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Mechanism of Selective Inhibition of Human Prostaglandin G/H Synthase-1 and -2 in Intact Cells

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ABSTRACT. Selective inhibitors of prostaglandin synthase-2 (PGHS-2) possess potent anti-inflammatory, antipyretic, and analgesic properties but demonstrate reduced side-effects (e.g. gastrotoxicity) when compared with nonselective inhibitors of PGHS-1 and -2. We investigated the mechanism of the differential inhibition of human PGHS-1 (hPGHS-1) and -2 (hPGHS-2) in intact cells by nonsteroidal anti-inflammatory drugs (NSAIDs) and examined factors that contribute to the increased potency of PGHS inhibitors observed in intact cells versus cell-free systems. In intact Chinese hamster ovary (CHO) cell lines stably expressing the hPGHS isozymes, both PGHS isoforms exhibited the same affinity for arachidonic acid. Exogenous and endogenous arachidonic acid were used as substrates by both CHO [hPGHS-1] and CHO [hPGHS-2] cell lines. However, differences were observed in the ability of the hPGHS isoforms to utilize endogenous arachidonic acid released intracellularly following calcium ionophore stimulation or released by human cytosolic phospholipase A_2 transiently expressed in the cells. Cell-based screening of PGHS inhibitors demonstrated that the selectivities and potencies of PGHS inhibitors determined using intact cells are affected by substrate concentration and differ from that determined in cell-free microsomal or purified enzyme preparations of PGHS isozymes. The mechanism of inhibition of PGHS isozymes by NSAIDs in intact cells involved a difference in their time-dependent inhibition. Indomethacin displayed time-dependent inhibition of cellular hPGHS-1 and -2. In contrast, the selective PGHS-2 inhibitor NS-398 exhibited time-independent inhibition of hPGHS-1 but time-dependent inhibition of hPGHS-2 in intact cells. Reversible inhibition of cellular CHO [hPGHS-1] and CHO [hPGHS-2] was observed with the nonselective NSAIDs ibuprofen and indomethacin, whereas inhibition by the selective PGHS-2 inhibitor DuP-697 was reversible against hPGHS-1 but irreversible against hPGHS-2. BIOCHEM PHARMACOL 52;7:1113–1125, 1996.

KEY WORDS. cyclooxygenase; prostaglandin G/H synthase; prostaglandin; NSAID; inflammation; phospholipase A_2

Most anti-inflammatory, antipyretic, and analgesic effects of NSAIDs† result from the inhibition of production of prostanoids from arachidonic acid [1]. The target of NSAID action is PGHS, the key rate-limiting enzyme in the production of prostanoids. This enzyme catalyzes the conversion of arachidonic acid to PGH_2 via a two-step reaction mechanism involving sequential cyclooxygenase and peroxidase activities [2]. Two isoforms of human PGHS, designated hPGHS-1 and hPGHS-2, have been identified [3, 4]. The human PGHS isoforms share 60% amino acid sequence identity, a common reaction mechanism, and similar affinities for arachidonic acid [2–5]. However, the two enzymes differ markedly in their patterns of expression and sensitivities to NSAID inhibition. PGHS-1 is normally expressed constitutively in many different cells and tissues in contrast to the highly regulated and inducible expression of PGHS-2 in a subset of cell types [3, 4, 6, 7]. Although many standard NSAIDs exhibit nonselective inhibition of both PGHS isoforms [8–13], new compounds, such as NS-398, DuP-697, L-745,337, and SC-58125 have been shown recently to exhibit potent and selective inhibition of hPGHS-2 versus hPGHS-1 [14–20].

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† *Abbreviations:* CHO, Chinese hamster ovary; CHO [hPGHS-1], Chinese hamster ovary cells stably transfected with human prostaglandin G/H synthase-1; CHO [hPGHS-2], Chinese hamster ovary cells stably transfected with human prostaglandin G/H synthase-2; $cPLA_2$, cytosolic phospholipase A_2; EIA, enzyme-linked immunoassay; FBS, fetal bovine serum; GMEM-S, Glasgow modified Eagle's medium; HBSS, Hanks' balanced salt solution; hPGHS-1, human prostaglandin G/H synthase-1; hPGHS-2, human prostaglandin G/H synthase-2; MSX, L-methionine sulfoximine; NSAID, nonsteroidal anti-inflammatory drug; PG, prostaglandin; and PGHS, prostaglandin G/H synthase.

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The discovery of two PGHS isoforms and the development of selective PGHS inhibitors will permit clinical investigation of whether the therapeutic effects of NSAIDs result from their inhibition of one or both of the PGHS isoforms. The current hypothesis is that prostaglandins produced by PGHS-2 play a central role in mediating the onset of the inflammatory response, whereas PGHS-1 is involved in prostanoid production required for the majority of physiological functions [8–15, 18–20]. The undesirable side-effects of NSAIDs (e.g. gastric ulceration) are presumed to result largely from mechanism-based events due to the nonselective inhibition of PGHS-1. Recent studies of PGHS inhibitors have demonstrated that selective PGHS-2 inhibitors are as effective as nonselective PGHS inhibitors in limiting adverse effects mediated by prostanoids in several *in vivo* models of inflammation, pyresis, and hyperalgesia [14, 15, 18–20]. Most importantly, the selective PGHS-2 inhibitors display a substantially improved gastrotoxicity profile over nonselective PGHS inhibitors [14, 15, 18–20].

A number of studies have reported the characterization of PGHS inhibitors using PGHS isoforms isolated from recombinant and natural sources [8–13, 21–24]. Cell-free preparations of PGHS isoforms have been crucial in establishing at least three mechanisms of PGHS inhibition and the basis for the selective inhibition of PGHS-2 [1, 16, 17, 25]. In one type of NSAID inhibition, aspirin irreversibly blocks PGH_2 synthesis in both PGHS-1 and -2 via an acetylation of a specific serine residue in the cyclooxygenase active site. In a second type of inhibition, NSAIDs such as indomethacin act as time-dependent inhibitors of both PGHS-1 and -2. Finally, in a third type of inhibition, the PGHS-2 selective inhibitors NS-398 and DuP-697 act as time-independent, reversible inhibitors of PGHS-1 but as time-dependent, irreversible inhibitors of PGHS-2 [16, 17]. However, the mechanism of selective inhibition of PGHS isoforms in intact cells has not yet been investigated.

Although crude and purified preparations of PGHS isozymes have been used in characterizing inhibitors, several studies indicate that for unknown reasons the potency and selectivity of inhibitors determined using intact cells expressing PGHS isozymes differ from values established using cell-free PGHS preparations [1, 12, 13, 22]. For example, ibuprofen is approximately 10-fold more potent against PGHS-2 in intact cells than against PGHS-2 activity in broken cells [13]. Previous investigations have used a variety of cell types from different species as sources of the PGHS isoforms [13, 22, 24]. To accurately compare the mechanism of inhibition of PGHS isozymes in intact cells, we established a system in which the human isoforms were compared in the same cellular background under controlled conditions of PGHS expression.

In this study we show, using intact CHO cell lines stably expressing the human PGHS isozymes, that the mechanism of selective NSAID-mediated inhibition of the PGHS isoforms involves a difference in their sensitivity to time-dependent inhibition. Selective PGHS-2 inhibition by compounds such as DuP-697 and NS-398 in intact cells involves a time-dependent, irreversible inhibition of hPGHS-2 and a time-independent, reversible inhibition of hPGHS-1. Furthermore, although the hPGHS isoforms display the same K_m for arachidonic acid in cells, these enzymes differentially utilize arachidonic acid released (a) intracellularly following calcium ionophore stimulation, and (b) by $cPLA_2$ transiently expressed in cells following transfection. The potencies of inhibitors of PGHS isozymes were greater in intact cells than in cell-free systems; however, the potency and selectivity of certain inhibitors were affected by the concentration of arachidonic acid.

