JPET Fast Forward, Published on April 29, 2003 as DOI:10.1124/jpet.103.050955

JPET #50955

Expression and molecular pharmacology of the mouse CRTH2 receptor

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JPET #50955

Running Title:

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text pages: 33

tables: 2

figures: 5

references: 40

Words in the abstract: 230

Words in the introduction: 553

Words in the discussion: 1484

Abbreviations:

$[cAMP]_i$: intracellular cAMP

COX: cyclooxygenase

CRTH2: chemoattractant receptor-homologous molecule expressed on Th2 cells

DK-PGD_2: 13,14-dihydro-15-keto-PGD_2

DP: D prostanoid receptor

GPCR: G-protein coupled receptor

IBMX: isobutylmethylxanthine

NSAID: non-steroidal anti-inflammatory drug

OVA: ovalbumin

PGD_2: prostaglandin D_2

15d-PGJ_2: 15-deoxy-$\Delta^{12,14}$-PGJ_2

ponA: ponasterone A

PI 3-kinase: phosphatidylinositol 3-kinase

PPAR: peroxisome proliferator-activated receptor

PTX: pertussis toxin

Section: Cellular and Molecular

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Abstract

Prostaglandin D_2 (PGD_2), the predominant prostanoid produced by activated mast cells, is implicated in a variety of allergic diseases. PGD_2 exerts its effects through two G-protein coupled receptors, DP and CRTH2. PGD_2 mediates chemotaxis of eosinophils, basophils and Th2 cells via CRTH2-evoked signaling, suggesting a role for this receptor in allergic disease. To characterize the mouse CRTH2 ortholog (mCRTH2), we amplified the mCRTH2 receptor gene and expressed it in HEK293 cells. Saturation ligand binding isotherms demonstrated high affinity binding of [^{3}H]PGD_2 with a K_d of 8.8 ± 0.8 nM. Competition binding assays with a panel unlabeled prostanoids demonstrated an order of affinity of 13,14-dihydro-15-keto-PGD_2 (DK-PGD_2) ≥ 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) ≥ PGD_2 ≥ PGJ_2. [^{3}H]PGD_2 binding was also displaced by the Non-Steroidal Anti-Inflammatory Drug (NSAID) indomethacin, with a K_i value of 1.04 ± 0.13 μM. No [^{3}H]PGD_2 displacement was detected using fluribrofen, ibuprofen or aspirin as competitors at concentrations of up to 30 μM. PGD_2, DK-PGD_2, 15d-PGJ_2 and indomethacin each inhibited intracellular cAMP generation in stable transfectant ER293/mCRTH2 cells through a pertussis toxin (PTX) sensitive pathway, consistent with mCRTH2 coupling to a G_i heterotrimeric G-protein. Activation of mCRTH2 elicited chemotaxis of ER293/mCRTH2 cells in response to PGD_2, indomethacin and 15d-PGJ_2. mCRTH2-dependent chemotaxis was inhibited by PTX and wortmannin, indicating dependence on G_i and PI 3-kinase signal transduction pathways. These data provide the first pharmacological and functional characterization of the mouse CRTH2 receptor.

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Prostaglandin D_2 (PGD_2) is the predominant prostanoid produced by activated mast cells and has been implicated in the pathogenesis of allergic diseases such as allergic asthma and atopic dermatitis (Lewis et al., 1982; Hardy et al., 1984; Murray et al., 1986; Barr et al., 1988). Increased production of PGD_2 leads to elevated Th2-type cytokines and eosinophilic inflammation in the murine ovalbumin (OVA)-induced experimental asthma model (Fujitani et al., 2002), while administration of PGD_2 to the canine trachea leads to accumulation of lumenal eosinophils (Emery et al., 1989). However, the molecular mechanism of PGD_2 in the pathogenesis of allergic disease remains only partially characterized.

PGD_2 exerts its effects through two G-protein coupled receptors (GPCRs), DP and CRTH2. The DP receptor, a member of the prostanoid sub-family of GPCRs, couples to the G_s-type G protein, and activation of this receptor leads to increases in intracellular cAMP ($[cAMP]_i$) and calcium (Hirata et al., 1994; Boie et al., 1995). In contrast, CRTH2 shows greatest sequence similarity to chemoattractant GPCRs, and CRTH2-evoked responses include inhibition of $[cAMP]_i$ and increases in intracellular calcium via G_i-dependent pathways (Hirai et al., 2001; Sawyer et al., 2002). CRTH2 has been recently shown to mediate PGD_2-stimulated chemotaxis of Th2 cells, eosinophils and basophils, suggesting that CRTH2 may play a pro-inflammatory role in allergic disease (Hirai et al., 2001). Indeed, increased numbers of circulating T cells expressing CRTH2 have been correlated with severity of atopic dermatitis (Cosmi et al., 2000).

In vivo, PGD_2 undergoes degradation to form J-series cyclopentenone prostaglandins such as 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) (Shibata et al., 2002). 15d-PGJ_2 has been the subject of intense investigation since it was discovered to bind and activate the peroxisome proliferator-activated receptor-γ (PPARγ) and promote adipocyte differentiation, albeit at concentrations in the micromolar range (Kliewer et al., 1995). Several PPARγ-independent actions of 15d-PGJ_2

have also been described including activation of MAP kinase (Lennon et al., 2002), induction of apoptosis (Ward et al., 2002), and upregulation of IL-8 expression in T cells (Harris et al., 2002). Recently, 15d-PGJ_2 has also been shown to be an agonist at the human CRTH2 receptor (Sawyer et al., 2002) and activate eosinophils *in vitro* (Monneret et al., 2002), suggesting that activation of CRTH2 may be responsible for some of the PPARγ-independent effects of 15d-PGJ_2. Interestingly, the affinity of 15d-PGJ_2 for CRTH2 is several orders of magnitude greater than for PPARγ, with binding and activation occurring at low nanomolar concentrations. At this time, however, the precise role 15d-PGJ_2 plays in inflammatory processes is not clear.

Although the sequence of the mouse CRTH2 receptor ortholog (mCRTH2) has been reported, its pharmacology and function are uncharacterized (Abe et al., 1999). Given the importance of *in vivo* mouse models such as the OVA-induced experimental asthma model in elucidating the molecular pathogenesis of allergic asthma, characterization of mCRTH2 is essential to understanding its role in allergic airway inflammation. In this study, we describe initial pharmacological and functional characterization of mCRTH2. Radioligand binding experiments reveal that mCRTH2 binds PGD_2 and PGD_2 metabolites with high affinity, as well as indoleacetic acid based Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) such as indomethacin. Activation of mCRTH2 expressed in ER293 cells activates the classical G_i-coupled pathway resulting in a reduction of $[cAMP]_i$ levels. Furthermore, mCRTH2 is capable of mediating chemotaxis of ER293/mCRTH2 cells in response to mCRTH2 agonists via G_i and PI 3-kinase dependent pathways.

Methods

Reagents

Prostaglandins and BW245C were purchased from Cayman Chemicals (Ann Arbor, MI). Isoproterenol, IBMX, indomethacin, sulindac, aspirin, salicylate and acetaminophen were from Sigma (St. Louis, MO). [^{3}H]PGD_2 was purchased from Amersham Biosciences (Piscataway, NJ). Pertussis toxin and wortmannin were purchased from Calbiochem (La Jolla, CA). DMEM, Opti-MEM I and hygromycin B were from Invitrogen (Carlsbad, CA). FBS was obtained from Hyclone (Logan, UT). G418 was purchased from Mediatech (Herndon, VA). L-glutamine and penicillin/streptomycin were from BioWhittaker (Walkersville, MD). Ponasterone A was purchased from Stratagene (La Jolla, CA).

Construction of pEGSH/mCRTH2 and pRc/CMV/mCRTH2 expression vectors

The full length mCRTH2 coding exon was amplified by PCR from mouse embryonic stem cell genomic DNA (129SvEv) using the primers 5'-CATATGGCCAACGTCACACTGAAG-3' (sense) and 5'-CTCCAGGGTGTCTCCCAGACT-3' (anti-sense) and ligated into the pCRII vector (Invitrogen). The coding region sequence was verified by sequencing and was identical to the previously published sequence (Abe et al., 1999). The mCRTH2 coding exon was sequentially subcloned into NotI/SacI in the pEGSH (Stratagene) and NotI/XbaI in the pRc/CMV (Invitrogen) mammalian expression vectors.

