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CUTTING EDGE

Cutting Edge: Agonistic Effect of Indomethacin on a Prostaglandin D_2 Receptor, CRTH2

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Indomethacin is a widely used nonsteroidal anti-inflammatory drug and is generally known to exhibit its multiple biological functions by inhibiting cyclooxygenases or activating peroxisome proliferator-activated receptors. In this study, we present evidence demonstrating that the novel PGD_2 receptor chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) is another functional target for indomethacin. Indomethacin induced Ca^{2+} mobilization in CRTH2-transfected K562 cells at submicromolar concentrations (approximate EC_{50}, 50 nM) in a $G_{\alpha i}$-dependent manner as PGD_2 did. Other nonsteroidal anti-inflammatory drugs (aspirin, sulindac, diclofenac, and acemetacin) had no such effect even at micromolar concentrations. In chemotaxis assay, three CRTH2-expressing cell types, Th2 cells, eosinophils, and basophils, were all significantly attracted by indomethacin (EC_{50}, 50–500 nM) as well as by PGD_2 (EC_{50}, 2–20 nM), and the effects of indomethacin were blocked by anti-CRTH2 mAb. These results suggest the involvement of CRTH2 in mediating some of therapeutic and/or unwanted side effects of indomethacin, independently of cyclooxygenases and peroxisome proliferator-activated receptors. ***The Journal of Immunology*****, 2002, 168: 981–985.**

Prostanoids, including PGs and thromboxanes, are a series of metabolites of arachidonic acid and are known to exert a variety of physiological and pathophysiological functions through their specific receptors (1–3). PGD_2 is the major prostanoid produced by allergen-activated mast cells and has been implicated in various allergic diseases as a proinflammatory lipid mediator (3, 4). In contrast, PGD_2 is recently revealed to possess an anti-inflammatory property in some animal models (5, 6). The actual roles of PGD_2 in various inflammatory diseases are thus currently unclear. To date, two G protein-coupled receptors, PG D receptor (DP)[2] (3, 7) and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) (8), have been identified to serve as selective receptors for PGD_2. DP is coupled with Gαs-type G protein, induces cAMP generation, and is generally thought to be involved in relaxant/inhibitory activities of PGD_2 such as inhibition of platelet aggregation, relaxation of various smooth muscles, and inhibition of cell migration (3, 7–9). Moreover, DP was also shown to contribute to the formation of allergic inflammations by unknown mechanisms in studies with DP-deficient mice (10). In contrast to DP, CRTH2 is coupled with $G_{\alpha i}$-type G protein and is thought to be involved in stimulatory activities of PGD_2 such as induction of cell migration and up-modulation of adherent molecules (8, 11). CRTH2 is intriguing in that it is selectively expressed in allergy-related cell types, including Th2 cells, T cytotoxic 2 cells, eosinophils, and basophils, and in yet unidentified monocyte/dendritic cell-like leukocytes (12–15). However, exact roles of CRTH2 in vivo still remain to be examined due to the lack of selective agonists and antagonists.

Prostanoids including PGD_2 are synthesized in various tissues by the constitutive enzyme cyclooxygenase (COX)-1 and its inducible isoform COX-2 (16, 17). Indomethacin and other nonsteroidal anti-inflammatory drugs (NSAIDs) have the ability to inhibit the activity of COX, a property that accounts for their shared therapeutic effects (18). In the course of identification of PGD_2 as a sole CRTH2 agonist produced by mast cells (8), indomethacin was used to suppress PGD_2 production. We unexpectedly found that indomethacin by itself has an agonistic effect on CRTH2. In this paper, we present evidence that indomethacin appears to be unique among various NSAIDs in that it can effectively activate CRTH2 to induce cell migration in Th2 cells, eosinophils, and basophils, independently of its anti-COX activity.

It has been well appreciated that indomethacin and eicosatetraynoic acid, an inhibitor of lipoxygenases, can inhibit the binding of some chemotactic peptides to leukocytes at high concentrations (19, 20). Our finding is interesting in view of the clinical significance because it shows that a widely used inhibitor of COXs can stimulate rather than inhibit a chemoattractant receptor and actually lead to cell migration in particular cell types of leukocytes at usual therapeutic concentrations.

