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# Genetic Evidence for Distinct Roles of COX-1 and COX-2 in the Immediate and Delayed Phases of Prostaglandin Synthesis in Mast Cells

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**Activation of mast cells by aggregation of their high-affinity IgE receptors stimulates prostaglandin (PG) $D_2$ synthesis and secretion. An immediate phase of $PGD_2$ synthesis, complete within 30 min, is followed by a delayed, second phase of $PGD_2$ production that reaches a maximum 4 to 8 h after activation. Activation of mast cells from COX-2 (−/−) mice stimulates the release of $PGD_2$ during the first 30 min, whereas activation of mast cells from COX-1 (−/−) mice does not generate any $PGD_2$ in the first 2 h. On the other hand, COX-2 (−/−) cells do not participate in delayed phase of $PGD_2$ synthesis, while COX-1 (−/−) cells secrete low levels of $PGD_2$ between 2 and 4 h after activation. These data demonstrate that (i) the first phase of PG synthesis is COX-1 dependent and (ii) the second, delayed phase of PG synthesis is dependent on activation-induced synthesis and activity of COX-2.**


Prostaglandins (PGs) play important roles in many biological processes, including cell division, immune responses, ovulation, bone development, wound healing and water balance. Altered PG production is associated with a variety of illnesses, including acute and chronic inflammation, cardiovascular disease, colon cancer and allergic diseases (1, 2). Cyclooxygenase (COX), which exists in two isoforms, COX-1 and COX-2, is the rate limiting enzyme in the biosynthesis of PGs. COX mediates the conversion of arachidonic acid, released from the membrane phospholipid stores by phospholipases, into $PGH_2$, which is then converted to various PGs by specific synthases (3, 4). COX-1 is constitutively expressed in nearly all cells, and is involved in cellular homeostasis. COX-2 is induced in a variety of cell types by diverse stimuli including cytokines, growth factors, mitogens and tumor promoters (5–9). COX-2 is also induced in cell types involved in inflammation, immune and allergic responses, including lipopolysaccharide induced macrophages and activated mast cells (10, 11), suggesting that COX-2 is a mediator of inflammatory processes.

Mast cells play a critical role in immune responses and allergic disease. Activation of mast cells, either by aggregation of their high affinity IgE receptors or by other stimulators, results in the release of stored inflammatory mediators such as histamine and serotonin. Aggregation of IgE receptors on mast cells also mediates the induced synthesis and release of inflammatory mediators such as leukotrienes and prostaglandin $D_2$ ($PGD_2$) (12). $PGD_2$ production in activated mast cells occurs in two distinct phases, an immediate activation-induced release that is completed within 10–15 min and a delayed phase of $PGD_2$ synthesis and secretion that peaks at 4–6 h following activation (11, 13). Studies utilizing chemical inhibitors of COX-1 and COX-2 suggested that the immediate phase of $PGD_2$ synthesis in mast cells following activation is due to the conversion of arachidonic acid to $PGD_2$ by COX-1 whereas the delayed phase of $PGD_2$ synthesis and secretion following activation is mediated by COX-2 (11).

The availability of COX-1 and COX-2 knockout mice (14, 15) provides a distinct experimental tool with which to examine the roles of COX-1 and COX-2 in the generation of $PGD_2$ in activated mast cells. In this

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report, we demonstrate that (i) activation of mast cells results in distinct phases of PG synthesis, (ii) the immediate phase of PG synthesis in activated mast cells is COX-1 dependent and (iii) the second, delayed phase of PG synthesis is COX-2 dependent in activated mast cells.

## MATERIALS AND METHODS

*Reagents.* Murine IgE and monoclonal anti-IgE were purchased from PharMingen (San Diego, CA). $PGD_2$ assay kits and Hybond-N membranes were purchased from Amersham Corp. (United Kingdom). RPMI 1640 medium was purchased from ICN (Cleveland, OH). Fetal bovine serum was from Gemini Products Inc. (Calabasas, CA). Arachidonic acid and 12-episcalaradial were purchased from Sigma (St. Louis, MO). SB 203347, inhibitor of sPLA2, was a gift from Dr. Lisa Marshall (SmithKline Beecham Pharmaceuticals, King of Prussia, PA).