Expression of mCRTH2 in HEK293 and ER293 cells

Cells were maintained at 37^{o}C in humidified air containing 5.5% CO_2 in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 units ml^{-1} penicillin, 100 μg ml^{-1}

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streptomycin (media for ER293 cells also contained 300 μg/ml G418). HEK293 cells were transiently transfected with pRc/CMV/mCRTH2 or pRC/CMV using Lipofectamine 2000 (Invitrogen). ER293 cells (Stratagene) were transfected with pEGSH/mCRTH2 or pEGSH and cells expressing CRTH2 were selected by addition of media containing 100 μg/ml hygromycin B at 48 hours post-transfection. Clonal cell lines were selected by two rounds of manual colony isolation using cloning rings. Expression of mCRTH2 was induced by addition of 10 μM ponasterone A (ponA) 24 hours prior to harvesting cells and verified by radioligand binding.

Preparation of membranes

Membranes for radioligand binding experiments were harvested 48 hours post-transfection. Cells were rinsed once with ice-cold PBS containing 1 mM EDTA and lysed by scraping in lysis buffer (15 mM HEPES, pH 7.6, 5 mM EDTA, 5 mM EGTA, 2 mM PMSF). and passage through a 21 ga needle five times. To collect membranes, the cell lysate was layered on a 60% sucrose cushion and centrifuged at 150,000 x *g* for one hour at 4^{o}C. The membrane fraction was passed through a 26 ga needle five times and frozen at –80^{o}C. Membranes from stable transfectant cell lines ER293/mCRTH2 and ER293/pEGSH were prepared following incubation of the cells with 10 μM ponA for 24 hours.

Radioligand binding assay

Membranes were incubated with [^{3}H]PGD_2 at 4^{o}C for 1.5 hours in binding buffer (25 mM HEPES (pH7.4), 1 mM EDTA, 5 mM $MgCl_2$, 140 mM NaCl, 5 mM KCl). The binding reaction was terminated by the addition of 3 ml ice-cold binding buffer and rapidly filtered under vacuum over Whatman GF/F filters. Filters were washed three times with 3 ml ice-cold binding

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buffer, dried and counted in 4 ml Ultima Gold scintillation fluid (Packard Biosciences, Groningen, The Netherlands). For saturation binding experiments, non-specific binding was determined in the presence of 10 μM 13,14-dihydro-15-keto-PGD_2 (DK-PGD_2). Competition binding experiments were performed in the presence of 3 nM [^{3}H]PGD_2 and varying concentrations of competing ligands.

Intracellular Ca^{2+} assay

ER293/mCRTH2 cells were plated in 96-well plates (50,000 cells/well) and mCRTH2 expression was induced with 10 μM ponA for 24 hours. Mobilization of intracellular calcium was measured on a FLEXstation system (Molecular Devices, Sunnyvale, CA) using the FLEXstation Calcium Assay Kit according to the manufacturers instructions (Molecular Devices). Briefly, cells were labeled with Calcium Assay Reagent resuspended in Hanks BSS/20 mM HEPES, pH 7.4, for one hour at 37^{o}C prior to measurement. PGD_2, indomethacin and carbachol were added to parallel wells in a volume equivalent to 10% of the final well volume while fluorescence was monitored, λ_{ex} = 485 nm, λ_{em} = 525 nm. In each case, the experiment was terminated by addition of 10 μM ionomycin to determine maximum Ca^{2+} response.

$[cAMP]_i$ assay

ER293/mCRTH2 cells were grown to 80% confluence in 6-well plates and incubated for 24 hours in the presence of 10 μM ponA. Thirty minutes prior to addition of ligands, media was replaced with Opti-MEM I containing 0.5 mM IBMX. Cells were incubated with ligands for 15 minutes, washed once with PBS, and the reaction was terminated by the addition of 0.1 M HCl.

Cells were scraped free and the resulting cell suspension was centrifuged for 10 minutes at 1000 x *g*. Supernatants were assayed for protein content by BCA assay (Pierce, Rockford, IL). After normalization to protein content, $[cAMP]_i$ levels were determined by an enzyme-linked immunoassay according to the manufacturer's instructions (Cayman Chemical).

Cell migration assay

ER293/mCRTH2 cells were incubated with 10 μM ponA for 24 hours prior to harvesting. Cells were trypsinized, washed three times in PBS and resuspended in DMEM. Cells (100,000) were added to the upper chamber of 24-well 0.8 μm polycarbonate transwell inserts (Costar, Cambridge, MA) that had been previously treated overnight with 5 μg/ml Matrigel (BD Biosciences, Bedford, MA) in PBS at 4°C and blocked in the presence of 2% BSA in PBS for one hour at 37°C. Ligands were diluted in DMEM and added to the lower chamber. After incubating for 4 hours at 37°C, inserts were removed and cells adhering to the top of the membrane were removed with a cotton swab. Cells on the bottom of the membrane were fixed with 3.7% formaldehyde for one hour, washed twice with PBS and stained overnight with crystal violet. For each insert, five independent fields were counted in blinded fashion at 200x magnification. In some studies, cells were incubated for 12 hours with 100 ng/ml pertussis toxin prior to harvesting. In other studies, cells were treated with 100 nM wortmannin for 10 minutes, which was maintained at the indicated concentration throughout the chemotaxis assay. For chemokinesis control experiments, 100 nM PGD_2 was added to both sides of the transwell membrane, and transwell inserts were processed and counted as above.

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Data analysis

All data are presented as the mean ± S.E.M. K_d and B_{max} values for saturation isotherm radioligand binding experiments were calculated based on a one-site binding model using Prism 3.0 software (GraphPad Software, Inc., San Diego, CA). Competition binding curves and IC_{50} values were calculated using a one-site competition model: Y (fraction bound) = (*max*+(*max*-*min*))/(1+10^(X-log(*IC*$_{50}$))) where X = log[*competitor*] (Prism). K_i values were calculated according to the method of Cheng and Prusoff (Cheng and Prusoff, 1973). EC_{50} values for $[cAMP]_i$ dose-response experiments were calculated using a fixed slope sigmoidal dose-response model: Y (% max stimulation) = (*max*+(*max*-*min*))/(1+10^(log(*EC*$_{50}$)-X)) where X = log[*agonist*] (Prism). Differences between means were tested for statistical significance using Dunnett's multiple comparison test (ANOVA) for inhibitor and dose-response chemotaxis experiments and two-tailed unpaired t-test for all other comparisons, with $P < 0.5$ considered significant (InStat 3 software, GraphPad).

Results

Cloning of mCRTH2

To characterize the mouse CRTH2 receptor, we amplified the coding region from mouse genomic DNA by PCR using 5' sense and 3' antisense primers based on the published sequence of mCRTH2 (Abe et al., 1999). The resulting fragment contained an 1149 base pair intronless open reading frame that was identical in sequence to the previously published sequence (GenBank NM 009962) as well as an equivalent region of sequence in the Celera mouse genome database.

Radioligand binding

To characterize mCRTH2 ligand binding, membranes were prepared from HEK293 cells that had been transiently transfected with pRc/CMV/mCRTH2. Saturation isotherm binding experiments revealed single-site high affinity specific binding of [^{3}H]PGD_2 with a K_d of 8.8 ± 0.8 nM and a B_{max} of 2.6 ± 0.6 pmol/mg membrane protein (Figure 1). Membranes from HEK293 cells transfected with the empty pRc/CMV vector exhibited no specific [^{3}H]PGD_2 binding (data not shown).