MATERIALS AND METHODS

Materials

Diclofenac (2-[2,6-dichlorophenyl)amino]benzeneacetic acid), acetaminophen (*N*-[4-hydroxyphenyl]acetamide), niflumic acid (2-[[3-(trifluoromethyl)phenyl]-amino]-3-pyridinecarboxylic acid), salicyclic acid (2-hydroxybenzoic acid), ibuprofen (α-methyl-4-[2-methylpropyl]benzeneacetic acid), and flurbiprofen ([±]-2-fluoro-α-methyl-4-biphenylacetic acid) were purchased from Sigma (St. Louis, MO). Aspirin (2-acetoxybenzoic acid), indomethacin (1-[*p*-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid), and peroxide-free arachidonic acid were purchased from Cayman (Ann Arbor, MI). Toradol [5-benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-carboxylic acid] was purchased from Syntex (Palo Alto, CA). DuP-697 (5-bromo-2[4-fluorophenyl]-3-[4-methylsulfonylgphenyl]-thiophene) [15], NS-398 (*N*-[2-cyclohexyloxy-4-nitrophenyl]methanesulfonamide) [14], and L-745,337 (5-methanesulfonamido-6-[2,4-difluorothiophenyl]-1-indanone) [20] were synthesized by the Department of Medicinal Chemistry, Merck Frosst.

Construction of hPGHS-1 and -2 Cell Lines

The human cDNAs used for these experiments have been described previously [11]. The parental hPGHS-1 and -2 cDNAs were subcloned into the multiple cloning site of the eucaryotic expression vector pcDNA3 (Invitrogen, San Diego, CA) to yield pcDNA3::hPGHS-1 and pcDNA3::hPGHS-2, respectively. The hPGHS-2 cDNA was also subcloned into the eukaryotic expression vector pEE14 [26, 27] to yield pEE14::hPGHS-2.

Stable expression of hPGHS-1 and hPGHS-2 was achieved by calcium phosphate-mediated transfection of the respective pcDNA3 or pEE14 plasmid constructs into Chinese hamster ovary cells (CHO-K1; American Type Culture Collection CCL-61), followed by clonal selection [28]. Cells were maintained in culture for 48 hr after transfection and then trypsinized, diluted, and replated in the presence of a selective agent. For cells transfected with the pcDNA3 constructs, cell lines were grown and selected using 500 μg/mL G418 (Gibco-BRL, Grand Island, NY) in HyQ-CCM 5 medium (HyClone Laboratories, Logan, UT)

supplemented with 2% heat-inactivated FBS, 100 μg/mL streptomycin, 100 units/mL penicillin, and 100 μg/mL gentamicin. Cell lines transfected with pEE14::hPGHS-2 were selected using 25 μM MSX in GMEM-S without tryptose phosphate broth and without glutamine and supplemented with 10% dialyzed FBS, 100 μg/mL streptomycin, 100 units/mL penicillin, 100 μg/mL gentamicin, 1.0 mM sodium pyruvate, 60 μg/mL each of L-glutamate and L-asparagine, 7 μg/mL each of ribonucleosides (adenosine, cytidine, guanosine, and uridine), and nonessential amino acids. Individual colonies were isolated by limiting dilution, then expanded, and clonal CHO-K1 cell lines expressing PGHS activity were identified by PGHS activity assays as described below. Cell lines were grown as monolayer cultures in a humidified environment with 6% CO_2 at 37°. In the MSX expression system, gene amplification was obtained by further selection of clonal cell lines resistant to 50, 150, and 300 μM MSX.

The expression construct containing the human $cPLA_2$ cDNA in the plasmid pSG5, and designated pSG5::$hcPLA_2$, has been described previously [29]. Transient expression of $hcPLA_2$ in the CHO [hPGHS] stable cell lines was achieved by transfection of pSG5::$hcPLA_2$ by cationic liposome-mediated transfer of DNA using Lipofectamine reagent, according to the manufacturer's instructions (Gibco-BRL). Briefly, for each 175 cm^2 flask containing ~8 × 10^6 cells, a transfection solution of 35 μg of pSG5::$hcPLA_2$ or control plasmid was mixed with 110 μL of Lipofectamine in 20 mL of serum-free Optimem medium (Gibco-BRL). The cells were maintained in culture for 48 hr after transfection before being harvested by trypsinization. Harvested cells were then recovered by centrifugation at 300 *g* for 5 min, washed once with medium containing 10% FBS, washed twice in HBSS, and resuspended in 1.5 × 10^6 cells/mL in HBSS.

Determination of PGHS Activity

PGHS activity in intact cells was determined by incubation of cells with 0.5 or 10 μM arachidonic acid and measuring the production of PGE_2 using an EIA for PGE_2 (Correlate PGE_2 EIA kit, Assay Designs, Inc., Ann Arbor, MI). Cells were prepared by trypsinization of adherent cultures, harvested by centrifugation (300 *g*, 5 min), washed once in HEPES-buffered HBSS, and resuspended in HBSS at a cell concentration of 1–1.5 × 10^6 cells/mL. PGHS inhibitors were dissolved in DMSO and tested at eight concentrations in duplicate using 3-fold serial dilutions in DMSO of the highest drug concentration. Cells (0.2–0.3 × 10^6 cells in 200 μL) were preincubated with 3 μL of the test drug or DMSO vehicle for 15 min at 37°. Then cells were challenged for 15 min at 37° in the presence or absence of drug with peroxide-free arachidonic acid, added to a final concentration of 0.5 or 10 μM from a 1 mM working stock solution dissolved in 10% ethanol/90% HBSS. Each experiment included positive control reactions (no drug addition, + DMSO preincubation, + arachidonic acid challenge) and negative control reactions (no drug addition, + DMSO preincubation, no arachidonic acid challenge). The reaction was terminated by the addition of 10 μL of 1 N HCl followed by neutralization with 20 μL of 0.5 N NaOH. The samples were centrifuged at 300 *g* at 4° for 5 min, and an aliquot of the clarified supernatant was appropriately diluted for the determination of PGE_2 levels by EIA. PGHS activity in the absence of test compounds was determined as the difference in PGE_2 levels of cells challenged with arachidonic acid versus the PGE_2 levels in control cells challenged with ethanol vehicle. Inhibition of PGE_2 synthesis by test compounds was calculated as a percentage of the activity in the presence of drug versus the activity in the positive control samples.

Measurement of PGHS Activity Using a Fluorescent Reducing Agent

CHO cells expressing hPGHS-1 or hPGHS-2 were trypsinized and resuspended in HBSS containing 15 mM HEPES at a final concentration of 1.0 × 10^6 cells/mL. Cells were incubated for 1 hr at 37° in the presence of 5 μM dichlorodihydrofluorescein diacetate bisacetoxymethyl ester [30]. After the incubation period, cells were pelleted by centrifugation and resuspended in HBSS containing HEPES as a washing step. The reaction was initiated by the addition of arachidonic acid as a 100-fold concentrated solution in ethanol. The concentration of ethanol never exceeded 1% of the final volume. The fluorescent intensity was measured at an excitation wavelength of 495 nm (slit width of 5 nm) and an emission wavelength of 525 nm (slit width of 10–20 nm) using a spectrofluorometer (Perkin Elmer L550B). The velocity of the reaction was measured from the initial change of the fluorescent intensity which was evaluated by linear fit of the first 30 sec of the reaction. K_m determinations were made by nonlinear fit of the experimental values to the Michaelis–Menten equation using computer software (Sigmaplot for Windows version 2.0).

Inhibitor Reversibility in Whole Cells

CHO cells stably expressing hPGHS-1 or hPGHS-2 were incubated for 15 min at 37° in HEPES-buffered HBSS containing 20 μM cycloheximide and one of the following inhibitors: indomethacin (300 nM final concentration); ibuprofen (100 μM final concentration); DuP-697 (18 μM final concentration for CHO [hPGHS-1] and 5.4 μM final concentration for CHO [$hPGHS\text{-}2_{int}$]). Following this inhibitor preincubation, aliquots of inhibitor- or DMSO-treated cells were challenged with 10 μM arachidonic acid for 15 min at 37° to determine PGE_2 synthesis at a time point defined as t = 0. The remainder of inhibitor- or DMSO-treated cells were pelleted at 300 *g* for 5 min, washed three times in HBSS, and then resuspended in HEPES-buffered HBSS containing 20 μM cycloheximide. Cells were plated in 24-well dishes and incubated in a humidified environment of 5% CO_2 at 37°. At each subse-

quent time point, cells were incubated in the presence of 10 μM arachidonic acid for 15 min at 37°, and the reaction was terminated by addition of HCl. Quantitation of PGE_2 production was determined by specific PGE_2 EIA as described above.