*Cell culture.* Mast cell lines from COX-1 (−/−) and COX-2 (−/−) mice were prepared as described previously for wild type mice (16). Briefly, bone marrow from the femurs was flushed out with RPMI medium containing 5% fetal bovine serum. The cells were centrifuged, resuspended, and plated in RPMI 1640 medium containing 10% fetal bovine serum and 50% WEHI-conditioned medium. Culture medium was changed twice each week. By 3–4 weeks in culture, this procedure yielded more than 95% pure mast cell cultures. Murine MMC-34 mast cells, COX-1 (−/−) mast cells, and COX-2 (−/−) mast cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum.

*Genotyping by PCR.* COX-1 and COX-2 null alleles were determined by PCR. The forward primer for the identification of the COX-1 null allele is 5′-GCAGCCTCTGTTCCACATACAC-3′, the forward primer for the COX-1 wild-type allele is 5′-AGGAGATGGCTGCTGAGTTGG-3′ and the reverse primer for both COX-1 reactions is 5′-AATCTGACTTTCTGAGTTGCC-3′. The forward primer for the COX-2 null allele is 5′-ACGCGTCACCTTAATATGCG-3′, the forward primer for the COX-2 wild-type allele is 5′-ACACACTCTATCACTGGCACC-3′ and the reverse primer for both COX-2 reactions is 5′-ATCCCTTCACTAAATGCCCTC-3′. PCRs in the presence of 10% dimethyl sulfoxide, in a Perkin–Elmer 9600 PCR machine, using one cycle at 94°C for 30 s followed by 30 cycles at 94°C for 30 s/55°C for 30 s/72°C for 30 s, and finally one cycle at 72°C for 7 min. The expected sizes of the PCR products were 601 bp for COX-1 (+/+), 646 bp for Cox1 (−/−), 760 bp for COX-2 (+/+), and 905 bp for Cox2 (−/−).

*Northern blot analysis.* Ten micrograms of total RNA from each sample was subjected to electrophoresis in a 1% agarose gel, transferred to Hybond-N membranes, and hybridized with cDNA probes for murine COX-1 or COX-2. Electrophoresis and hybridization protocols have been described previously (17, 18).

*Western analysis.* COX-1 (−/−) and COX-2 (−/−) mast cells were activated as described previously (11). Briefly, mast cells were treated with 1 $\mu$g/ml of mouse IgE (PharMingen, San Diego, CA) for 1 h, washed and further treated with 1 $\mu$g/ml of anti-IgE (PharMingen, San Diego, CA) for 4 h. Control cells were given medium alone after IgE treatment. Following incubation, cells were washed with PBS, and isolated in SDS loading buffer. Samples were boiled for 10 min and 75 $\mu$g of protein extracts were subjected to SDS–PAGE. The proteins were electroblotted onto nitrocellulose membranes using a semi-dry transfer apparatus from Bio-Rad (CA). The nitrocellulose membranes were blocked with PBS/10% non-fat dried milk for 60 min, then washed and incubated with primary and secondary antibodies. All washes and antibody incubations were done in PBS/1% non-fat milk. COX-1 (goat) and COX-2 (rabbit) polyclonal antibodies were purchased from Oxford Research, Inc. (Oxford, MI), and used at 1/100 dilutions. Rabbit anti-goat IgG and goat anti-rabbit IgG (Sigma) were used at 1/8000 dilutions. The membranes were washed extensively after the secondary antibody incubation, and proteins were detected using the enhanced chemiluminiscence reagents (Amersham, UK). The filters were exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) at room temperature.