Binding affinities (K_i) for a variety of prostanoids were evaluated by their ability to displace [^{3}H]PGD_2 in competition binding experiments. PGD_2 bound to mCRTH2 with the highest affinity, with an order of affinity of PGD_2 >> $PGF_{2\alpha}$ > PGE_2 (Figure 2a). Several PGD_2 metabolites and analogs also bound to CRTH2 with high affinity, with an order of affinity of DK-PGD_2 ≥ 15d-PGJ_2 ≥ PGD_2 ≥ PGJ_2 ≥ 15-deoxy-$\Delta^{12\text{-}14}$-PGD_2 (Table 1). Indomethacin has been reported to bind and activate the human CRTH2 receptor (Hirai et al., 2002; Sawyer et al., 2002). We tested the ability of indomethacin and other commonly used NSAIDs to bind to

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mCRTH2. Of those tested, only indomethacin and sulindac displaced [^{3}H]PGD_2 at concentrations less than 30 μM in competition binding experiments, with indomethacin exhibiting the highest affinity (Figure 2b, Table 1).

mCRTH2 Intracellular Signaling

To characterize the intracellular signaling pathways activated by mCRTH2, we generated a stable transfectant ER293 cell line expressing mCRTH2. In this cell line, expression of mCRTH2 is under the control of a modified ecdysone receptor promoter system and is induced by pretreatment with ponasterone A (ponA). After 24 hour incubation with 10 μM ponA, mCRTH2 expression was determined to be 0.7 ± 0.4 pmol/mg membrane protein by radioligand binding. We tested the ability of mCRTH2 to activate the classical G_i-mediated pathway leading to inhibition of $[cAMP]_i$ in ER293/mCRTH2 cells, as has been observed for the human CRTH2 receptor (Sawyer et al., 2002). PGD_2, indomethacin and PGD_2 metabolites inhibited increases in $[cAMP]_i$ in isoproterenol-stimulated cells in a dose dependent manner (Figure 3a and Table 2). This response was abolished following pretreatment of the cells with pertussis toxin (PTX), demonstrating that mCRTH2 couples to a G_i-type G protein (Figure 3b). Vector transfected cells showed no response to mCRTH2 agonists (data not shown).

In addition to inhibition of $[cAMP]_i$, activation of CRTH2 has been demonstrated to lead to increases in intracellular calcium (Hirai et al., 2001). Therefore, we investigated if mCRTH2 ligands could stimulate increases in intracellular calcium in ER293/CRTH2 cells. Despite the ability to bind to mCRTH2, PGD_2 and indomethacin were unable to stimulate increases in intracellular calcium in this cell line. In control experiments, stimulation of endogenous

muscarinic cholinergic receptors in ER293/CRTH2 and ER293/vector cells with carbachol led to a robust calcium response (data not shown).

*Cell migratio*n

Because activation of the human CRTH2 receptor has been shown to mediate chemotaxis of Th2 cells, basophils and eosinophils (Gervais et al., 2001; Hirai et al., 2001; Hirai et al., 2002), we tested whether mCRTH2 was able to mediate a chemotactic response of ER293/mCRTH2 cells to mCRTH2 agonists. In transwell cell migration assays, nanomolar concentrations of both PGD_2 and indomethacin were able to stimulate migration of ER293/mCRTH2 cells in a dose dependent manner (Figure 4a). The PGD_2 metabolites PGJ_2 and 15d-PGJ_2 also stimulated migration (Figure 4c). No migration was observed for vector-transfected cells (Figure 4b) or ER293/mCRTH2 cells incubated with ibuprofen (data not shown). In chemokinesis control experiments, no significant increase in migration of ER293/CRTH2 cells was observed, demonstrating that the observed migration is true chemotaxis (data not shown). These data demonstrate that mCRTH2 is capable of functionally coupling to signal transduction pathways that mediate chemotaxis in ER293 cells.

Chemotaxis induced by activation of GPCRs has been shown to involve G_i- and inositol phosphate-dependent signal transduction (Neptune and Bourne, 1997; Hirsch et al., 2000). To investigate which signal transduction pathways mediate migration of ER293/mCRTH2 cells in response to PGD_2, we treated cells with PTX or wortmannin which inhibit the G_i-type G-protein and PI 3-kinase, respectively. While cell migration was completely abolished by pretreatment with PTX, treatment with wortmannin resulted in partial inhibition of PGD_2-stimulated migration

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(Figure 5). Taken together these results demonstrate that mCRTH2-evoked chemotaxis is mediated by G_i and PI 3-kinase dependent pathways.

Discussion

PGD_2 elicits a variety of physiological responses including modulation of smooth muscle tone, renin secretion, sleep induction and the inflammatory response (Morrow and Roberts, 2001). PGD_2 is the predominant prostanoid produced by activated mast cells and has been implicated in Th2-mediated atopic and inflammatory diseases such as allergic asthma (Lewis et al., 1982; Murray et al., 1986). The murine OVA-induced experimental asthma model has become widely used to elucidate the molecular pathogenesis of allergic asthma (Foster et al., 1996; Wills-Karp et al., 1998). Although the molecular mechanisms in the pathogenesis of asthma are complex, the emerging picture suggests that PGD_2 may play a central role in this disease. Transgenic mice overexpressing lipocalin-type prostaglandin D synthase produced increased levels of PGD_2 and Th2 cytokines and exhibited greater bronchoalveolar infiltration of lymphocytes and eosinophils upon OVA challenge compared to wild type mice (Fujitani et al., 2002). *In vitro* chemotaxis assays have demonstrated that chemotaxis of human Th2 cells, eosinophils and basophils in response to PGD_2 is mediated by CRTH2 (Hirai et al., 2001), however it has not been established if mCRTH2 plays a role in mediating the effects of PGD_2 in murine asthma models. Indeed, mCRTH2 has been reported to have a much wider pattern of mRNA expression than that observed for the human CRTH2 receptor (Abe et al., 1999), and it is unclear if they play the same physiological roles *in vivo.*

As a first step in defining the role of mCRTH2 in mouse physiology, we have cloned and characterized the pharmacology of the receptor. mCRTH2 binds PGD_2 and PGD_2 metabolites with high affinity. The mouse DP receptor, a member of the prostanoid subfamily of G-protein coupled receptors, also binds PGD_2 with high affinity (K_d = 40 nM) (Hirata et al., 1994). DP null mice have an attenuated asthmatic response when challenged with intermediate levels of OVA,

but at higher levels their response is similar to wild type (Matsuoka et al., 2000). This suggests that while the DP receptor plays a role in OVA-induced airway hyperreactivity, activation of this receptor may not account for all of the effects of PGD_2 in this model.

Several metabolites of PGD_2 bind to mCRTH2 with similar affinity to PGD_2. DK-PGD_2 is the product of the NADP-linked15-hydroxyprostaglandin dehydrogenase pathway (Giles and Leff, 1988) and its biological role, if any, has not been established. In contrast, the cyclopentenone prostaglandins PGJ_2 and 15d-PGJ_2 are capable of activating the PPARγ nuclear receptor and promote adipocyte differentiation (Kliewer et al., 1995). Numerous PPARγ-independent effects of 15d-PGJ_2 have also been described including activation of MAP kinase (Lennon et al., 2002), induction of apoptosis (Ward et al., 2002) and upregulation of IL-8 expression in activated T cells (Harris et al., 2002). *In vivo*, 15d-PGJ_2 has been detected in the cytoplasm of foamy macrophages in human aortic atherosclerotic plaques, and LPS-stimulation of macrophages *in vitro* leads to accumulation of both intracellular and extracellular 15d-PGJ_2 (Shibata et al., 2002). The role of 15d-PGJ_2 in inflammatory processes appears to be complex. 15d-PGJ_2 has been shown to exert anti-inflammatory effects in the acute inflammatory carrageenan-induced pleurisy and chronic collage-induced arthritis murine models (Cuzzocrea et al., 2002). In contrast, the observed upregulation of IL-8 in activated T cells in response to 15d-PGJ_2 would be expected to be pro-inflammatory. 15d-PGJ_2 binds to both human (Sawyer et al., 2002) and mouse CRTH2 receptors with an affinity several orders of magnitude greater than that observed for PPARγ (Kliewer et al., 1995), raising the possibility that 15d-PGJ_2 may play a pro-inflammatory role through activation of CRTH2. Consistent with this possibility, nanomolar concentrations of 15d-PGJ_2 have been shown to lead to calcium mobilization, actin polymerization, CD-11b expression in human eosinophils (Monneret et al., 2002). In addition,

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data presented here provide the first direct evidence that 15d-PGJ_2 can also stimulate chemotaxis via mCRTH2.

mCRTH2 is closely related to peptide chemoattractant receptors such as FPR and C5aR (Abe et al., 1999) which mediate neutrophil chemotaxis (Pellas et al., 1998; Gao et al., 1999). Nanomolar concentrations of mCRTH2 agonists stimulated chemotaxis of ER293/mCRTH2 cells, which was inhibited by pretreatment with the G_i inhibitor PTX or the PI 3-kinase inhibitor wortmannin. Involvement of G_i and PI 3-kinase in the chemotactic response mediated by GPCRs has been well established in a number of systems, including neutrophils (Niggli and Keller, 1997; Hirsch et al., 2000), *Dictyostelium* (Meili et al., 1999) and HEK293 cells (Neptune and Bourne, 1997). Our studies further confirm these observations and demonstrate that mCRTH2 couples to the classic signaling pathways that mediate chemotaxis.