Immunoblot Analysis

Protein samples from cells (equal cell numbers for each cell type) and two concentrations of purified protein standards (PGHS-1, PGHS-2, or $cPLA_2$) were resolved by SDS–PAGE followed by electrophoretic transfer to nitrocellulose membranes as previously described [31]. Nonspecific sites were blocked with 5% skim milk powder in TTBS (20 mM Tris, pH 7.5, 0.5 M NaCl, 0.1% Tween 20) for 1 hr at 23°. The filters were then incubated for 2 hr at 23° with a dilution of the primary antiserum in 5% milk powder/TTBS. The primary antisera of PGHS-1 and -2 and $cPLA_2$, which have been described previously [29, 31], were used at final dilutions of 1:5000, 1:5000, and 1:1000, respectively. The secondary horseradish peroxidase-linked anti-rabbit IgG antibody (Amersham Life Sciences, Oakville, Ontario, Canada) was used at a dilution of 1:3000 in 5% milk/TTBS. Immunodetection was performed using enhanced chemiluminescence according to the manufacturer's instructions (Amersham). All immunoblot analyses were carried out simultaneously.

RESULTS

Stable Expression of hPGHS Isozymes in CHO Cells

Stable CHO cell lines expressing hPGHS isoforms were developed using the chromosomally integrating expression vectors pcDNA3 and pEE14, and screened by determining PGE_2 production after challenge with 10 μM arachidonic acid. One cell line designated CHO [hPGHS-1] exhibited a high level of PGE_2 synthesis (20–40 ng $PGE_2/10^6$ cells; Table 1) that remained stable even after 4 months of continuous suspension culture in serum-free medium. Of the CHO [pcDNA3::hPGHS-2] cell lines characterized, one clone that maintained a level of PGHS production of approximately 1.2 ng $PGE_2/10^6$ cells after 8 weeks as a continuous, adherent culture was selected for further characterization, and designated CHO [hPGHS-2_{low}] (Table 1). To obtain higher levels of hPGHS-2 expression in CHO cells, an expression system that uses selection and gene-amplification based on the expression of vector-encoded glutamine synthetase and growth in the presence of the glutamine synthetase inhibitor MSX was used [26, 27]. Using this system, three clones were isolated that synthesized greater than 10 ng $PGE_2/10^6$ cells and were subjected to further gene amplification at 150 and 300 μM MSX. One clonal cell line, designated CHO [hPGHS-2_{high}], was obtained at 300 μM MSX that produced in excess of 500 ng $PGE_2/10^6$ cells following challenge with 10 μM arachidonic acid (Table 1). For further characterization of cellular hPGHS-2, a clonal cell line constructed with the glutamine synthetase expression system that produced an intermediate level of PGHS-2 activity relative to CHO [hPGHS-2_{low}] and CHO [hPGHS-2_{high}], was chosen for further study and designated CHO [hPGHS-2_{int}] (Table 1). The CHO [hPGHS-2_{int}] and CHO [hPGHS-2_{high}] clonal cell lines exhibited a 20–30% increased doubling-time as compared with the parental CHO cells. Addition of 100 μM ibuprofen, a reversible PGHS inhibitor, to the growth medium did not alter significantly the growth-rate of the over-producing CHO [hPGHS-2_{int}] and CHO [hPGHS-2_{high}] cell lines (data not shown).

The gas chromatographic–mass spectrometric profiles of prostanoids produced by the recombinant cell lines showed that significant concentrations of eicosanoids were not pro-

TABLE 1. Conversion of arachidonic acid to PGE_2 by CHO stable cell lines expressing hPGHS-1 and -2

Cell line	Vector	Selection*	PGHS activity† (ng $PGE_2/10^6$ cells) -AA	+AA	N
CHO	pcDNA3	G418	0.01–0.07	0.04–0.25	2
CHO [hPGHS-1]	pcDNA3	G418	0.06–0.1	20–40	10
CHO [hPGHS-2_{low}]	pcDNA3	G418	0.06–0.1	1.0–1.2	10
CHO	pEE14	MSX (25 μM)	0.03–0.07	0.1–0.4	2
CHO [hPGHS-2_{int}]	pEE14	MSX (25 μM)	0.1–0.4	10–20	10
CHO [hPGHS-2_{high}]	pEE14	MSX (300 μM)	3.0	580	2

* Cells transfected with the pcDNA3 vector were selected for growth in medium containing 500 μM G418; cells transfected with the pEE14 vector were selected for growth in medium containing 25 or 300 μM MSX.

† Control CHO cells or CHO cells expressing PGHS-1 or PGHS-2 or vector control were grown in suspension or monolayer cultures. Prior to arachidonic acid challenge, cells growing on monolayers were trypsinized. All cells were rinsed in HBSS, resuspended in HBSS, and incubated in the presence of ethanol vehicle (-AA) or 10 μM arachidonic acid (+AA) for 15 min at 37°. Reactions were terminated by the addition of HCl, and quantitation of PGE_2 production was determined by specific PGE_2 EIA as described in Materials and Methods. Values represent the range of PGE_2 production for two to ten separate experiments (N), with each determination performed in duplicate.

duced by the parental CHO cells, whereas in the CHO [hPGHS-1], CHO [hPGHS-2_{low}], CHO [hPGHS-2_{int}], and CHO [hPGHS-2_{high}] cell lines PGE_2 and PGD_2 each accounted for greater than 40% of the total prostaglandins produced (data not shown).

Kinetics of Exogenous Arachidonic Acid Utilization of CHO [hPGHS] Cell Lines

Although PGHS isozymes can utilize a number of fatty acids as substrates, arachidonic acid, which is normally released by phospolipase A_2, is the main substrate for PGHS isozymes [2]. Since the endogenous production of arachidonic acid is low in CHO cells, we first characterized the use of exogenously added arachidonic acid by the CHO [hPGHS] cell lines with respect to substrate concentration, time–course of PGE_2 synthesis, and K_m of the cellular hPGHS isoforms for arachidonic acid. Stable cell lines were challenged with 0.5, 1, 2, 5, 10, 20, and 50 μM arachidonic acid at time intervals of 1, 2, 4, 8, 10, 25, 35, 45, and 65 min followed by measuring PGE_2 production using a PGE_2 EIA. For CHO [hPGHS-1], CHO [hPGHS-2_{low}], and CHO [hPGHS-2_{int}] cells, maximal PGE_2 production was obtained with 10 μM arachidonic acid 15 min following addition of substrate to the cells (results not shown).

The K_m values for arachidonic acid were determined for the hPGHS isozymes in cells using a continuous fluorometric assay that monitors the rate of the appearance of the oxidized form of the peroxidase cosubstrate 5- (and 6-)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate [30]. In the stable cell lines expressing hPGHS isoforms, values of 1.1 and 0.74 μM were obtained for hPGHS-1 and hPGHS-2, respectively (Fig. 1, panels B and D). A range of K_m values between 0.9 and 12 μM has been reported for the purified hPGHS-1 and -2 isozymes as measured by oxygen consumption and fluorometric assays using other peroxidase cosubstrates such as homovanillic acid [5, 9, 10, 24].