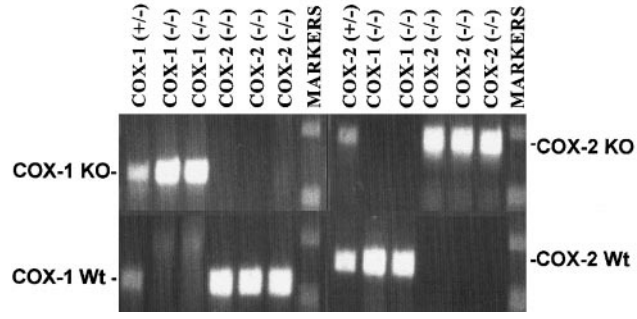


**FIG. 1.** Genotyping of COX-1 (−/−) and COX-2 (−/−) mice. High-molecular-weight genomic DNA was isolated from 1-cm clips of mouse tails. Primers and PCR conditions were as described under Materials and Methods. DNA from heterozygous animals, either COX-1 (+/−) or COX-2 (+/−), was used as positive control. Primers were designed to generate the following sizes of PCR products: COX-1 (−/−), 646 base pairs; COX-2 (−/−), 905 base pairs; COX-1 (+/+), 601 base pairs; and COX-2 (+/+), 760 base pairs.

*Cell activation and $PGD_2$ assays.* COX-1 (−/−) mast cells, COX-2 (−/−) mast cells and MMC-34 cells were treated with 1 $\mu$g/ml of mouse IgE for 1 h, washed and further treated with 1 $\mu$g/ml of anti-IgE for further periods of time as described in the text. Control cells were given medium alone after IgE treatment. Following incubation, supernatants were collected, centrifuged and analyzed for $PGD_2$ according to the manufacturer's protocol (Amersham).

## RESULTS

*Preparation and characterization of COX-1 (−/−) and COX-2 (−/−) mast cell lines.* We used 6 week old, female COX-1 (−/−) and COX-2 (−/−) mice to isolate mast cells. To be certain that the mice used for mast cell preparation are COX-1 (−/−) and COX-2 (−/−), we genotyped the mice by PCR prior to isolation of cells. PCR amplification of genomic DNA, isolated from tail clips of the mice, generated the expected COX-1 (−/−) and COX-2 (−/−) specific products, confirming the genotypes of the mice (Fig. 1).

Bone-marrow-derived mast cell lines were prepared from 6 week old COX-1 (−/−) and COX-2 (−/−) mice as described under Materials and Methods. The cell lines were further characterized by northern (Fig. 2A) and western (Fig. 2B) analyses for the expression of COX-1 and COX-2 gene products. As expected, (i) COX-1 message (Fig. 2A) and protein (Fig. 2B) are present in COX-2 (−/−) cells but are not detected in COX-1 (−/−) mast cells and (ii) there is no detectable COX-2 mRNA or protein expression in both cell lines prior to activation. Following activation of COX-1 (−/−) and COX-2 (−/−) mast cells by aggregation of their high affinity IgE receptors, COX-2 message and protein are detected

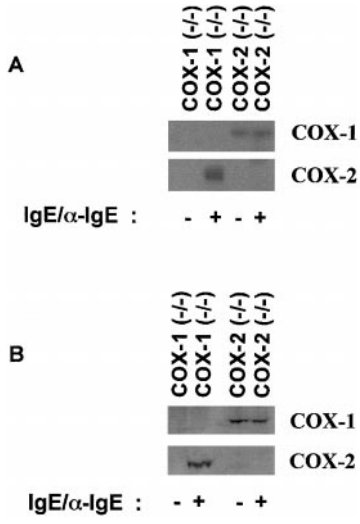


**FIG. 2.** COX-1 and COX-2 expression in COX-1 (−/−) and COX-2 (−/−) mast cells. A. IgE/α-IgE aggregation induces COX-2 message in COX-1 (−/−) mast cells but not in COX-2 (−/−) mast cells. Confluent cultures of COX-1 (−/−) and COX-2 (−/−) mast cells were activated as described under Materials and Methods. Four hours later cells were harvested and total RNA was isolated. 10 μg of total RNA from each sample was subjected to electrophoresis on a 1% agarose gel. RNA was transferred to nitrocellulose, hybridized with labeled cDNA probes, and subjected to autoradiography. B. IgE/α-IgE aggregation induces COX-2 protein in COX-1 (−/−) mast cells but not in COX-2 (−/−) mast cells. COX-1 (−/−) and COX-2 (−/−) mast cells were activated as described under Materials and Methods. Four hours later, cells were harvested and analyzed for COX-1 and COX-2 protein by Western analysis as described under Materials and Methods.

in the COX-1 (−/−) mast cells but not in the COX-2 (−/−) mast cells.