In addition to PGD_2 and PGD_2-derived prostanoids, mCRTH2 is capable of binding non-prostaglandin molecules such as indomethacin, a commonly prescribed NSAID. Indomethacin is a non-specific cyclooxygenase (COX) inhibitor (Mitchell et al., 1993) that also possesses COX-independent activity such as activation of PPARγ (Lehmann et al., 1997) and CRTH2 (Hirai et al., 2002). In the present studies, we found that indomethacin is a potent activator of mCRTH2, approximately 2.5-fold less potent than PGD_2. In contrast indomethacin bound to the mCRTH2 receptor with an affinity 25-fold lower than that of PGD_2. One possible explanation for this discrepancy is that indomethacin has a very high intrinsic activity at mCRTH2 compared to PGD_2. In this case, a smaller fraction of receptor occupancy would be required for a given response. Alternatively, differential transport or metabolism of the endogenous PGD_2 ligand versus indomethacin may effectively lower the PGD_2 concentration in live cell assays such as $[cAMP]_i$ signaling, though not in binding assays on membranes. This would result in a

rightward shift in the PGD_2 dose-response curve relative to the indomethacin curve. The ability of indomethacin to bind and activate CRTH2 leading to chemotaxis of Th2 cells, eosinophils and basophils, as well as stimulation of shape change, CD11b expression, and respiratory burst in eosinophils, is not shared by other NSAIDs (Hirai et al., 2002; Stubbs et al., 2002). In the present studies, both indomethacin and sulindac were capable of binding mCRTH2, and indomethacin acted as an agonist at mCRTH2. Indomethacin and sulindac share a common indoleacetic acid core molecular structure, which appears to be fundamental for mCRTH2 recognition. Other NSAIDs such as ibuprofen, that are not indoleacetic acid derivatives, do not bind or activate mCRTH2. Indomethacin is commonly used in a number of mouse models because of its potent action in inhibiting both COX-1 and COX-2. In these models, the effects of indomethacin as a COX inhibitor may be confounded by its potent agonist activity at the mCRTH2 receptor, and other NSAIDs such as ibuprofen, that do not activate mCRTH2, may be a more advantageous choice.

Stimulation of CRTH2 has been demonstrated to lead to both increases in intracellular calcium and inhibition of $[cAMP]_i$ via PTX-sensitive mechanisms (Hirai et al., 2001; Sawyer et al., 2002). Activation of mCRTH2 in ER293 cells inhibited isoproterenol-induced increases in $[cAMP]_i$ but did not elicit an observable change in intracellular calcium. One possibility is that mCRTH2 is inherently incapable of coupling to the required signal transduction machinery for raising intracellular calcium. It is likely, however, that ER293 cells, a derivative of HEK293 cells, do not possess the appropriate G proteins for this response. Differences in the complement of heterotrimeric G-proteins expressed in a particular cell type have been observed to lead to differences in the ability for a given GPCR to activate a particular signal transduction pathway. For instance, activation of the G_i-coupled sphingosine-1-phosphate receptor Edg-1 leads to an

increase in intracellular calcium in CHO but not HEK293 cells (Okamoto et al., 1998; Van Brocklyn et al., 1998). In accordance with this possibility, PGD_2 stimulation of HEK293 cells transfected with the human CRTH2 causes only a slight increase in intracellular calcium; this response was greatly enhanced upon transfection of the G-protein $G_{\alpha 15}$ (Sawyer et al., 2002).

In this study, we have described pharmacological characterization of the mouse CRTH2 receptor and demonstrated that mCRTH2 is G_i-coupled, can be activated by PGD_2, PGD_2 metabolites and indoleacetic acid NSAIDs and mediates chemotaxis of ER293/CRTH2 cells via G_i and PI 3-kinase signal transduction pathways. In contrast to the human receptor, which is expressed in Th2 but not Th1 cells (Nagata et al., 1999), mCRTH2 mRNA has been detected at low levels in both Th1 and Th2 cells (Abe et al., 1999). Although the significance of this expression difference is not clear, it has been shown that G_i-coupled chemoattractant receptors expressed on T cells play an important role in the pathogenesis of allergic airway inflammation in mice. When mice received allo-transfer of PTX-treated Th2 cells, they exhibited greatly reduced infiltration of lymphocytes and eosinophils in the OVA-induced experimental asthma model (Mathew et al., 2002). Eosinophilic inflammation did occur when the cells were directly instilled into the airway, indicating the importance of G_i-coupled chemoattractant signaling and resulting migration of Th2 cells in the pathogenesis of allergic airway inflammation. Based on the pharmacology of mCRTH2, it is expected that future studies using mCRTH2 knock-out mice will provide insight into the molecular pathogenesis of allergic diseases.

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Footnotes

This work was supported by grants from the NIH GM15431 (RMB), DK46205 (RMB), DK37097 (MDB) as well as the Vanderbilt-Ingram Cancer Center. ANH is supported by a PhRMA Foundation pre-doctoral fellowship. RZ is a clinician scientist of the NKF and has an Advanced Career Development Award from the Veterans Administration.

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Figure 1

Saturation isotherm analysis of [^{3}H]PGD_2 binding to mCRTH2 expressed in HEK293 cells. Membranes isolated from HEK293 cells transiently transfected with mCRTH2 were incubated with varying concentrations of [^{3}H]PGD_2 in the absence (total binding) or presence (non-specific binding) of 10 μM DK-PGD_2 as described in Methods. Specific binding was determined to be the difference between total and non-specific binding and is shown above. The K_d and B_{max} were determined using a one-site binding model (Prism 3.0). A Scatchard plot (inset) is shown for reference. Each data point was determined in triplicate; error bars represent S.E.M. These data are representative of three independent experiments.

Figure 2

Competition binding analysis of mCRTH2 ligands. Membranes isolated from HEK293 cells transiently transfected with mCRTH2 were incubated with 3 nM [^{3}H]PGD_2 in the presence of varying concentrations of prostanoids (A) or NSAIDs (B) as described in Methods. In Panel B, PGD_2 binding is plotted for comparison. Binding curves were generated using a one-site competition model (Prism 3.0). Each data point was determined in triplicate; error bars represent S.E.M. These data are representative of three independent experiments.

Figure 3

mCRTH2-evoked inhibition of $[cAMP]_i$. (A) ER293/mCRTH2 cells were stimulated with 500 nM isoproterenol/50 nM sodium ascorbate in the presence of varying concentrations of mCRTH2 agonists and $[cAMP]_i$ levels were determined as described in Methods. (B) Cells were additionally treated with or without 100 ng/ml PTX for 12 hours prior to stimulation with 500

nM isproterenol and 100 nM PGD_2. Each data point was determined in duplicate for dose-response studies and triplicate for PTX studies (error bars in Panel B represent S.E.M.); the data are representative of 3-4 independent experiments.

Figure 4

mCRTH2 mediates chemotaxis of ER293/mCRTH2 cells. Migration of ER293/mCRTH2 (A and C) or ER293/pEGSH (B) cells in response to mCRTH2 agonists was assessed as described in Methods. (A) PGD_2 and indomethacin elicit a characteristic bell-shaped dose response. (B) PGD_2 (100 nM) and indomethacin (100 nM) were unable to stimulate migration of vector-transfected cells; however, these cells migrate in response 5% FBS, a non-specific chemoattractant. (C) PGD_2 and metabolites PGJ_2 and 15d-PGJ_2 (100 nM) are equipotent in stimulating chemotaxis of ER293/mCRTH2 cells. Data are expressed as percentage of cells migrating in the absence of added ligand (control). Each data point represents the average of 5 high power fields of a single experimental condition; error bars represent S.E.M. These data are representative of 3-4 independent experiments. In Panel A, data points within brackets are significantly different from baseline control; in panels B and C, $^{**}P < 0.01$ compared with control.