Endogenous Arachidonic Acid Utilization by CHO [hPGHS] Cell Lines

Previous experiments using antisense oligonucleotides to selectively block PGHS isozyme expression suggested that murine PGHS-2, but not murine PGHS-1, can utilize arachidonic acid released in response to mitogen or endotoxin stimulation [32]. Since CHO cells contain a phospholipase activity that can be activated by a number of stimuli, we first investigated whether the hPGHS isoforms in the CHO cell lines could utilize endogenous arachidonic acid liberated by the CHO phospholipase following stimulation of the cells with the calcium ionophore A23187. In the absence of transfected $cPLA_2$, no significant increase in PGE_2 production in CHO [hPGHS-1] cells was observed following ionophore challenge (Fig. 2, panels B and D), although these cells had high levels of PGE_2 production when provided with 10 μM arachidonic acid (40,000–70,000 pg $PGE_2/10^6$ cells) (Fig. 2, panels A and C). In CHO

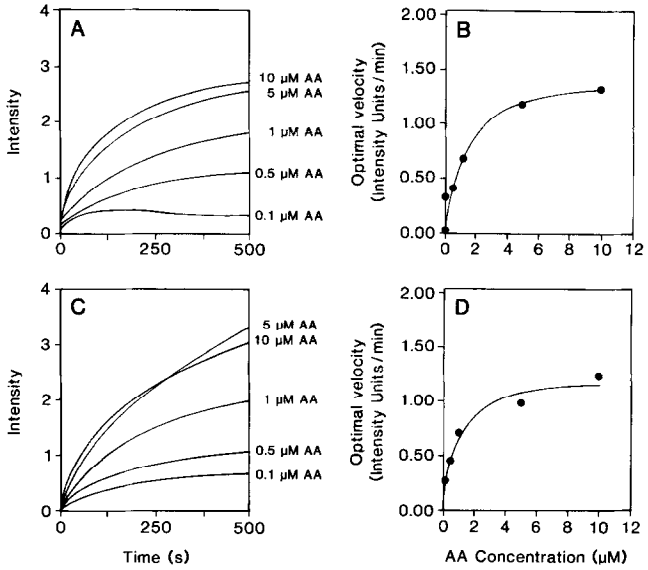

FIG. 1. Determination of the K_m for arachidonic acid in CHO cells stably expressing hPGHS-1 and hPGHS-2. CHO cells expressing hPGHS-1 (panels A and B) or hPGHS-2 (panels C and D) were incubated in the presence of 5 μM dichlorodihydrofluorescein diacetate bisacetoxymethyl ester for 1 hr. Reactions were initiated by the addition of five different concentrations of arachidonic acid, and the fluorescent intensity was measured at excitation and emission wavelengths of 495 and 525 nm, respectively, as described in Materials and Methods (panels A and C). The velocity of the reaction was measured from the initial change of the fluorescent intensity which was evaluated by linear fit of the first 30 sec of the reaction. K_m determinations were made by nonlinear fit of the experimental values to the Michaelis–Menten equation. The data from one of two experiments are presented. Identical K_m values for arachidonic acid were obtained for PGHS-1 (1.1 μM) and PGHS-2 (0.74 μM) in both experiments.

[hPGHS-2_{int}] cells not transfected with $cPLA_2$, ionophore challenge produced a 20-fold increase in PGE_2 production (2300–2600 pg $PGE_2/10^6$ cells) (Fig. 2, panels B and D) that was only 4-fold less than when cells were provided with 10 μM arachidonic acid (8000–10,000 pg $PGE_2/10^6$ cells) (Fig. 2, panels A and C). These results indicate that the CHO [hPGHS-2] cells, but not the CHO [hPGHS-1] cells, can convert endogenous arachidonic acid into PGE_2 liberated following ionophore challenge by coupling to an endogenous phospholipase in the CHO cells. Immunoblot analysis using an anti-$cPLA_2$ antiserum revealed that the CHO cells also contain a low level of a protein that is immunologically related to $cPLA_2$ and comigrates on SDS–polyacrylamide gels with the same molecular weight as human $cPLA_2$ (Fig. 3).

Recent experiments in human synovial cells and embryonic mouse cells reveal that ligand-induced PGE_2 production involves the activation and coordinate regulation of the expression of PGHS-2 and cytosolic phospholipase A_2 ($cPLA_2$) [33, 34]. We next examined whether the hPGHS isozymes expressed in the CHO stable cell lines could utilize arachidonic acid released by human $cPLA_2$ following

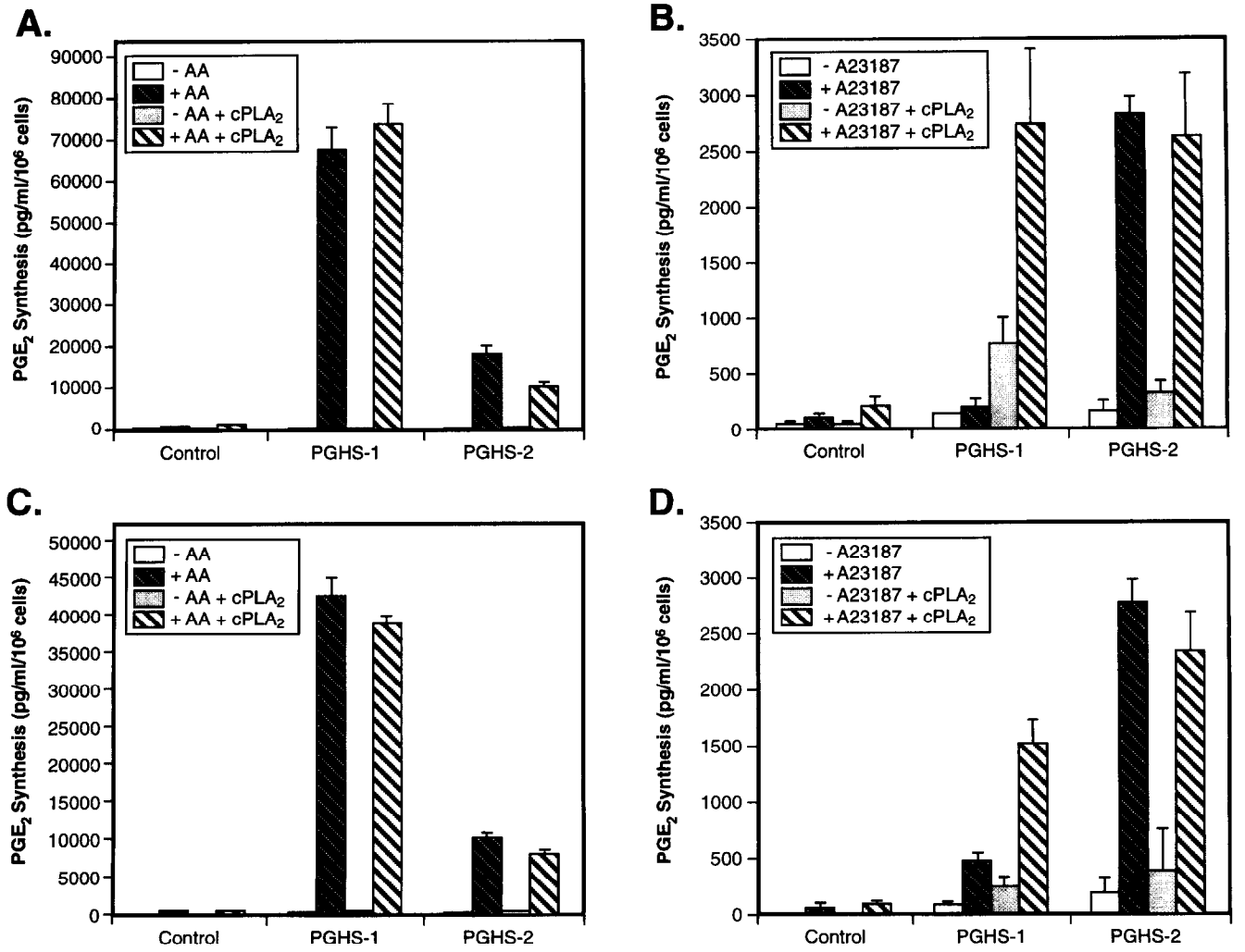

FIG. 2. Effect of $cPLA_2$ expression on the conversion of endogenous and exogenous arachidonic acid to PGE_2 by CHO stable cell lines expressing hPGHS-1 and -2. Control CHO cells or CHO cells stably expressing PGHS-1 or PGHS-2 were transiently transfected with human $cPLA_2$ (indicated as + $cPLA_2$) or mock transfected with control DNA. Forty-eight hours following transfection, cells were incubated in the presence of 10 μM arachidonic acid (or ethanol as control) (panels A and C) or 10 μM calcium ionophore A23187 (or DMSO vehicle as control) (panels B and D) for 15 min at 37°. Reactions were terminated by the addition of HCl, and PGE_2 production was quantitated by specific PGE_2 EIA, as described in Materials and Methods. The values shown represent the means ± SD for quadruplicate determinations in two separate experiments (experiment 1, panels A and B; experiment 2, panels C and D).