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[2] Abbreviations used in this paper: DP, PG D receptor; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; PPAR, peroxisome proliferator-activated receptor; PTX, pertussis toxin; CTX, cholera toxin; MCP-1, monocyte chemotactic protein-1.

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Materials and Methods

Reagents

Aspirin, diclofenac, sulindac, and acemetacin (1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid carboxy-methyl ester) were purchased from Sigma-Aldrich (St. Louis, MO). Indomethacin was purchased from Wako Pure Chemical (Osaka, Japan) and Sigma-Aldrich. PGD_2 was obtained from Cayman Chemicals (Ann Arbor, MI). Thrombin and monocyte chemotactic protein-1 (MCP-1) were obtained from Sigma-Aldrich and PeproTech (Rocky Hill, NJ), respectively. CRTH2-specific rat mAbs BM7 and BM16 were described previously (12, 13). PerCP-conjugated anti-CD4 mAb (clone Leu-3a) and FITC-conjugated anti-CD3 mAb (Lue-4) were purchased from BD Biosciences (San Jose, CA). PE-labeled streptavidin was obtained from Life Technologies (Long Island, NY).

Cells

K562 and Jurkat lines were transfected with a CRTH2 expression vector pRc/B19 (12), a DP expression vector pRc/DP (8), or a control vector pRc/CMV (Invitrogen, San Diego, CA) and cloned as described (12). Human Th1 and Th2 lines were generated as described in our previous report (12). PBMCs and granulocytes were isolated from heparinized peripheral blood of consenting healthy subjects by density gradient centrifugation on Mono-Poly Resolving Medium (Dainippon Pharmaceutical, Osaka, Japan). $CD16^-$ leukocytes were purified with anti-CD16 microbeads on MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) as described (8).

Ligand binding, Ca^{2+} mobilization, and chemotaxis assays

Ligand binding, Ca^{2+} mobilization, and chemotaxis assays were performed as described (8, 13). For inactivation of G proteins, cells (2.5×10^6/ml) were incubated in RPMI 1640 medium containing 10% FBS (growth medium) with 1 μg/ml of either pertussis toxin (PTX; Sigma-Aldrich) or cholera toxin (CTX; Sigma-Aldrich) at 37°C for 2 h. In some cases, cells were pretreated with 600 μg/ml of either BM7 or normal rat IgG for 20 min at room temperature before being subjected to the assays.

Receptor down-modulation assay

Th2 cells were incubated in growth medium with various concentrations of test compounds for 60 min at 37°C. The cells were washed twice with PBS and then stained with biotinylated BM16 and PE-labeled streptavidin, along with PerCP-conjugated mAb to CD4 and FITC-labeled mAb to CD3, as described (13). The stained cells were analyzed by three-color flow cytometry on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences).

Results

Induction of Ca^{2+} mobilization by indomethacin in CRTH2-transfected K562 cells

As reported previously (8), PGD_2 induced Ca^{2+} mobilization in both CRTH2-transfected (K562/CRTH2) and DP-transfected (K562/DP) K562 cells at nanomolar concentrations (EC_{50}, 1–5 nM) but not in mock-transfected K562 (K562/neo) cells even at 2.5 μM (Fig. 1*A*). Indomethacin induced Ca^{2+} mobilization in K562/CRTH2 cells at submicromolar concentrations (approximate EC_{50}, 50 nM) with around one order of magnitude lower potency than that of PGD_2; however, it induced no significant Ca^{2+} mobilization in K562/DP and K562/neo cells even at 2.5 μM (Fig. 1*A*). Similar results were obtained in transient transfection experiments with the parental K562 line (data not shown). Four different lots of indomethacin samples (the purity of each was >99%) showed identical potencies, and following their reversed-phase chromatography purification on μRPC C2/C18 SC 2.1/10 column (Amersham Pharmacia Biotech, Uppsala, Sweden), the agonist activity was exclusively confined in the major peak fraction of each indomethacin sample, indicating that indomethacin by itself was the active substance. Interestingly, no Ca^{2+} mobilization was induced in K562/CRTH2 cells by up to 10 μM of other NSAIDs tested (aspirin, dichlofenac, acemetacin, and sulindac) or of an indomethacin analog indoleacetic acid (Fig. 1*A* and data not shown). Furthermore, prior inactivation of COXs by aspirin (500 μg/ml) or diclofenac (500 μg/ml) did not inhibit the indomethacin-induced Ca^{2+} mobilization in K562/CRTH2 cells (Fig. 1*A* and data not shown). These results indicated that COX products were not involved in this system.