Figure 5

PGD_2-stimulated chemotaxis of ER293/mCRTH2 cells is inhibited by PTX and wortmannin. Cells were pretreated with or without 100 ng/ml PTX for 12 hours or 100 nM wortmannin for 10 minutes prior to performing migration assay. Migration of cells in response to 100 nM PGD_2 was determined as described in Methods. Data are expressed as percentage of untreated cells

migrating in the absence of added ligand (control). Each data point represents the average of 5 high power fields of a single experimental condition; error bars represent S.E.M. These data are representative of 3 independent experiments.

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Table 1

Binding affinities of prostanoid and NSAID ligands for mCRTH2 expressed in HEK293 cell membranes.

Major Prostanoids	K_i
PGD_2	38 ± 6 nM
$PGF_{2\alpha}$	600 ± 50 nM
PGE_2	3200 ± 400 nM
PGD_2 metabolites and analogs	
DK-PGD_2	20 ± 6 nM
15d-$\Delta^{12,14}$-PGJ_2	24 ± 4 nM
PGJ_2	45.7 ± 0.2 nM
Δ^{12}-PGJ_2	410 ± 20 nM
15d-$\Delta^{12,14}$-PGD_2	50 ± 2 nM
PGD_3	37 ± 12 nM
BW245C	> 10,000 nM
NSAIDs	
indomethacin	1.04 ± 0.13 μM
sulindac	8.8 ± 0.6 μM
flurbiprofen	> 30 μM
ibuprofen	> 30 μM
aspirin	> 30 μM
sodium salicylate	> 30 μM
acetaminophen	> 30 μM

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Competition binding experiments were performed as described in Methods. IC_{50} values were determined using a one-site competition binding model (Prism 3.0). K_i values were calculated according to the equation $K_i = IC_{50}/(1+[D]/K_d)$ where [D] is the concentration of [^{3}H]PGD_2 (Cheng and Prusoff, 1973). Results are mean ± S.E.M. of 3-7 independent experiments each performed in triplicate.

Table 2

Potency of mCRTH2 ligands for inhibiting isoproterenol-induced increases in $[cAMP]_i$ in ER293/mCRTH2 cells.

Ligand	EC_{50} (nM)
PGD_2	0.9 ± 0.2
Indomethacin	2.4 ± 0.2
DK-PGD_2	0.73 ± 0.12
15d-$\Delta^{12,14}$-PGJ_2	0.8 ± 0.3
ibuprofen	*not detected*
BW245C	*not detected*

$[cAMP]_i$ signaling assays were performed as described in Methods. Results are expressed as per cent of $[cAMP]_i$ in uninhibited isoproterenol-stimulated cells. EC_{50} values were determined using a fixed-slope sigmoidal dose-response model (Prism 3.0). Results are mean ± S.E.M. of 3-4 independent experiments each performed in duplicate.

Figure 1

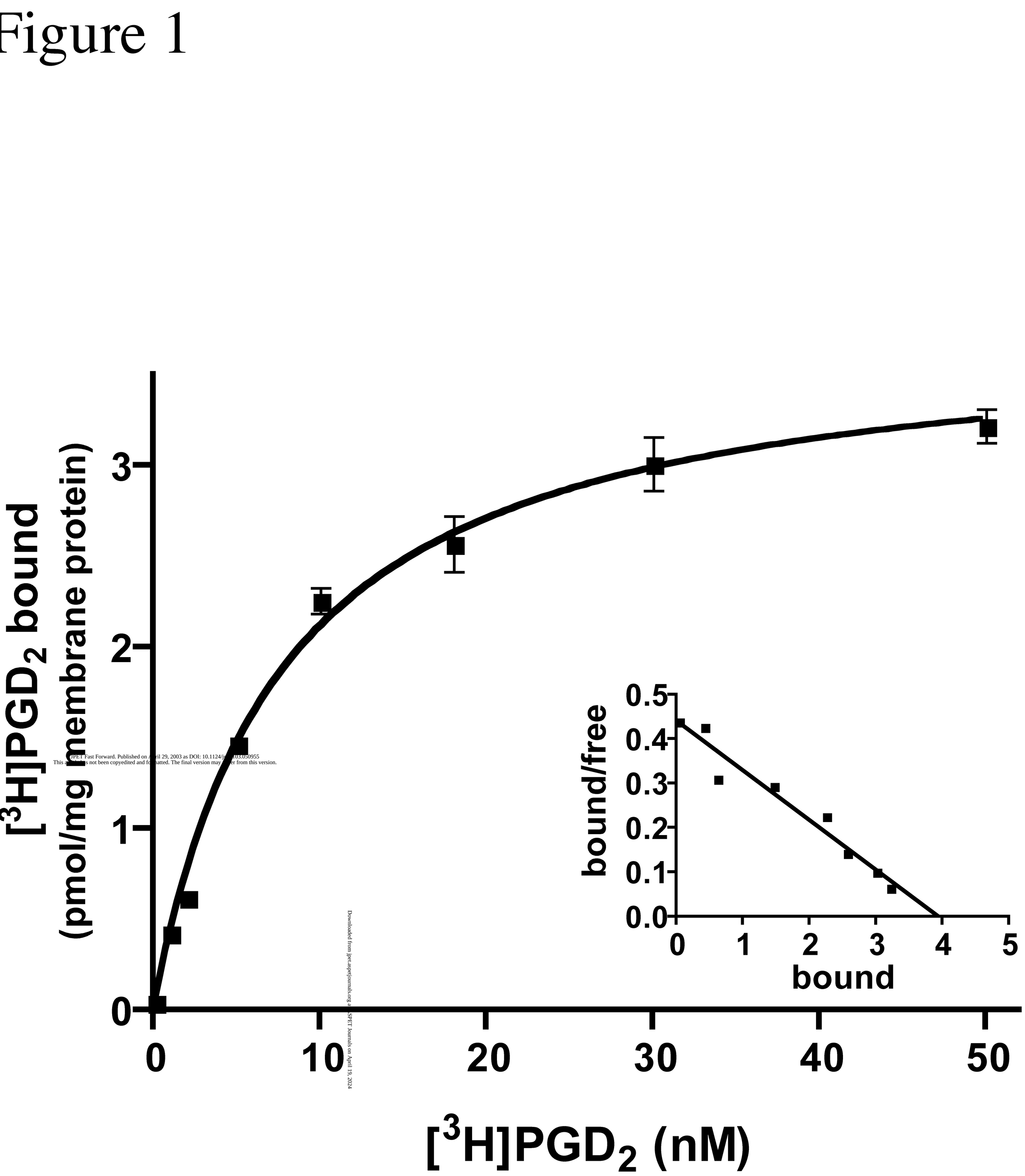

Figure 2

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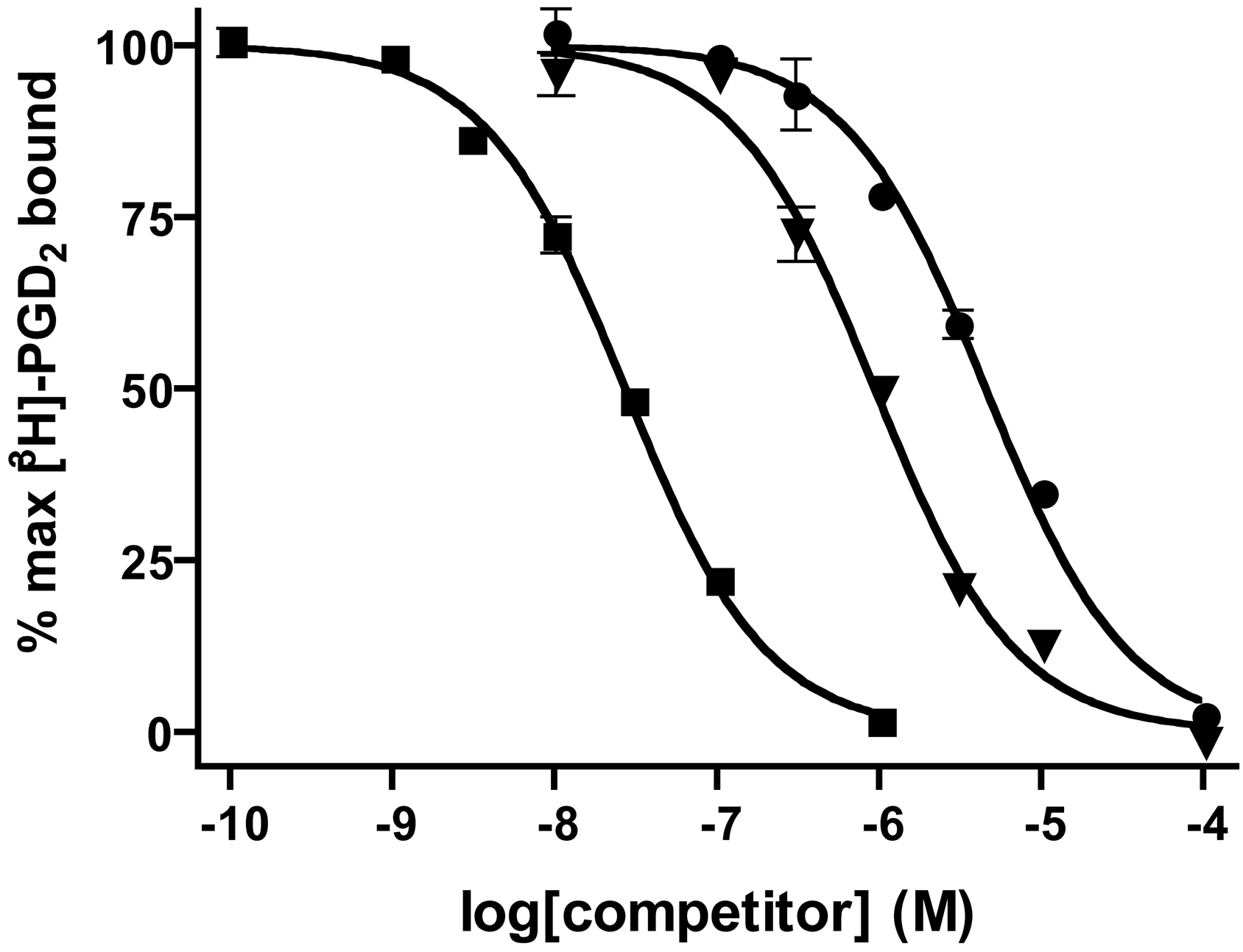

