Secreted Phospholipase A_2 Inhibitors Are Also Potent Blockers of Binding to the M-Type Receptor†

Eric Boilard,‡ Morgane Rouault,‡ Fanny Surrel,‡ Catherine Le Calvez,‡ Sofiane Bezzine,‡ Alan Singer,§ Michael H. Gelb,§ and Gérard Lambeau*,‡

Institut de Pharmacologie Moléculaire et Cellulaire, CNRS-UMR 6097, 660 route des Lucioles, Sophia-Antipolis, 06560 Valbonne, France, and Departments of Chemistry and Biochemistry, University of Washington, Seattle, Washington 98195

Received July 7, 2006; Revised Manuscript Received September 4, 2006

ABSTRACT: Mammalian secreted phospholipases A_2 (sPLA$_2$s) constitute a family of structurally related enzymes that are likely to play numerous biological roles because of their phospholipid hydrolyzing activity and binding to soluble and membrane-bound proteins, including the M-type receptor. Over the past decade, a number of competitive inhibitors have been developed against the inflammatory-type human group IIA (hGIIA) sPLA$_2$ with the aim of specifically blocking its catalytic activity and pathophysiological functions. The fact that many of these inhibitors, including the indole analogue Me-Indoxam, inhibit several other sPLA$_2$s that bind to the M-type receptor prompted us to investigate the impact of Me-Indoxam and other inhibitors on the sPLA$_2$−receptor interaction. By using a Ca^{2+} loop mutant derived from a venom sPLA$_2$ which is insensitive to hGIIA inhibitors but still binds to the M-type receptor, we demonstrate that Me-Indoxam dramatically decreases the affinity of various sPLA$_2$s for the receptor, yet an sPLA$_2$−Me-Indoxam−receptor complex can form at very high sPLA$_2$ concentrations. Me-Indoxam inhibits the binding of iodinated mouse sPLA$_2$s to the mouse M-type receptor expressed on live cells but also enhances binding of sPLA$_2$ to phospholipids. Because Me-Indoxam and other competitive inhibitors protrude out of the sPLA$_2$ catalytic groove, it is likely that the inhibitors interfere with the sPLA$_2$−receptor interaction by steric hindrance and to different extents that depend on the type of sPLA$_2$ and inhibitor. Our finding suggests that the various anti-inflammatory therapeutic effects of sPLA$_2$ inhibitors may be due not only to inhibition of enzymatic activity but also to modulation of binding of sPLA$_2$ to the M-type receptor or other as yet unknown protein targets.

Secreted phospholipases A_2 (sPLA$_2$s)[1] are enzymes that catalyze the hydrolysis of the *sn*-2 ester of glycerophospholipids to release free fatty acids and lysophospholipids (*1*). To date, up to 10 mammalian active sPLA$_2$s and two structurally related inactive variants have been identified (*2*, *3*). These sPLA$_2$s form a family of low-molecular mass proteins (∼14−20 kDa) that are likely to be involved in various biological settings, including inflammation and associated diseases, host defense, and cancer (*4*−*11*). One major interest in sPLA$_2$ comes from its role in the control of biosynthesis of eicosanoids and other lipid mediators which are important in numerous physiological and pathophysiological conditions. Current evidence indicates that group IIA, V, and X sPLA$_2$s work together with or in a manner independent of intracellular group IV and VI PLA$_2$s to produce lipid mediators from various types of agonist-activated cells (*12*−*20*). The role of other sPLA$_2$s (groups IB, IIC, IID, IIE, IIF, III, XIIA, and XIIB) in lipid mediator release remains uncertain or unknown (*15*, *21*−*23*).