*Utilization of exogenous arachidonic acid for the synthesis of $PGD_2$ by unstimulated COX-1 (−/−) and COX-2 (−/−) mast cells.* To test cyclooxygenase activity in unstimulated COX-1 (−/−) and COX-2 (−/−) mast cells, we added arachidonic acid (10 μm) to the medium and measured the levels of $PGD_2$ in the supernatants after 10 min. Neither COX-1 (−/−) nor COX-2 (−/−) cells produced $PGD_2$ in the absence of exogenous arachidonic acid. Unstimulated COX-2 (−/−) cells secreted 2 ± 0.2 ng/ml of $PGD_2$ into the medium after 10 min of incubation with arachidonic acid (Fig. 3). These values were very similar to prostaglandin synthesis from both the MMC-34 mast cell line and from mast cells prepared from B6 mice (16). In contrast, COX-1 (−/−) cells failed to produce $PGD_2$, even when provided with exogenous arachidonic acid. These data further demonstrate that mast cells derived from COX-1 (−/−) mice have neither a functional COX-1 protein nor an alternative means of producing prostaglandin prior to stimulation.

*Time course of $PGD_2$ synthesis in activated COX-1 (−/−) and COX-2 (−/−) mast cells.* If COX-1 is involved only in the immediate phase of $PGD_2$ synthesis,

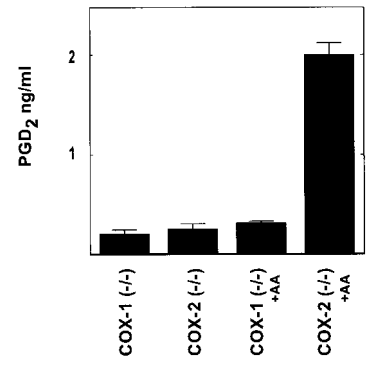


**FIG. 3.** Exogenous arachidonic acid can be converted to $PGD_2$ by unstimulated COX-2 (−/−) mast cells but not by unstimulated COX-1 (−/−) mast cells. COX-1 (−/−) and COX-2 (−/−) mast cells were cultured for 4 h. Cells were washed and plated in fresh medium with or without arachidonic acid (10 μm). Ten minutes later, cell culture supernatants were collected and centrifuged at 4000 rpm for 5 min at 4°C. The supernatants were analyzed for $PGD_2$ concentrations. This experiment was repeated three times with similar results.

we expect to see only a delayed phase of $PGD_2$ synthesis in COX-1 (−/−) mast cells following activation. On the other hand, if COX-2 is involved only in the delayed phase of $PGD_2$ synthesis we expect to see the immediate, but not the delayed phase of $PGD_2$ synthesis in COX-2 −/− mast cells following activation. To test this proposal, COX-1 (−/−), COX-2 (−/−) and MMC-34 mast cells were activated with IgE/α-IgE. $PGD_2$ accumulation in the supernatants of these activated cells was analyzed at various times (Fig. 4). $PGD_2$ accumulation in MMC-34 mast cells occurs in two temporal phases (Fig. 4), as reported previously (11). For activated mast cells from COX-2 (−/−) mice, an immediate phase of $PGD_2$ synthesis is also observed within 10 min