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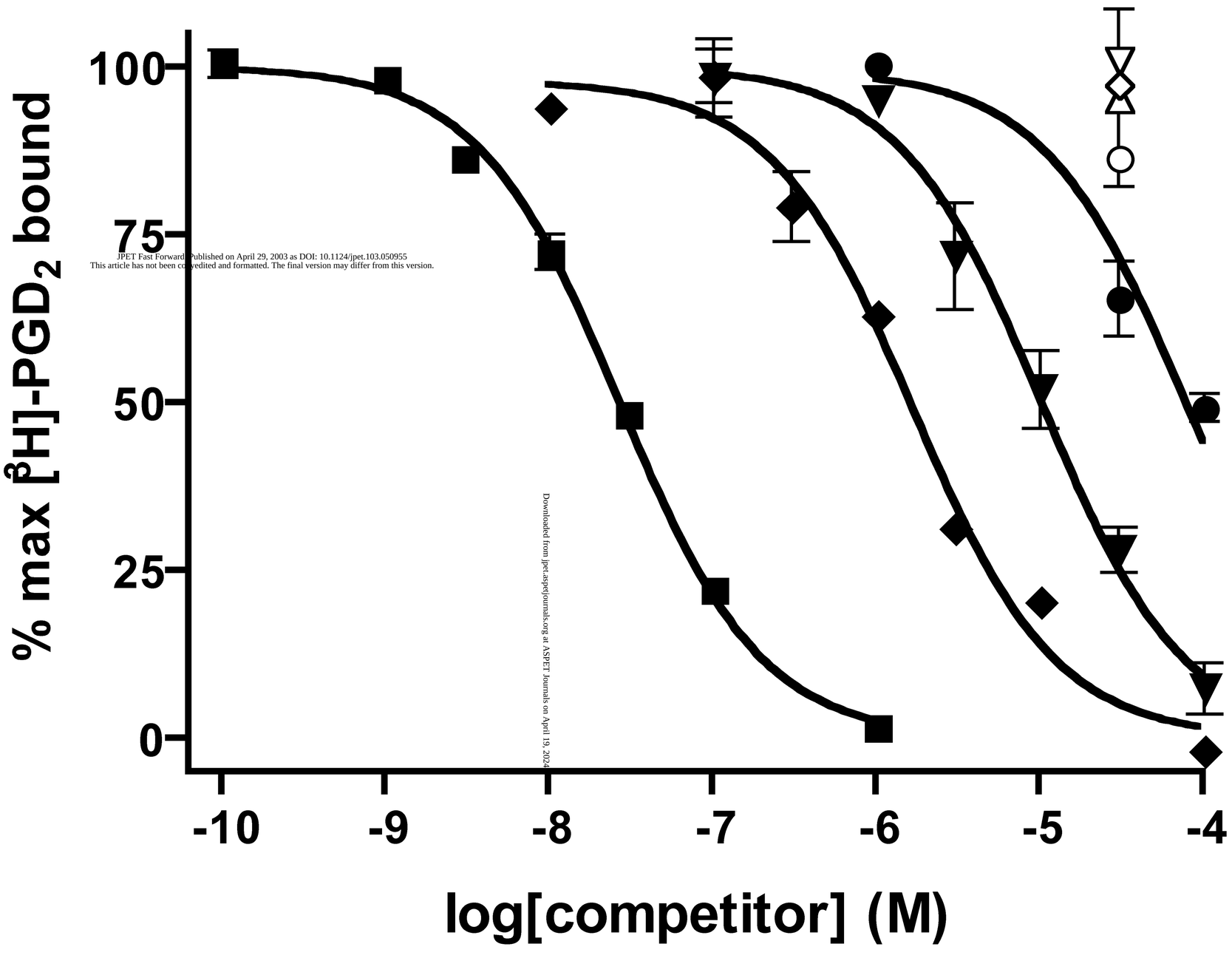

Figure 3

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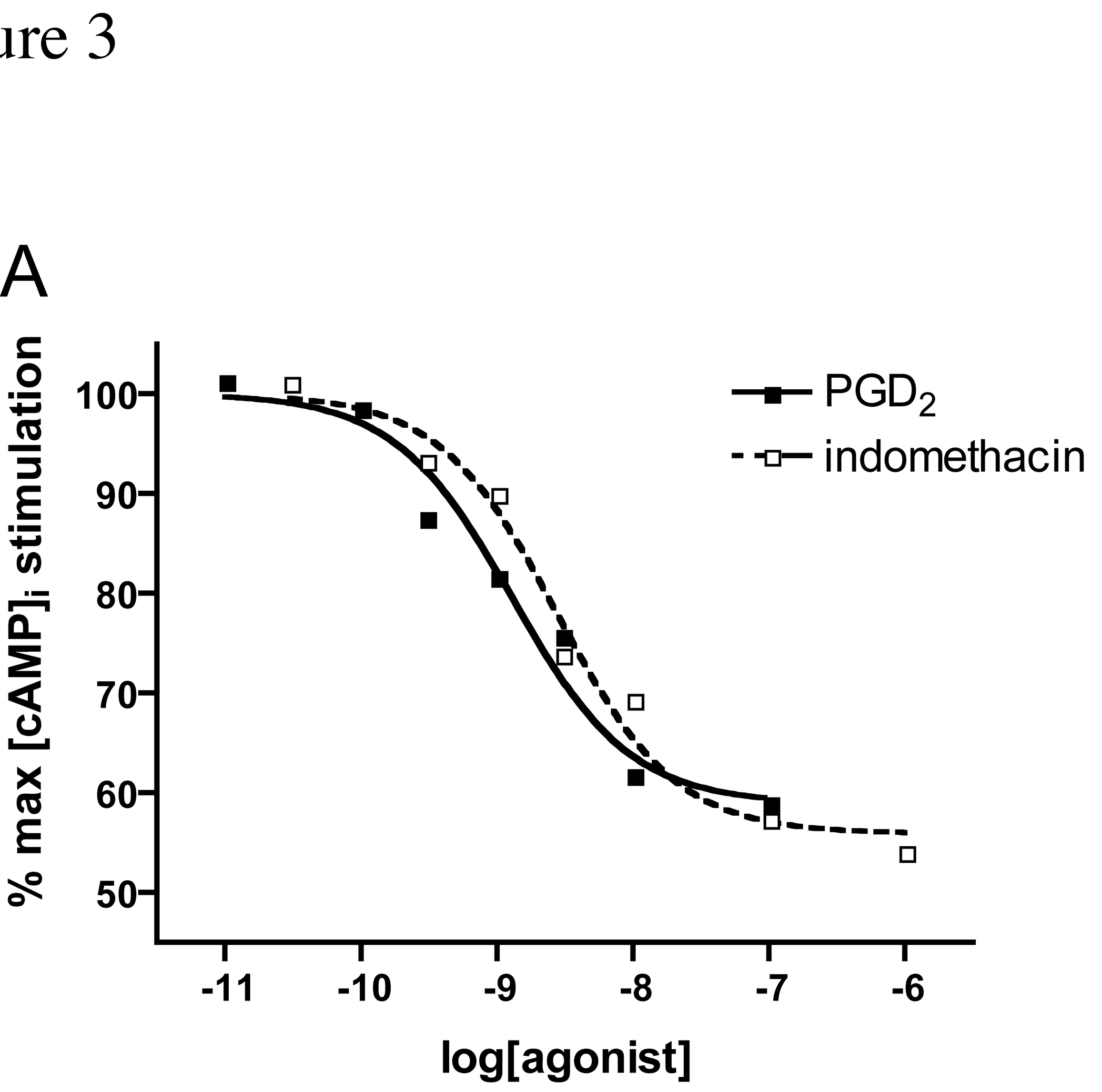

