Table II). The V509A mutant was susceptible to time-dependent inhibition by all of the agents tested except nimesulide, whereas only DuP697 produced time-dependent inhibition in the V509K mutant, and none of the agents produced time-dependent inhibition in the V509E and V509I mutants. The V509I and V509E mutations thus induced a qualitative response to these inhibitors most like that seen with PGHS-1, whereas the V509A mutant behaved much like PGHS-2. The differences between the behavior of wild-type PGHS-2 and the V509I mutant demonstrate that even subtle changes at position 509 influenced the susceptibility to time-dependent inhibition. The small number of mutants tested make generalizations difficult, but increases in bulk or change (Val → Ile, Glu, or Lys) decreased the tendency to time-dependent inhibition, whereas a decrease in bulk (Val → Ala) had little effect.

It is important to note that each of the Val509 mutants was obviously inhibited by the selective agents, even in the absence of a time-dependent effect (Fig. 4 and Table II). For example, the cyclooxygenase activity of the Val509 mutants was decreased by 24–57% at 25 μM nimesulide. This behavior is characteristic of reversible inhibition (3) and confirms the competence of the mutants for initial binding of the agents in the cyclooxygenase active site (governed by K<sub>i</sub> in Equation 1). Precise evaluation of K<sub>i</sub> values was not practical because of the relatively low control activity levels and the complex nature of the enzyme preparations. However, rough estimates of reversible inhibition can be obtained for time-dependent inhibitors by extrapolating the surviving cyclooxygenase activity data (as for NS398 in Fig. 4, A and B) back to zero time. These values can be compared with the level of reversible inhibition seen for other mutants with the same concentration of agent. In this manner, reversible inhibition by 5 μM NS398 can be estimated from the data in Fig. 4 to be about 35% for PGHS-2, 30% for V509A, 10% for V509E, V509I, and V509K, and less than 2% for PGHS-1. Considering that the inhibitor is competing with 100 μM substrate for reversible binding, the observed levels of inhibition indicate that the Val509 mutants retain a strong affinity for inhibitor, certainly much more than with PGHS-1. The modest effects of the Val509 mutations on inhibitor binding are quite consistent with those on substrate binding (Table I). The absence of time-dependent action in several of the Val509 mutants despite inhibitor binding competence (Fig. 4 and Table II) indicates that the inhibitor–enzyme complex does not undergo the further transition to the tighter complex (governed by K<sub>e</sub> in Equation 1). Val509 thus seems to have a major role, not in the initial inhibitor binding, but rather in the subsequent structural transformation.

The nature of the structural transformation induced by non-covalent time-dependent agents (E1 → E′ in Equation 1) is not known for either isoform. In PGHS-1, bound inhibitor blocks proteolytic attack near Arg<sup>777</sup>, in a distant part of the protein (9, 29). This long-range effect is not easily rationalized from the crystal structure of inhibited PGHS-1. Inhibitor binding also stabilizes PGHS-1 to extremes of temperature and pH (30). These observations suggest that local conformational changes in the cyclooxygenase active site can trigger a general increase in rigidity of the protein. In this interpretation, a particular combination of inhibitor structure and accessible protein motion in the cyclooxygenase site would give rise to the time-dependent transition of the inhibitor–enzyme complex.

Mutation of Arg<sup>320</sup>, the putative carboxyl group ligand in the PGHS-1 cyclooxygenase site, does diminish susceptibility to the time-dependent action of acidic inhibitors (31, 32). However, the Arg<sup>320</sup> mutant had a K<sub>i</sub> value for fatty acid, at least 100-fold higher than that of wild-type PGHS-1, so the decreased time-dependent inhibition in the mutants is probably due to a very large drop in affinity for the acidic inhibitors. This situation contrasts with the Val509 mutants of PGHS-2, which retained considerable affinity for the inhibitors (Table II). The present results with the Val509 mutants of PGHS-2 are thus the first to identify, in either isoform, a protein structural feature involved in the transition from an initial enzyme-inhibitor complex to a very high affinity complex. This represents an important step toward characterizing the structural transition in the enzyme–inhibitor complex, which is the hallmark of the time-dependent agents and makes them such potent and pharmacologically useful cyclooxygenase inhibitors.
REFERENCES
1. Samuelsson, B., Goldyne, M., Granstrom, E., Hamberg, M., Hammarstrom, S., and Malmsten, C. (1978) Annu. Rev. Biochem. 47, 997–1029.
2. Herschman, H. R. (1996) Biochim. Biophys. Acta 1299, 125–140.
3. Rone, L. H., and Lands, W. E. M. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 4863–4865.
4. Stanford, N., Roth, G. J., Shen, T. Y., and Majerus, P. W. (1977) Prostaglandins 13, 669–675.
5. Kulmacz, R. J., and Lands, W. E. M. (1985) J. Biol. Chem. 260, 12572–12578.
6. Barnett, J., Chow, J., Ives, D., Chiou, M., Mackenzie, R., Osan, E., Nguyen, B., Tseng, S., Bach, C., Fréres, J., Chan, H., Sigal, E., and Ramesha, Chakk. (1994) Biochim. Biophys. Acta 1209, 130–139.
7. Ouellet, M., and Percival, M. D. (1995) Biochem. J. 306, 247–251.
8. Seibert, K., Zhang, Y., Leahy, K., Hauser, S., Masferrer, J., Perkins, W., Lee, L., and Isakson, P. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12013–12017.
9. Callan, O. H., So, O.-Y., and Swinney, D. C. (1996) J. Biol. Chem. 271, 3548–3554.
10. Kulmacz, R. J. (1989) J. Biol. Chem. 264, 14136–14144.
11. Mizuno, K., Yamamoto, S., and Lands, W. E. M. (1982) Prostaglandins 23, 743–757.
12. Mancini, J. A., Riendeau, D., Falgueyret, J.-P., Vickers, P. J., and O'Neill, G. P. (1995) J. Biol. Chem. 270, 29372–29377.
13. Bhattacharyya, D. K., Lecomte, M., Rieke, C. J., Garavito, R. M., and Smith, W. L. (1996) J. Biol. Chem. 271, 2179–2184.
14. Barnes, W. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2216–2220.
15. Peterson, G. L. (1983) Methods Enzymol. 91, 95–119.
16. Hla, T., and Neilson, K. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7384–7388.