ionophore challenge. The cell lines were transiently transfected with a human $cPLA_2$ expression construct, and PGE_2 production in the intact cells was determined following challenge with either A23187 ionophore or 10 μM arachidonic acid. Immunoblot analysis (Fig. 3), using specific antisera for PGHS-1, PGHS-2, and $cPLA_2$ and equivalent amounts (50 ng) of purified PGHS-1, PGHS-2, and $cPLA_2$ protein standards, demonstrated that in cells transfected with human $cPLA_2$, this enzyme is expressed to levels at least as high as the stably expressed human PGHS isozymes. In both CHO [hPGHS-1] and CHO [hPGHS-2] cells transfected with human $cPLA_2$ and challenged with 10 μM arachidonic acid, there was no significant difference in the level of prostanoid synthesis as compared with control CHO [hPGHS-1] and CHO [hPGHS-2] cells not transiently transfected with $cPLA_2$ (Fig. 2, panels A and C). In the CHO [hPGHS-1] cells transfected with human $cPLA_2$, A23187 challenge yielded a 3- to 6-fold higher level of PGE_2 production than did non-$cPLA_2$ transfected CHO [hPGHS-1] cells also challenged with A23187 ionophore (Fig. 2, panels B and D). In contrast, there was no difference in the PGE_2 production following ionophore challenge in the CHO [$hPGHS\text{-}2_{int}$] cells transfected with human $cPLA_2$ as compared with mock-transfected CHO [$hPGHS\text{-}2_{int}$] cells (Fig. 2, panels B and D). These results demonstrate that the stable CHO [hPGHS-1] cell line, but not the CHO [$hPGHS\text{-}2_{int}$] cell line, can utilize arachidonic acid released by the transiently transfected human $cPLA_2$ enzyme following ionophore challenge. The rate-limiting step in PGE_2 synthesis in both the stable CHO

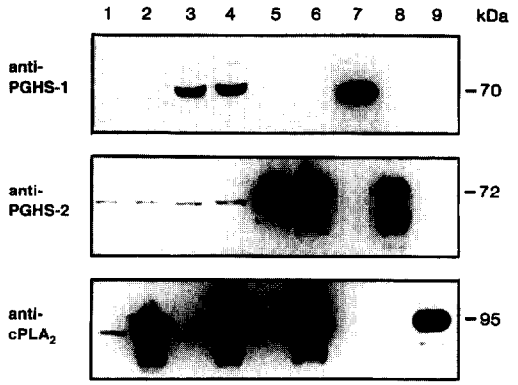

FIG. 3. Immunoblot analysis of hPGHS-1, hPGHS-2, and $cPLA_2$ expression in CHO cell lines. Protein samples from 0.28×10^6 control CHO cells, CHO cells stably expressing hPGHS-1 or hPGHS-2 and transiently co-expressing $cPLA_2$ and equal concentrations of purified protein standards were analyzed by SDS–PAGE, transferred to nitrocellulose, and immunoblotted simultaneously with anti-PGHS-1 (upper panel), anti-PGHS-2 (middle panel), or anti-$cPLA_2$ (lower panel) antisera, with detection by chemiluminescence, as described in Materials and Methods. Lane 1, control CHO cells; lane 2, CHO cells transfected with $cPLA_2$; lane 3, CHO cells expressing hPGHS-1; lane 4, CHO cells expressing hPGHS-1 and transfected with $cPLA_2$; lane 5, CHO cells expressing hPGHS-2; lane 6, CHO cells expressing hPGHS-2 and transfected with $cPLA_2$; lane 7, 50 ng ovine PGHS-1 standard; lane 8, 50 ng ovine PGHS-2 standard; and lane 9, 50 ng human $cPLA_2$.

[hPGHS-1] and CHO [hPGHS-2] cell lines appears to be the availability of arachidonic acid, as even in the cell lines transfected with human $cPLA_2$ (Fig. 2, panels B and D), PGE_2 production in the presence of ionophore was less than 30% of the PGE_2 synthesis in the presence of exogenous 10 μM arachidonic acid (Fig. 2, panels A and C).

Time-Dependency of Inhibition of hPGHS Isoforms in Intact Cells

Studies using purified preparations of PGHS isozymes have demonstrated that the selective inhibition of hPGHS-2 by compounds such as NS-398 and DuP-697 results from a difference in the nature of the time-dependency of inhibition of the two isoforms [16, 17]. We investigated the effect of inhibitor time-dependency on the hPGHS isoforms in intact cells by preincubation of cells in the presence of drug for increasing periods of time before initiation of the reaction by addition of arachidonic acid. With no preincubation of drug with cells, indomethacin had an IC_{50} of 1.1 μM on hPGHS-1 and showed no significant inhibition of hPGHS-2 in intact cells. However, following a 15-min preincubation period of drug with cells, indomethacin became a potent inhibitor of both cellular hPGHS-1 and -2, with IC_{50} values of 15 and 70 nM, respectively (Fig. 4). Thus, in intact cells as with purified enzyme, the nonselective NSAID indomethacin exhibits time-dependent inhibition of PGHS activity in both CHO [hPGHS-1] and CHO [$hPGHS\text{-}2_{int}$] cell lines. The potency of NS-398 on cellular hPGHS-2 activity was increased markedly following a preincubation period of as short as 1 min (Fig. 4). The time-dependent inhibition of hPGHS-1 and hPGHS-2 by indomethacin and of hPGHS-2 by NS-398 was apparent over a wide range of inhibitor concentrations but was most evident at the higher concentrations of inhibitors tested. In contrast, preincubation of CHO [hPGHS-1] cells with NS-398 for 0–15 min had little effect on inhibitor potency over a drug concentration range of 10 nM to 50 μM (Fig. 4). These results demonstrated that in cells, as with purified enzyme, NS-398 is a time-independent inhibitor of hPGHS-1 but a time-dependent inhibitor of hPGHS-2.

Reversibility of NSAID Inhibition of hPGHS Isoforms in Intact Cells

We next investigated whether the reversibility of inhibition of the nonselective NSAIDs ibuprofen, flurbiprofen and indomethacin, and the selective PGHS-2 inhibitors DuP-697 and NS-398 on the hPGHS isoforms also occurred in intact cells. Cells were pretreated with the protein translation inhibitor cycloheximide and with a PGHS inhibitor for 15 min. At the end of the inhibitor incubation, an aliquot was removed, and PGE_2 production was determined. The remainder of the cells were washed extensively and incubated in inhibitor-free buffer containing cycloheximide. At intervals from 0 to 9 hr following the drug washout, the PGHS activity in the cells was determined by assaying PGE_2 production after challenging the cells for 15 min with 10 μM arachidonic acid and comparing it with PGE_2 production in control, non-drug-treated cells. Three separate experiments were performed with indomethacin, flurbiprofen, ibuprofen, NS-398, and DuP-697. The results shown are representative of one experiment for DuP-697 and ibuprofen and two experiments for indomethacin. The initial inhibition of CHO [hPGHS-2] at t = 0 by ibuprofen was 60%; a flat concentration–response curve is characteristic of ibuprofen analogs, with maximal inhibition frequently reaching only 60–80% (S. Kargman, unpublished results; and Ref. 24). The inhibition of PGHS activity by ibuprofen was rapidly reversible in both CHO [hPGHS-1] and CHO [$hPGHS\text{-}2_{int}$] cell lines with essentially 100% of the PGHS activity recovered following drug washout (Fig. 5). Inhibition by indomethacin was also reversible on both CHO [hPGHS-1] and CHO [$hPGHS\text{-}2_{int}$] cell lines, with over 80% of the hPGHS-1 activity recovered 3 hr after removal of the inhibitor, and approximately 40% of hPGHS-2 activity regained 9 hr after drug washout. The inhibition mediated by the selective PGHS-2 inhibitor DuP-697 was partially reversible in CHO [hPGHS-1] cells (50% activity recovered at 9 hr post drug washout) but essentially irreversible in CHO [$hPGHS\text{-}2_{int}$] cells (Fig. 5).