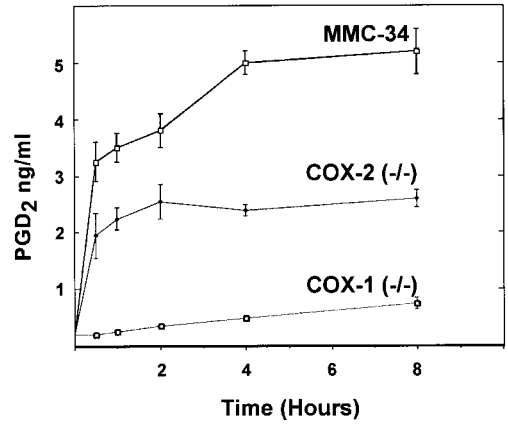


**FIG. 4.** Time course of $PGD_2$ synthesis in MMC-34, COX-1 (−/−) and COX-2 (−/−) mast cells. MMC-34, COX-1 (−/−) and COX-2 (−/−) mast cells were passively sensitized for 1 h with IgE (1 μg/ml) and then treated with 1 μg/ml of anti-IgE. Media were collected at the times shown and $PGD_2$ concentrations were determined. Data are represented as average ± SD of three dishes.

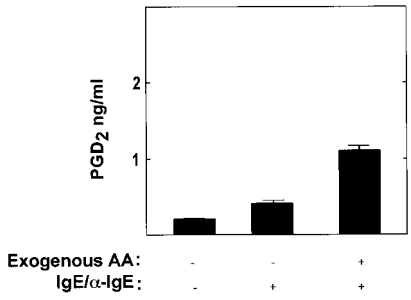


**FIG. 5.** COX-1 (−/−) mast cells do have a functional COX-2 protein. COX-1 (−/−) mast cells were activated as described under Materials and Methods. Following activation, arachidonic acid (10 $\mu$m) was added for 10 min to some of the plates. Supernatants from control cells (no addition), activated cells and cells that were activated and treated with arachidonic acid, were analyzed for $PGD_2$ concentrations. This experiment was repeated three times with similar results.

after activation. However, unlike the MMC-34 mast cells, there was no further increase in $PGD_2$ up to 8 h after activation. In contrast, an immediate phase of $PGD_2$ accumulation does not occur during the first several hours after activation of mast cells derived from COX-1 (−/−) mice. Although there is a small amount of $PGD_2$ produced at later times in activated mast cells derived from COX-1 (−/−) mice (~0.3 ng/ml), the delayed phase of $PGD_2$ production in activated MMC-34 mast cells is substantially greater (~1–2 ng/ml).

*COX-1 (−/−) mast cells do have a functional COX-2 protein.* Although the production of $PGD_2$ in the delayed phase of activation is not extensive in mast cells derived from COX-1 (−/−) mice, both COX-2 message and protein are induced within 4 h following activation of COX-1 (−/−) mast cells (Fig. 2). To test whether the COX-2 protein made in the COX-1 (−/−) cells is functional, COX-1 (−/−) mast cells were activated for 4 h and synthesis of $PGD_2$ was measured after a 10-min incubation with exogenously added arachidonic acid (Fig. 5). As observed previously (Fig. 4), activated COX-1 (−/−) mast cells show only a small increase in $PGD_2$ synthesis and accumulation in the absence of exogenous arachidonic acid. However, in the presence of exogenous arachidonic acid, the same cells are able to synthesize substantial amounts of $PGD_2$, suggesting that COX-2 enzyme in these mast cells is functional. The presence of COX-2 message and protein (Figs. 2A and 2B) and a functional COX-2 enzyme activity suggest that lack of a substantial delayed phase of $PGD_2$ synthesis in COX-1 (−/−) mast cells is likely to result from factors other than activation-induced expression of a functional COX-2 enzyme (see Discussion).

*Effect of $sPLA_2$ inhibitors on $PGD_2$ accumulation in activated COX-2 (−/−) mast cells.* We have previously shown that the immediate phase of $PGD_2$ synthesis in activated mast cells is mediated by an $sPLA_2$-dependent pathway (20). To test whether $sPLA_2$ is necessary in COX-2 (−/−) mast cells for the immediate phase of $PGD_2$ synthesis, COX-2 (−/−) mast cells were activated in the presence of the $sPLA_2$ inhibitors SB 203347 (10 $\mu$M) and 12-episcalaradial (1 $\mu$M). $PGD_2$ accumulation is blocked in the presence of either inhibitor (Fig. 6), suggesting that $sPLA_2$ is involved in the immediate phase of $PGD_2$ synthesis in activated mast cells.