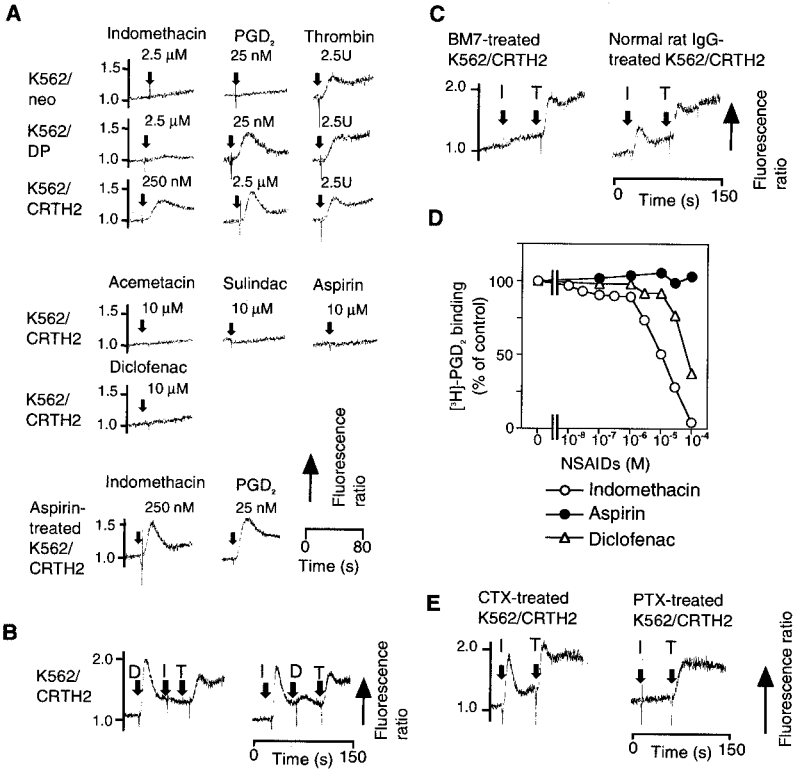

FIGURE 1. CRTH2 mediates indomethacin-induced Ca^{2+} mobilization in K562/CRTH2 cells. *A*, Abilities of PGD_2 and various NSAIDs to induce Ca^{2+} mobilization in K562 transfectants. Thrombin was used as an irrelevant stimulant. Arrows indicate the time of stimulant addition. For blocking COXs, cells were incubated with aspirin at 500 μg/ml for 1 h at 37°C before use. *B*, Cross-desensitization effects between PGD_2 and indomethacin in K562/CRTH2 cells. D, I, and T represent 250 nM of PGD_2, 2.5 μM of indomethacin, and 2.5 U/ml of thrombin, respectively. *C*, Specific inhibition of indomethacin-induced Ca^{2+} mobilization by anti-CRTH2 mAb BM7. I and T represent 250 nM of indomethacin and 2.5 U/ml of thrombin, respectively. *D*, Competitive inhibition of $[^3H]PGD_2$ (1 nM) binding to CRTH2 by NSAIDs. Data points are the mean of duplicate experimental values and are representative data from more than two experiments giving similar results. Acemetacin and sulindac showed similar inhibitory potencies to diclofenac (data not shown). *E*, Indomethacin-induced Ca^{2+} mobilization is $G_{\alpha i}$ dependent. I and T represent 250 nM of indomethacin and 2.5 U/ml thrombin, respectively.