Inhibition of hPGHS Isoforms in Intact Cells by NSAIDs

Based on the experiments described above which characterize arachidonic acid utilization, the kinetics of PGE_2

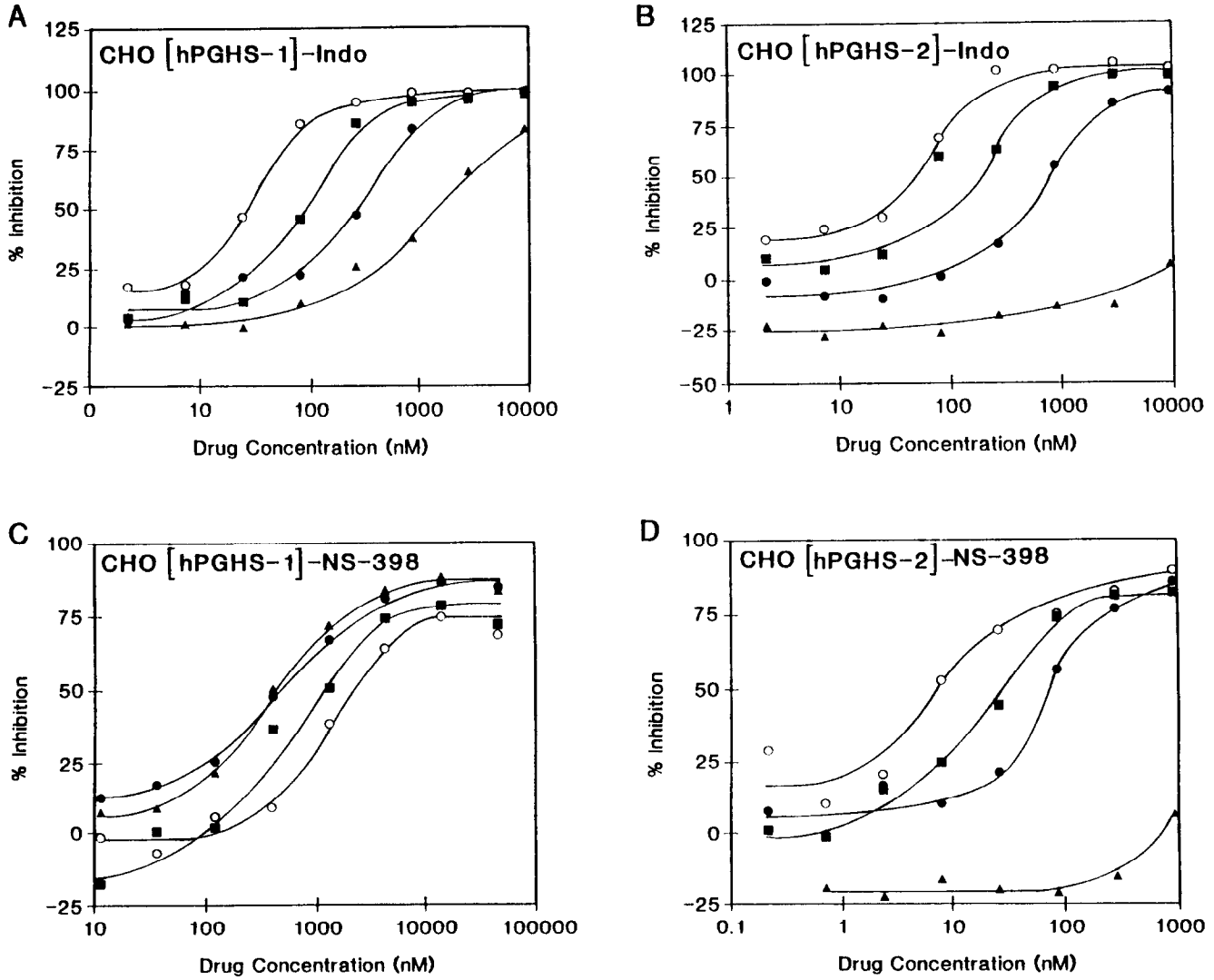

FIG. 4. Effect of inhibitor preincubation period on inhibition of hPGHS-1 and hPGHS-2 in intact CHO cells. CHO cells expressing hPGHS-1 (panels A and C) or hPGHS-2 (panels B and D) were preincubated at 37° in the presence of indomethacin (panels A and B) or NS-398 (panels C and D) for 0 min (▲), 1 min (●), 5 min (■) or 15 min (○) followed by the addition of 0.5 μM arachidonic acid for CHO [hPGHS-1] and 10 μM arachidonic acid for CHO [hPGHS-2$_{int}$] for 15 min at 37°. Reactions were terminated by the addition of HCl, and quantitation of PGE_2 production was determined by specific PGE_2 EIA as described in Materials and Methods. Values shown are representative of two separate experiments, each performed in duplicate.

synthesis, and the time-dependent inhibition by NSAIDs in the CHO [hPGHS] stable cells, we established the following screening assay: preincubation of cells with eight concentrations of drug for 15 min followed by challenge of cells with 10 μM arachidonic acid for 15 min and determination of PGE_2 production by a PGE_2 enzyme immunoassay. Under these assay conditions, initial experiments revealed that selective PGHS-2 inhibitors, such as NS-398, were weak inhibitors of hPGHS-1 (IC_{50} of 25 μM for inhibition of CHO [hPGHS-1]) but potent inhibitors of hPGHS-2 (IC_{50} of 3 nM for CHO[hPGHS-2]) (Table 2).

Since we were interested in identifying selective inhibitors with increased selectivity for inhibition of hPGHS-2 versus hPGHS-1, the CHO [hPGHS-1] cell-based assay was modified to make it maximally sensitive to inhibition by selective PGHS-2 inhibitors. We reasoned that because selective PGHS-2 inhibitors are time-independent, reversible inhibitors of hPGHS-1 which compete for binding with arachidonic acid [16, 17], the sensitivity of the CHO [hPGHS-1] assay to selective PGHS-2 inhibitors would be increased if the concentration of exogenously added arachidonic acid was decreased from 10 to 0.5 μM. Indeed, the IC_{50} values for NS-398 in the CHO [hPGHS-1] assay using either 10 or 0.5 μM arachidonic acid were 25.5 and approximately 1.1 μM, respectively. Similarly, the selective PGHS-2 inhibitor DuP-697 also showed a lower IC_{50} value for inhibition of hPGHS-1 when tested using 0.5 versus 10 μM arachidonic acid (Table 2). Among the nonselective