## DISCUSSION

We (11) and others (13) have previously shown that there are two distinct phases of prostaglandin synthesis in activated mast cells. Pharmacologic studies with cyclooxygenase inhibitors (11), glucocorticoids (11, 21) and antisense oligonucleotides (16) suggested that the immediate phase of prostaglandin production in activated mast cells is dependent on constitutive COX-1 and that the delayed phase of prostaglandin production in these cells is dependent on induced COX-2 expression. Pharmacologic, antisense and antibody inhibition studies to define the roles of the phospholipases that supply arachidonic acid to COX-1 in the immediate phase of prostaglandin production and to COX-2 in the delayed phase of prostaglandin production have lead to conflicting reports. We reported that the immediate phase of $PGD_2$ production in activated murine mast cells is inhibited by pharmacologic agents (11, 21), antisense oligonucleotides (16) and monoclonal antibodies (16, 21) for secretory phospholipase $A_2$, and also by pharmacologic agents that inactivate type IV cytoplasmic $PLA_2$ ($cPLA_2$) (20). We therefore concluded that COX-1 and both type V $sPLA_2$ and type IV $cPLA_2$ activity are required for the immediate phase of $PGD_2$

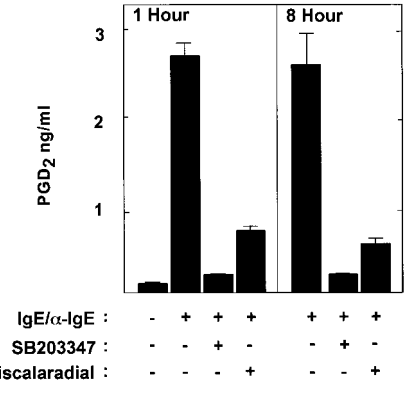


**FIG. 6.** Effect of secretory phospholipase $A_2$ inhibitors on $PGD_2$ synthesis in activated COX-2 (−/−) mast cells. COX-2 (−/−) mast cells were (i) untreated, (ii) activated with IgE/α-IgE, (iii) activated in the presence of SB203347 (10 $\mu$M) or (iv) activated in the presence of 12-episcalaradial (1 $\mu$m). Supernatants were collected after 1 and 8 h and analyzed for $PGD_2$ concentrations.

production in activated mast cells. Because, in our hands, only COX-2 inhibitors and $cPLA_2$ pharmacologic inhibitors blocked the delayed phase of $PGD_2$ synthesis in activated mast cells, we concluded (i) that COX-2 and $cPLA_2$ are required for the delayed phase of prostaglandin production and (ii) that $sPLA_2$ activity is not required. In contrast, Bingham *et al.* (19) reported that the immediate phase of $PGD_2$ synthesis in activated mast cells is not blocked by $sPLA_2$ inhibitors, and that the delayed phase of prostaglandin production is blocked by inhibitors of $sPLA_2$. Our laboratory and others (21) have now turned to mice deficient in cyclooxygenases and phospholipases as an alternative approach to define the enzyme pathways contributing to the immediate and delayed production of prostaglandins in activated mast cells.

*COX-1 is necessary for the immediate phase of $PGD_2$ synthesis in activated mast cells.* The complete lack of an immediate phase of $PGD_2$ synthesis in COX-1 (−/−) mast cells following activation (Fig. 4) clearly suggests a requisite role for COX-1 in the immediate phase of $PGD_2$ synthesis in activated mast cells. These data are in complete agreement with previous pharmacologic observations (11, 21) that COX-2 is not necessary for the immediate phase of $PGD_2$ synthesis in activated mast cells. Activated COX-2 (−/−) mast cells accumulate substantial $PGD_2$ in their media during the first 30 min following activation. There is no further increase or synthesis of $PGD_2$ in the following 8 h (Fig. 4). These data are strikingly similar to our previous results using NS-398, a COX-2 specific inhibitor (11). Our data with mast cells from COX-1(−/−) and COX-2(−/−) mast cells clearly suggest that the immediate phase of prostaglandin synthesis in activated mast cells is entirely COX-1 dependent; lack of COX-2 does not impair the immediate phase of $PGD_2$ synthesis in mast cells.