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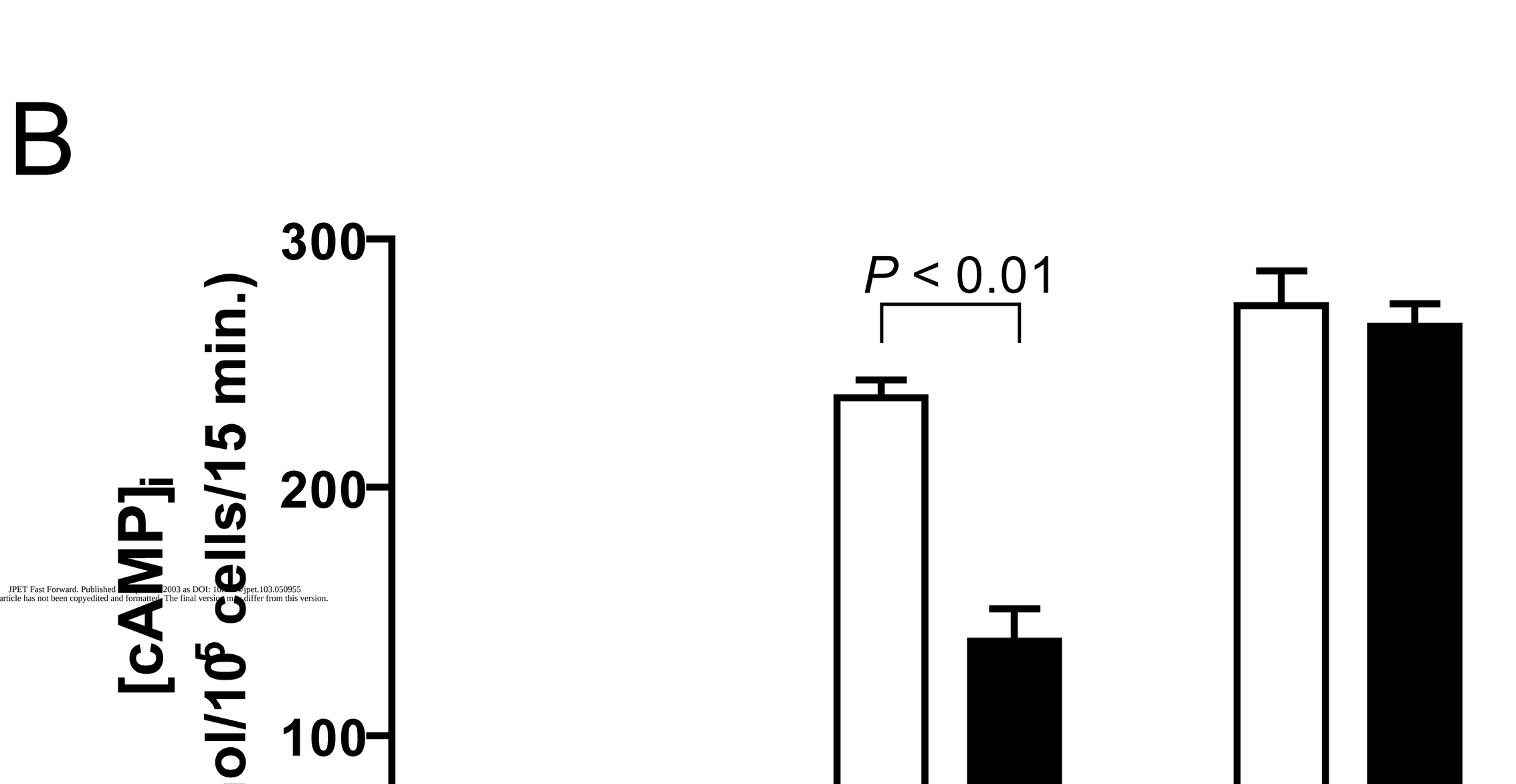

Figure 4

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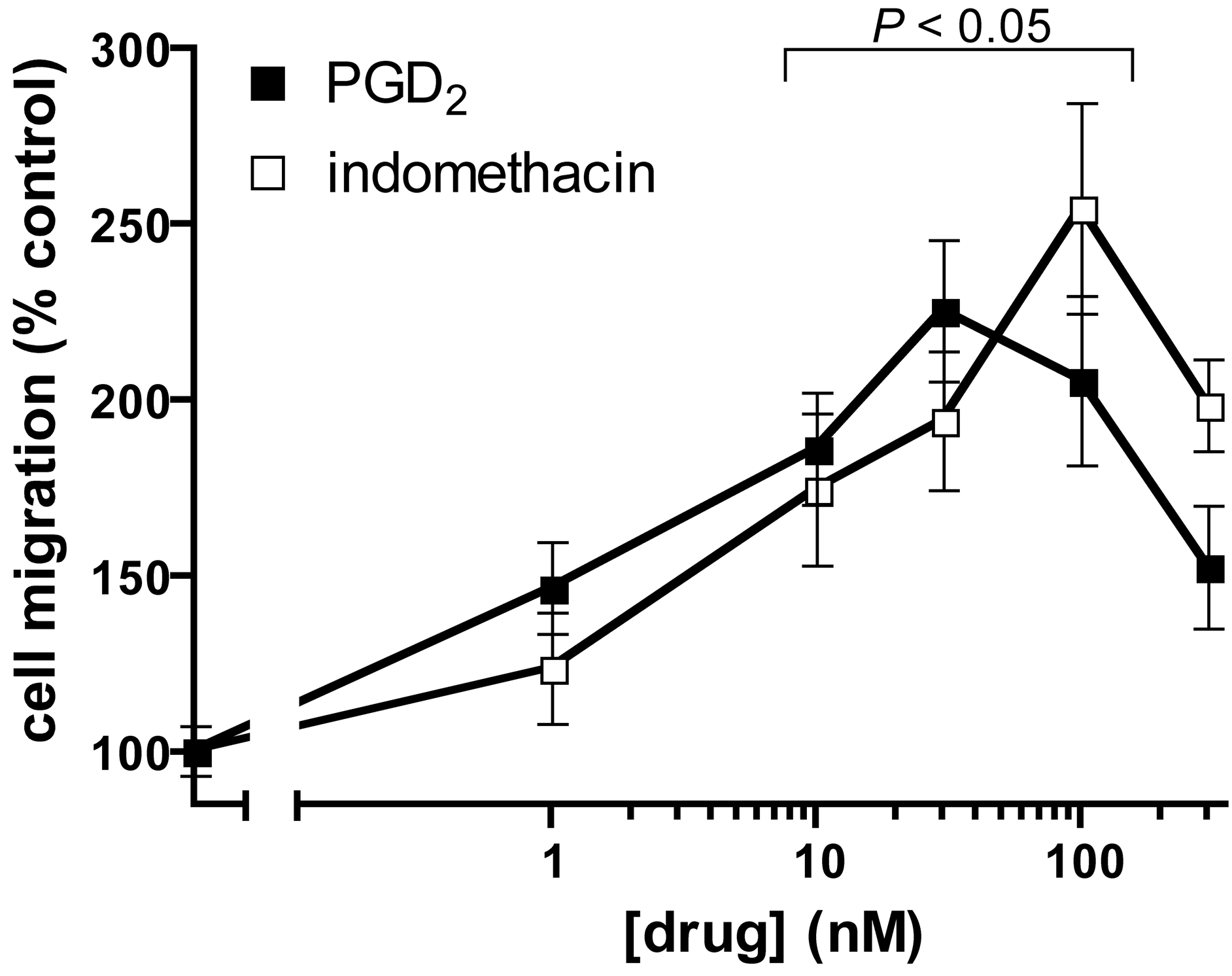