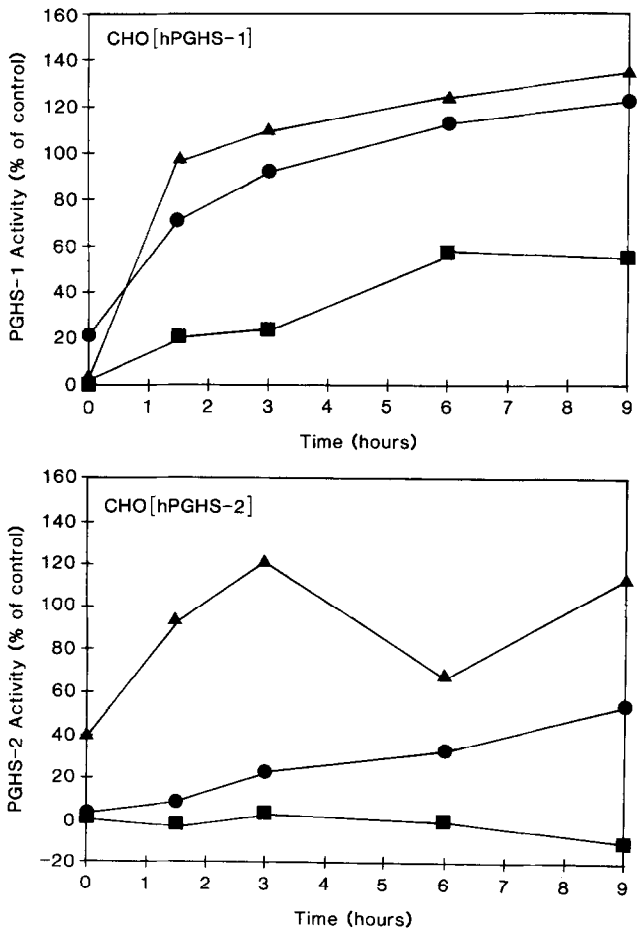

FIG. 5. Reversibility of NSAID inhibition in whole cells. CHO cells expressing hPGHS-1 or hPGHS-2 were incubated for 15 min at 37° in HBSS containing 20 μM cycloheximide in the presence or absence of one of the following inhibitors: 300 nM indomethacin (●); 100 μM ibuprofen (▲); 18 μM DuP-697 for CHO [hPGHS-1] or 5.4 μM DuP-697 for CHO [hPGHS-2$_{int}$] (■). Control reactions contained cells incubated in the absence of inhibitor but with DMSO vehicle. For t = 0, an aliquot of each sample was incubated in the presence of 10 μM arachidonic acid for 15 min followed by termination of the reaction by HCl. The remainder of each sample was pelleted, washed three times, resuspended in HBSS containing 20 μM cycloheximide, and incubated at 37°. At various times, 10 μM arachidonic acid was added to aliquots of inhibitor- or control-treated cells for 15 min at 37°, followed by termination of the reaction by addition of HCl. Quantitation of PGE_2 was determined by specific PGE_2 EIA. PGHS activity is expressed as a percentage of the PGE_2 produced at each time point in the presence of inhibitor versus the PGE_2 produced in the control DMSO-treated cells. The means of the levels of PGE_2 production in the control DMSO-treated CHO [hPGHS-1] cells at 0, 1.5, 3, 6, and 9 hr were 17.9, 15.3, 15.2, 11.2, and 9.7 ng $PGE_2/10^6$ cells, respectively. The means of the levels of PGE_2 production in the control DMSO-treated CHO [hPGHS-2] cells at 0, 1.5, 3, 6, and 9 hr were 9.2, 5.1, 3.7, 3.3, and 2.6 ng $PGE_2/10^6$ cells, respectively.

PGHS inhibitors (flurbiprofen, indomethacin, and diclofenac), there were no significant differences between the IC_{50} values for inhibition of PGHS-1 determined at 0.5 and 10 μM arachidonic acid (Table 2). Since the lower concentration of arachidonate increased the discrimination between selective and nonselective inhibitors in the CHO [hPGHS-1] assay, the CHO [hPGHS-1] assay was routinely carried out at 0.5 μM arachidonate. The IC_{50} values for twelve PGHS inhibitors of the hPGHS isoforms expressed in CHO cells using 0.5 and 10 μM arachidonic acid in the hPGHS-1 assay and 10 μM arachidonic acid in the hPGHS-2 assay are presented in Table 2. In the CHO cells, seven inhibitors (flurbiprofen, indomethacin, acetaminophen, diclofenac, salicylic acid, ibuprofen, and aspirin) with IC_{50} values ranging from 2 nM to greater than 50 μM showed no selectivity for either PGHS isoform. Five compounds showed a range of selective inhibition of hPGHS-2, with L-745,337 and NS-398 exhibiting over 200-fold selectivity for inhibition of hPGHS-2 (Table 2). In general, inhibitors were more potent in the cell-based system when compared with published IC_{50} values determined in cell-free preparations (Table 2).

DISCUSSION

Since the discovery of a second form of PGHS and the elucidation of the role of PGHS-2 in inflammation, many reports on the inhibitory activity of NSAIDs on PGHS-1 and PGHS-2 have been published [12]. For the same compound, the reported IC_{50} values for inhibition of PGHS-1 and -2 and the relative selectivity of the compound can vary widely (Table 2). For example, published IC_{50} values for inhibition of hPGHS-2 by indomethacin vary from 0.074 μM [21] to greater than 1000 μM [24]. Factors that could affect the potency of PGHS inhibition include (a) the use of purified or microsomal PGHS preparations versus intact cells, (b) substrate concentration, (c) source of substrate, (d) time of preincubation of inhibitor with enzyme prior to substrate addition, (e) method of determining inhibition of PGHS activity (e.g. rate versus endpoint production accumulation), (f) species differences, (g) subcellular localization of the PGHS isoform and/or inhibitor in cells, (h) glycosylation, (i) interaction with nitric oxide synthase, and (j) complexation with regulatory or accessory proteins. To develop a directly comparative assay to characterize selective PGHS inhibition, we report here a cell-based system in which we have assessed various factors that can influence inhibitor potency and selectivity.

The potency and selectivity of inhibitors of PGHS isoforms have been assayed in many different cell types [12, 13, 22–24]. The intact cellular sources used for assaying PGHS-1 inhibition have included human platelets, COS-1 monkey kidney cells transiently transfected with the hPGHS-1 cDNA, bovine aortic endothelial cells, and guinea pig macrophages. The intact cellular sources used for assaying PGHS-2 inhibition have included lipopolysaccharide-treated murine J774.2 macrophages, guinea pig macro-

TABLE 2. Inhibition of hPGHS-1 and hPGHS-2 activity in intact cells by NSAIDs

Inhibitor	IC_{50}* (μM) CHO [hPGHS-1] cell-based assay		IC_{50}* (μM) CHO [hPGHS-2] cell-based assay	IC_{50}† (μM) Cell-free assay	
	10 μM AA (N)	0.5 μM AA (N)	10 μM AA (N)	PGHS-1	PGHS-2
Flurbiprofen	0.005 (3)	0.001–0.003 (3)	0.002–0.012 (6)	0.04–0.5[8–10,24]	0.4–3.2[8–10,24]
Indomethacin	0.023 (3)	0.013–0.016 (3)	0.016–0.061 (9)	0.013–13.97[8–11,13,14,21,23,24,35]	0.074–>1000[8–11,13,21,23,24]
Acetaminophen		>50 (2)	>50 (2)		
DuP-697	0.257 (3)	0.041–0.125 (4)	0.002–0.007 (5)	0.8[9]	0.01[9]
Diclofenac	0.003 (3)	0.005–0.008 (2)	0.001–0.002 (5)	0.015–2.7[9–11,14,21,24]	0.022–1.5[9–11,21]
Niflumic acid		0.267–0.928 (2)	0.079–0.137 (2)	16[10]	0.1[10]
Toradol	0.070 (3)	0.081–0.083 (3)	0.010–0.015 (2)		
L-745,337		47.6–>50 (4)	0.02–0.098 (3)		
Salicylic acid		>50 (2)	>100 (2)	>1000[24]	>1000[24]
NS-398	25 (3)	0.864–1.46 (3)	0.003–0.004 (5)	11–>100[9,10,14,17]	0.1–1.77[9,10,17]
Ibuprofen		0.472–0.656 (3)	0.465–0.677 (2)	2.6–60[8–11,13,14,24]	1.53–>300[8–11,13,24]
Aspirin		14.9–60.8 (4)	5–69.6 (4)	27.75–200[13,35]	1166[13]

PGHS activities from CHO cells stably expressing PGHS-1 and PGHS-2 were determined by measuring the synthesis of PGE_2 using a specific PGE_2 EIA. Cells were incubated in the presence of inhibitor for 15 min at 37° in the absence of added arachidonic acid. Reactions were initiated by the addition of arachidonic acid (AA) to a final concentration of 0.5 or 10 μM for CHO [hPGHS-1] cells and 10 μM for CHO [hPGHS-2] cells. Values represent the range of two to nine separate experiments (N), each performed in duplicate. The ">" symbol denotes the highest concentration of drug tested.

* The IC_{50} values are derived from concentration–response curves at eight concentrations of compounds for the inhibition of PGE_2 production by CHO cells stably expressing hPGHS-1 and hPGHS-2.

† The IC_{50} values are those reported for various cell-free assays using crude microsomal preparations or purified preparations of human PGHS-1 and -2. The references from which the IC_{50} values were derived are indicated by superscripts.

phages, and human mononuclear cells, COS-1 monkey kidney cells transiently transfected with the hPGHS-2 cDNA, and interleukin-1-stimulated rat mesangial cells and murine calvarial cells [12, 13, 22–24]. Most of the selectivity data have been obtained by comparing inhibition of PGHS-1 and -2 in different cell types from various species. The cell-based screening system we have characterized here determines inhibitor potencies on the human PGHS isoforms expressed in the same cellular background. Therefore, variables such as differences in the uptake and subcellular distribution of both inhibitor and substrate are comparable.