*The delayed phase of $PGD_2$ synthesis in activated mast cells is mediated by COX-2.* We observe only a minimal increase in the delayed phase of $PGD_2$ synthesis in activated COX-1 (−/−) mast cells, compared to the delayed phase of $PGD_2$ synthesis in MMC-34 mast cells. The lack of a substantial delayed phase of $PGD_2$ synthesis in COX-1 (−/−) mast cells is not due to the absence of COX-2 message or protein (Figs. 2A and 2B). Although, in the absence of exogenous arachidonic acid, there is only a minimal increase in $PGD_2$ accumulation 4 h after activation, COX-1 (−/−) mast cells are able to produce substantial $PGD_2$ when arachidonic acid is supplied exogenously (Fig. 5). These data suggest that (i) COX-2 protein synthesized following activation of COX-1 (−/−) mast cells is functional and (ii) that the absence of a substantial delayed phase of $PGD_2$ synthesis in the COX-1 (−/−) mast cells is due to factors other than the lack of a functional COX-2 protein.

Bingham *et al.* (19) report that mast cells isolated from wild type 129/sv mice do not demonstrate a delayed phase of $PGD_2$ synthesis when compared to mast cells derived from other strains. The mice used in the current studies are on a mixed 129Ola/C57Bl/6 background. Consistent with our observations, Fujishima *et al.* (21) report that mast cells prepared from mice that are on a mixed background (129/B6) are capable of producing a delayed phase of $PGD_2$ synthesis, albeit substantially less than the delayed phase in mast cells derived from BALB/c strain (21). Thus, strain differences that are independent of COX-2 production might be the reason for our inability to observe a substantial delayed phase of $PGD_2$ synthesis in activated COX-1 (−/−) mast cells. Isolating COX-1 (−/−) mast cells from a mouse strain that demonstrates a more robust delayed phase of $PGD_2$ production might help to resolve this problem.

Studies with genetically altered mice should help to clarify the roles of the phospholipases that provide arachidonic acid for COX-1 and COX-2 in the immediate and delayed phases of prostaglandin production in activated mast cells. Mast cells derived from mice disrupted in the type IIa $sPLA_2$ gene are still able to mount effective immediate and delayed $PGD_2$ responses following activation (16). Therefore, type II $sPLA_2$ does not play a role in prostaglandin production in activated murine mast cells. Because (i) agents that block the activity of type V $sPLA_2$ prevent the immediate phase of $PGD_2$ production in activated mast cells (16), (ii) type V $sPLA_2$ mRNA is plentiful in murine mast cells and (iii) antisense oligonucleotides specific for type V $sPLA_2$ block the immediate phase of $PGD_2$ accumulation in activated mast cells (16), we concluded that type V $sPLA_2$ is the key phospholipase releasing arachidonic acid for the immediate phase of prostaglandin production in activated mast cells. However, several new $sPLA_2$ genes have recently been described in both mouse and man (22–24). Consequently, it will be necessary to re-investigate the identity of the low-molecular-weight $sPLA_2$ enzyme(s) that mediate prostaglandin production in activated mast cells.

Mast cells derived from mice with a homozygous disruption of the type IV $cPLA_2$ gene are incapable of mounting either an immediate or a delayed prostaglandin response following activation (21). These data, derived from genetically manipulated animals, are in agreement with our original proposal, based on pharmacologic studies, that suggested a role for type IV $cPLA_2$ in both the immediate and delayed prostaglandin production following mast cell activation (20). Continued development of both pharmacologic inhibitors and murine strains deficient in specific enzymes of prostaglandin synthesis, as described in this report for COX-1 and COX-2 and in studies for type IV $cPLA_2$ (21), should clarify the interactions of the phospholipases and cyclooxygenases responsible for these dis-

crete periods of prostaglandin production in activated mast cells.

## ACKNOWLEDGMENTS

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