Having once responded to PGD_2, K562/CRTH2 cells did not show any considerable increases in Ca^{2+} mobilization by indomethacin, and vice versa (cross-desensitization; Fig. 1*B*). A neutralizing anti-CRTH2 mAb BM7 completely blocked the indomethacin-dependent Ca^{2+} mobilization (Fig. 1*C*). In addition, indomethacin indeed inhibited [^{3}H]PGD_2 binding to K562/CRTH2 cells with the lowest IC_{50} value (8.1 ± 1.9 μM) among NSAIDs examined (Fig. 1*D*). Diclofenac, acemetacin, and sulindac showed around one order of magnitude higher IC_{50} values than that of indomethacin, and aspirin had no effect at up to 100 μM in the same assay (Fig. 1*D*). Furthermore, the Ca^{2+} mobilization effect of indomethacin was selectively inhibited by a Gαi inhibitor PTX but not by a Gαs inhibitor CTX, as that of PGD_2 (Fig. 1*E* and Ref. 8).

Induction of Ca^{2+} mobilization and CRTH2 down-modulation by indomethacin in Th2 cells

Cultured Th2 but not Th1 cells constitutively expressed CRTH2 (12) and showed Ca^{2+} mobilization in response to indomethacin as well as to PGD_2 (Fig. 2*A*). The effect of indomethacin was selectively inhibited by BM7 (Fig. 2*B*) and was again sensitive to PTX but not to CTX (data not shown). We further confirmed the selective interaction between CRTH2 and indomethacin by a ligand-induced receptor down-modulation assay. As shown in Fig. 2*C*, surface CRTH2 expression was selectively down-modulated by PGD_2 and indomethacin at nanomolar to micromolar concentrations without any significant change in the levels of other irrelevant cell surface molecules, CD4 and CD3 (data not shown).

Chemotactic responses of CRTH2-expressing cells to indomethacin

Previous studies showed that CRTH2 mediates PGD_2-dependent cell migration (8). Therefore, we next examined effects of indomethacin on cell migration using four different CRTH2-expressing cell types. CRTH2-transfected Jurkat cells (Jurkat/CRTH2) were indeed attracted by indomethacin and PGD_2 (approximate EC_{50},

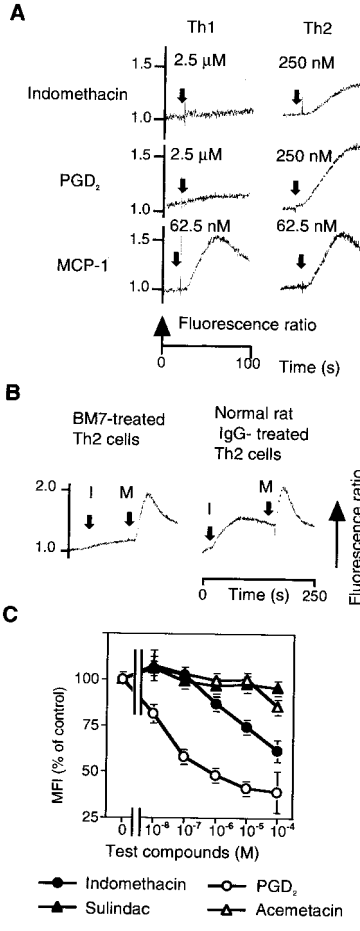

FIGURE 2. Indomethacin activates Th2 cells via CRTH2. *A*, Induction of Ca^{2+} mobilization in Th2 cells by PGD_2 and indomethacin. MCP-1 was used as an irrelevant stimulant. *B*, Specific inhibition of indomethacin-induced Ca^{2+} mobilization in Th2 cells by anti-CRTH2 mAb BM7. I and M represent 250 nM of indomethacin and 62.5 nM of MCP-1, respectively. *C*, Down-modulation of surface CRTH2 expression in Th2 cells by PGD_2, indomethacin, and indomethacin derivatives as determined by flow cytometry. MFI, Mean fluorescence intensity (mean ± SD, $n = 3$).