Arachidonic acid from both exogenous and endogenous sources was used as substrate by the hPGHS isoforms expressed in CHO cells, albeit differentially. Interestingly, following ionophore challenge, an increase in PGE_2 production was observed in the CHO [hPGHS-2] but not in the CHO [hPGHS-1] cells. This observation is in agreement with an earlier study by Reddy and Herschman which used antisense oligonucleotides to selectively inhibit PGHS-2 expression to show that murine PGHS-2, but not PGHS-1, could use arachidonic acid released by phospholipases following mitogenic stimulation of fibroblasts and macrophages [32]. More recently, Reddy and Herschman [36] reported that secretory phospholipase A_2 released from activated mast cells can mobilize arachidonate from cocultured fibroblast cells. This arachidonate is then used by PGHS-1 in the fibroblasts for prostanoid synthesis. These studies suggest at least two independent pathways for prostaglandin synthesis, including a secretory phospholipase A_2-mediated, PGHS-1-dependent transcellular pathway and an intracellular cytosolic phospholipase A_2-mediated, PGHS-2-dependent pathway [32, 36]. Other reports which demonstrate that the expression of $cPLA_2$ and PGHS-2 are coordinately regulated support the suggestion that a primary source of substrate for PGHS-2 is $cPLA_2$ [33, 34]. In our studies, although an increase in the level of prostanoid production following ionophore treatment occurred in CHO [hPGHS-2] cells but not in CHO [hPGHS-1] cells, the transient expression of human $cPLA_2$ combined with ionophore treatment resulted in an increased level of prostanoid production in CHO [hPGHS-1] cells but not in CHO [hPGHS-2] cells. This apparent ability of transiently transfected $cPLA_2$ to augment prostanoid production by hPGHS-1 but not hPGHS-2 does not necessarily indicate that hPGHS-1 normally couples to human $cPLA_2$. It is important to note that in both CHO [hPGHS-1] and [hPGHS-2] cell lines, the amount of product formed in response to exogenous arachidonic acid was at least 5-fold greater than that formed from endogenous arachidonic acid following ionophore challenge, even after overexpression of $cPLA_2$ in these cell lines by transient transfection. Previous studies (e.g. Refs. 37 and 38) have also reported that the level of prostanoid production can be many fold greater in cells utilizing exogenous arachidonate versus arachidonate generated following ligand or ionophore stimulation. Chepenik *et al.* [37] have suggested that exogenous arachidonate can be metabolized by either PGHS isoform, but only those PGHS molecules coupled or juxtaposed to intracellular sites of acyl mobilization can utilize endogenous arachidonate. The activation of $cPLA_2$ involves a Ca^{2+}-dependent translocation to intracellular membranes [39, 40]. The inability of transfected $cPLA_2$ to increase prostanoid synthesis in CHO [hPGHS-2] cells may indicate that there are limited intracellular sites for mobilization of arachidonate that are already fully occupied by $cPLA_2$ in the CHO [hPGHS-2] cells but not in the CHO [hPGHS-1]

cells. Immunocytofluorescent studies have demonstrated different subcellular locations of the PGHS isoforms, suggesting that PGHS-1 functions predominantly in the endoplasmic reticulum, whereas PGHS-2 also appears to function in the nuclear envelope [41]. In the case of $cPLA_2$, one study demonstrated translocation of $cPLA_2$ to the nuclear envelope but not the endoplasmic reticulum in rat mast cells [40]. However, a second study by Schievella *et al.* [39] reported translocation of $cPLA_2$ to the nuclear envelope and to the endoplasmic reticulum in CHO cells overexpressing $cPLA_2$, raising the possibility that the localization of $cPLA_2$ to the endoplasmic reticulum in CHO cells expressing recombinant $cPLA_2$ is a consequence of overexpression. Whereas previous studies have suggested an absolute preferential recognition of endogenous substrate by hPGHS-2 [32], our ionophore-challenge and $cPLA_2$ transfection experiments would tend to support a model in which $cPLA_2$ preferentially couples to hPGHS-2, but under certain conditions (i.e. overexpression of $cPLA_2$) it is clearly capable of coupling to hPGHS-1.

The effect of inhibitor time-dependency on selectivity towards the hPGHS isoforms demonstrated here in intact CHO cells parallels the effects previously observed with purified enzyme preparations [16, 17]. Inhibitor permeability should be identical in both CHO [PGHS-1] and CHO [PGHS-2] due to the fact that each isozyme is expressed in the same CHO cellular background. Indomethacin exhibited time-dependent inhibition of both cellular hPGHS-1 and -2, whereas NS-398 displayed time-dependent inhibition of cellular hPGHS-2 but time-independent inhibition of cellular hPGHS-1. There was a small loss of inhibitor potency on hPGHS-1 with the selective PGHS-2 inhibitor, NS-398, during the 15-min preincubation interval. It is not clear why NS-398 reproducibly loses potency with increasing preincubation time in CHO [PGHS-1] cells; it may be due to decreased availability of inhibitor due to nonspecific adsorption or sequestering into cellular compartments. Nevertheless, the pattern of time-dependent inhibition mediated by NS-398 differs strikingly for the two isozymes, with the inhibition of CHO [PGHS-2] increasing over 100-fold and the inhibition of CHO [PGHS-1] decreasing less than 5-fold during the 15-min incubation period. The difference in the nature of the time-dependent inhibition of PGHS isoforms by inhibitors such as NS-398 has been proposed to be the basis for the high degree of selective inhibition of hPGHS-2 by this compound and other PGHS-2 selective inhibitors [16, 17]. The mechanism of the time-dependent inhibition of hPGHS-2 involves an initial rapid, reversible equilibrium binding of the inhibitor to the enzyme, followed by the relatively slow formation of an essentially irreversible tightly bound enzyme–inhibitor complex. This complex formation was observed in CHO [hPGHS-2] cells with the selective PGHS-2 inhibitor DuP-697, as the inhibition was irreversible even after 9 hr following drug removal. Other nonselective PGHS inhibitors, including indomethacin and ibuprofen, displayed reversible inhibition in cells as expected from previous results in cell-free systems [16, 17]. It is important to note that the design of our inhibitor reversibility experiments assumes that inhibitor binding affects the half-life of the PGHS isozymes similarly. There is limited information on the turnover rates of PGHS isozymes in the presence or absence of inhibitors. In the absence of inhibitors, PGHS-2 protein appears to be much less stable than PGHS-1 protein, with a half-life of 4 hr in fibroblasts [42, 43]. Inhibitors of ovine PGHS-1 can protect the apoenzyme from cleavage by trypsin, indicating that inhibitor binding can affect the sensitivity of PGHS-1 apoenzyme to proteolytic cleavage [44]. Further studies will be necessary to clarify whether PGHS inhibitors differentially affect the turn-over rates of the PGHS proteins and their sensitivity to autoinactivation in various cell types.

The time-dependent nature of PGHS isoform inhibition by NSAIDs underlines the importance of the design of inhibitor-screening protocols. In our experiments, indomethacin does not exhibit inhibition of the cellular form of hPGHS-2 if inhibition is measured instantaneously, while following a 15-min preincubation of CHO [hPGHS-2] cells with inhibitor, the IC_{50} is lowered to 10–40 nM. Since previous reports of IC_{50} values for various NSAIDs are based on various assay protocols that incorporate drug preincubations ranging from 0 to 30 min (e.g. Refs. 8, 11, 21, and 22), it is difficult to compare our results to the potencies and selectivities of published IC_{50} values. Our results show that in intact cells time-dependent inhibition is a critical factor in determining NSAID potency and that at least a 15-min preincubation period is required to detect maximal inhibition for time-dependent inhibitors in cells.

Purified ram seminal vesicle PGHS-1 and recombinant cell-free preparations of PGHS isoforms have been essential in establishing mechanisms of inhibition and initial profiles of PGHS inhibitors. However, several groups have identified the requirement to test inhibitor potency in whole cells as cell-free systems appear to often underestimate the potency and selectivity of PGHS inhibitors [1, 12, 13, 22]. It seems reasonable to assume that the cellular environment encountered by an inhibitor is more representative of the situation *in vivo* than of a cell-free system. Both PGHS isoforms are membrane-associated proteins localized to the luminal side of the endoplasmic reticulum membrane and in the case of PGHS-2 also to the nuclear envelope [41]. In the whole cell, PGHS isoforms would be exposed to both substrate and PGHS inhibitors in the context of an intact membrane lipid bilayer. In the cell-based system described here, the selective inhibition of PGHS isozymes involves a difference in the nature of the time-dependent inhibition of the enzymes, but other factors such as substrate concentration can dramatically affect the observed potency of PGHS inhibitors in intact cells.

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