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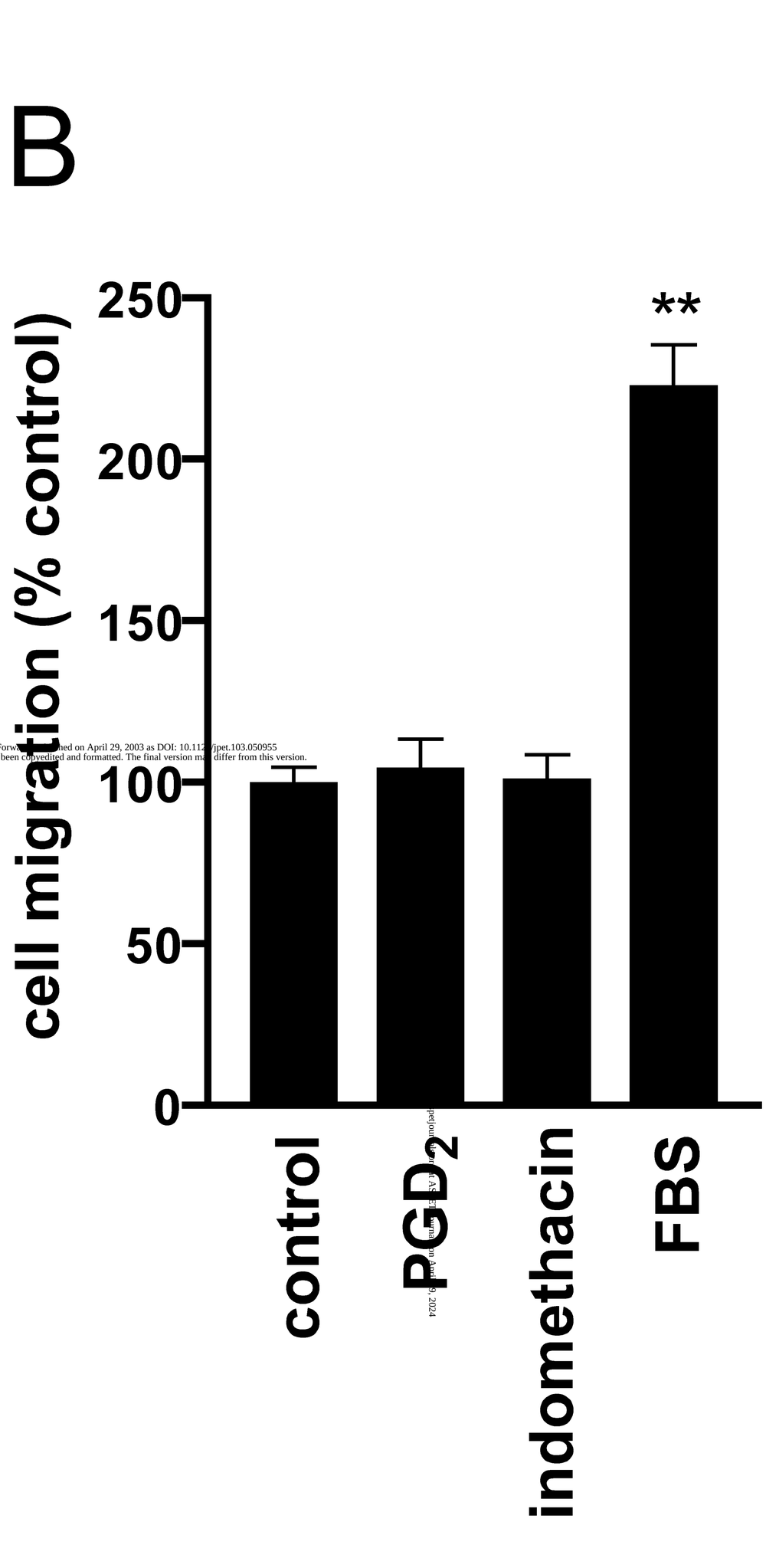

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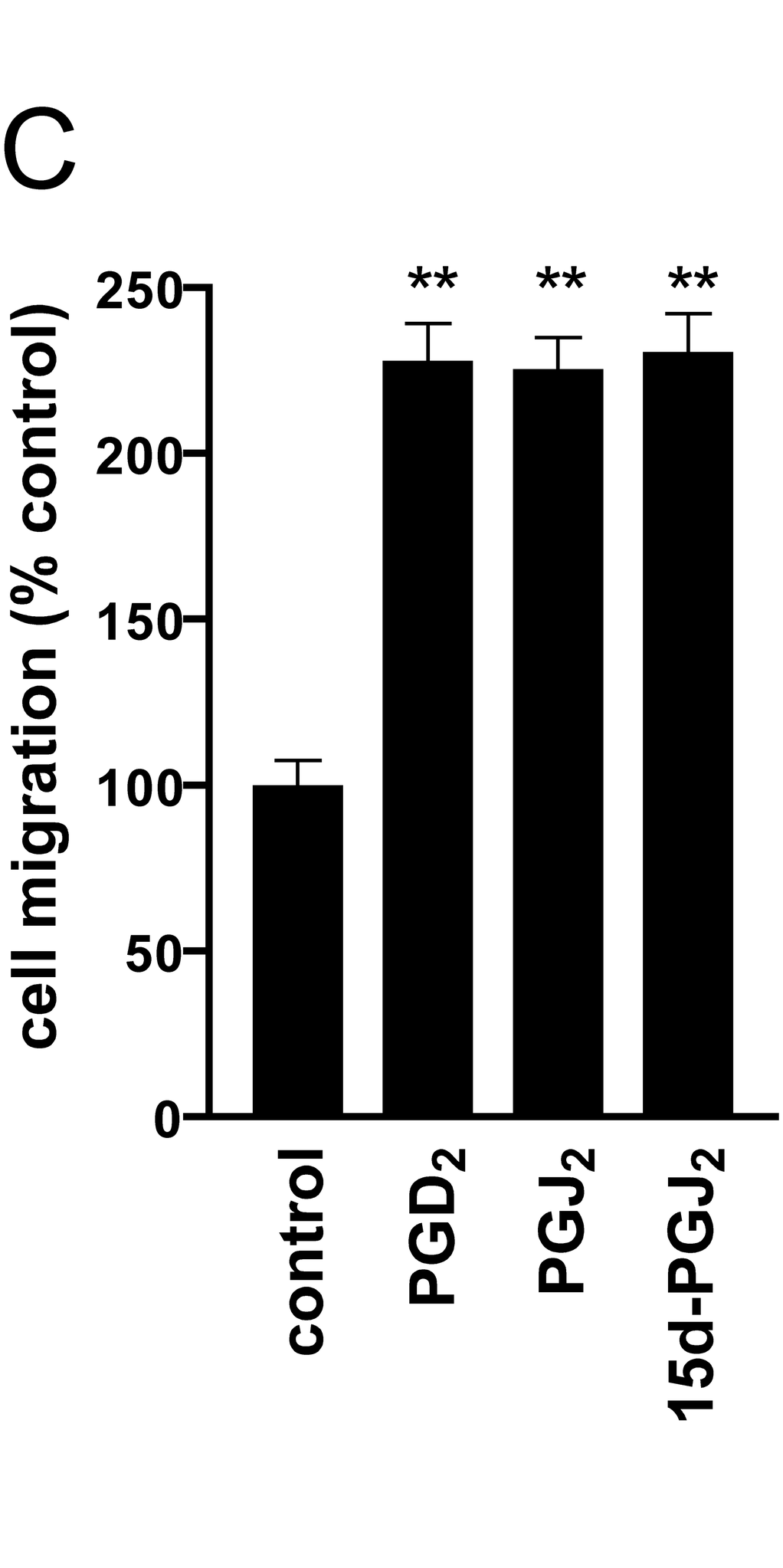

Figure 5