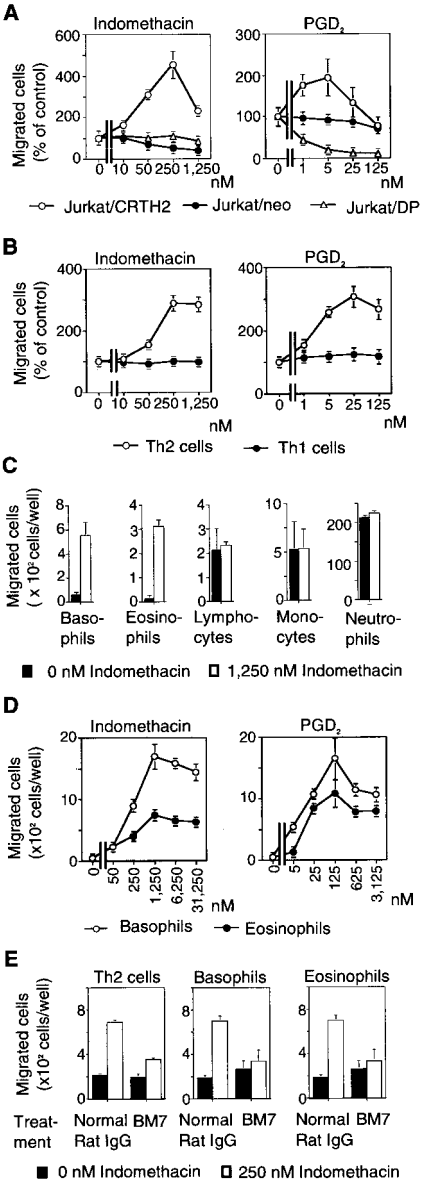

FIGURE 3. Indomethacin induces cell migration via CRTH2. *A*, Chemotactic responses of Jurkat/CRTH2 and Jurkat/DP cells to indomethacin and PGD_2 (mean ± SD, $n = 3$). *B*, Chemotactic responses of Th1 and Th2 cells to indomethacin and PGD_2 (mean ± SD, $n = 3$). *C*, Chemotactic responses of peripheral blood leukocytes to indomethacin (mean ± SD, $n = 3$). *D*, Chemotactic responses of eosinophils and basophils to indomethacin and PGD_2 (mean ± SD, $n = 3$). This experiment was performed using CD16$^-$ peripheral blood leukocytes as previously described (8). *E*, Specific inhibition of indomethacin-dependent migration of Th2 cells, basophils, and eosinophils by BM7.

50 nM and 1 nM, respectively) but mock-transfected Jurkat cells (Jurkat/neo) were not (Fig. 3*A*). DP-transfected Jurkat cells (Jurkat/DP) were suppressed in their spontaneous migration by PGD_2, as shown previously (8), but not by indomethacin (Fig. 3*A*). Indomethacin and PGD_2 also induced chemotactic migration of Th2 but not Th1 cells at submicromolar concentrations (EC_{50}, 50–100 nM and 2–3 nM, respectively; Fig. 3*B*). When total peripheral blood leukocytes were examined, only basophils and eosinophils were significantly attracted by indomethacin at 1.25 μM or lower concentrations as well as by PGD_2 (EC_{50}, 300–500 nM for indomethacin and 10–20 nM for PGD_2; Fig. 3, *C* and *D*). A neutralizing anti-CRTH2 mAb BM7 but not control rat IgG significantly blocked the migratory responses to indomethacin of Th2 cells, basophils, and eosinophils (Fig. 3*E*). Similar results were obtained in three independent pairs of Th1 and Th2 lines and leukocytes from two different donors. Taken together, these results demonstrate that, like PGD_2, indomethacin induces cell migration via CRTH2.

Discussion

Indomethacin is clinically used for its potent anti-inflammatory, antipyretic, and analgesic properties, although it also has unwanted side effects causing various complications such as gastrointestinal injury (21). Both of such pharmaceutical and unwanted side effects of indomethacin are primarily ascribed to its potent inhibitory activity against COXs (22). However, recent studies revealed that some of the indomethacin actions are independent of COXs. For example, antineoplastic action of indomethacin was observed in the absence of COXs (23). Furthermore, indomethacin directly binds and activates peroxisome proliferator-activated receptor (PPAR)γ and, possibly, PPARα and induces adipocyte differentiation at micromolar concentrations (24).

In this study we present several lines of evidence to demonstrate that indomethacin can directly activate CRTH2 independently of COXs and PPARs. First, induction of Ca^{2+} mobilization and/or chemotaxis by submicromolar concentrations of indomethacin was consistently observed in CRTH2-expressing cells regardless of cell types, while it was not observed in cells lacking CRTH2. Second, such effects of indomethacin were always blocked by an anti-CRTH2 mAb BM7, or the effects were completely canceled by prior desensitization with natural CRTH2 ligand PGD_2. Conversely, binding of [^{3}H]PGD_2 to CRTH2 was specifically inhibited by indomethacin. These findings demonstrate the direct interaction between indomethacin and CRTH2. Third, other potent COX inhibitors such as aspirin and diclofenac (22) showed little, if any, effect even at 10 μM, and prior inactivation of COXs could not affect indomethacin actions. These findings demonstrate lack of contribution of COXs to this system and uniqueness of indomethacin among tested NSAIDs, which may imply that the CRTH2 mechanisms could not contribute significantly to a wide range of adverse effects of indomethacin that are common to other NSAIDs. Finally, the reported concentrations of indomethacin required to induce adipocyte differentiation via PPARs (10–100 μM) (24) are two to three orders of magnitude higher than that required to induce Ca^{2+} mobilization or chemotaxis in CRTH2-expressing cells. Moreover, in the immune system, activation of PPARγ is generally associated with an inhibitory nature as shown in monocytes (25) and Th cells (26).

At present, clinical significance of the indomethacin-triggered CRTH2 activation is unclear. It was reported that IC_{50} values for indomethacin on COX-1 and COX-2 in intact cells were 0.01 μM and 0.6 μM, respectively (16), and that the peak plasma level of indomethacin after the usual therapeutic dose of 25 mg in humans was 0.8 μg/ml (2.2 μM) (21). In this study, we demonstrated that indomethacin can fully activate CRTH2 at submicromolar concentrations in Th2 cells, basophils, and eosinophils. Therefore, it is conceivable that therapeutic concentrations of indomethacin in tissues may be on levels sufficient to activate CRTH2. The direct effects of indomethacin in vivo on Th2 cells, eosinophils, or basophils have not been reported. It is interesting to learn the observation that infiltration of mucosal epithelium by eosinophils was one of the earliest histological features of indomethacin-induced intestinal injury in rats (27). Furthermore, in vitro studies showed that, like PGD_2, indomethacin can enhance Ag-induced histamine release from human basophils at submicromolar concentrations (28). The findings may be simply explained by the action of indomethacin on CRTH2. In contrast, PGD_2 has recently been shown to have an anti-inflammatory property in rats (5, 6). The action of PGD_2 was suggested to be mediated by DP because a DP-specific agonist, BW245C, could mimic the PGD_2 effect (6). Indomethacin, an agonist for CRTH2 but not for DP, had no such anti-inflammatory effect in the same report (6). These findings suggest that CRTH2 is not responsible for PGD_2-dependent anti-inflammatory action. We thus further speculate a concept that PGD_2 exhibits its inhibitory functions via DP and its stimulatory functions through CRTH2 (8, 11), even though the clinical consequences of CRTH2 activation remains largely to be elucidated.

Our present study, which indicates that indomethacin is a potent agonist for CRTH2, provides new insights into the pharmacological actions of indomethacin and, furthermore, the physiological and pathophysiological functions of CRTH2. Studies, especially with specific CRTH2 antagonists or CRTH2-deficient mice, are required to clarify the clinical significance of the CRTH2-mediated activities.

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