Among the various sPLA$_2$ members, the group IIA sPLA$_2$ has attracted the most interest over the past 15 years. It was the first enzyme to be discovered at high levels in inflammatory fluids with a proposed pro-inflammatory role (*24*). Its expression was subsequently found to be upregulated by various pro-inflammatory stimuli and to be elevated in numerous acute and chronic inflammatory diseases (*4*, *6*, *7*, *25*−*27*). This led to the early proposal that group IIA sPLA$_2$ may be central in lipid mediator release during inflammation and associated diseases (*28*). Consequently, the human group IIA (hGIIA) sPLA$_2$ has been the focus of intense efforts at several pharmaceutical companies, including Eli Lilly and Shionogi Laboratories (*6*, *29*−*31*). This collective work has led to the development of many hGIIA inhibitors among

† This work was supported in part by the CNRS (to G.L.), the Association pour la Recherche sur le Cancer (to G.L.), and National Institutes of Health Grant HL36236 (to M.H.G.). E.B. is a recipient of a postdoctoral fellowship from the Canadian Institute of Health Research in partnership with The Arthritis Society. M.R. is a recipient of a fellowship from the Fondation de la Recherche Médicale. F.S. is a recipient of a fellowship from the Région Provence Alpes Côte d'Azur-CNRS.

* To whom correspondence should be addressed. Telephone: 33(0) 4 93 95 77 33. Fax: 33(0) 4 93 95 77 04. E-mail: lambeau@ipmc.cnrs.fr.

‡ CNRS-UMR 6097.

§ University of Washington.

[1] Abbreviations: BSA, bovine serum albumin; HEK, human embryonic kidney; HPLC, high-performance liquid chromatography; HSPG, heparan sulfate proteoglycan; sPLA$_2$, secreted phospholipase A_2; OS$_1$, *Oxyuranus scutellatus* toxin 1; OS$_2$, *O. scutellatus* toxin 2; bvPLA$_2$, bee venom sPLA$_2$. A comprehensive abbreviation system for the various mammalian sPLA$_2$s is used. Each sPLA$_2$ is abbreviated with a lowercase letter indicating the sPLA$_2$ species (m and h for mouse and human, respectively), followed by uppercase letters identifying the sPLA$_2$ group (GIB, GIIA, GIIC, GIID, GIIE, GIIF, GIII, GV, GX, GXIIA, and GXIIB for groups IB, IIA, IIC, IID, IIE, IIF, V, X, XIIA, and XIIB, respectively).

10.1021/bi061376d CCC: $33.50
Published on Web 10/14/2006

which the indole-based derivatives appear to be the most potent inhibitors with druglike properties (*31*−*33*). Some of these inhibitors are active in various animal models of inflammation, suggesting that group IIA $sPLA_2$ may indeed be an attractive target for preventing inflammation (*34*−*39*). For example, Indoxam was found to be a potent inhibitor of the pro-inflammatory response induced by endotoxin challenge in mice and was able to prolong mouse survival (*40*). However, the current clinical trials showed no obvious benefit in patients with organ failure, severe sepsis, asthma, and rheumatoid arthritis (*6*, *31*, *41*−*44*). The fact that many hGIIA inhibitors, including Indoxam, can inhibit several other $sPLA_2$s (*33*, *45*−*48*) suggests that one or more $sPLA_2$s may be targeted in vivo by these inhibitors and, thus, may also participate in inflammatory processes. Thus, there is a need for more selective inhibitors that can discriminate between $sPLA_2$ members, especially among group IIA, V, and X $sPLA_2$s (*33*). Such inhibitors will be very useful in probing the role of each $sPLA_2$ in lipid mediator release and other biological functions.

There is a growing body of evidence showing that mammalian $sPLA_2$s, including group IB, IIA, V, or X, are also capable of modulating cell proliferation, cell survival, cell contraction, cell migration, apoptosis, or the release of peptides, hormones, and cytokines (*2*, *11*, *22*, *49*−*52*). Mammalian $sPLA_2$s can also trigger the activation of various intracellular signaling molecules, including MAP kinases, PI3K, Akt, $cPLA_2$ IVA, cyclooxygenase-2 (COX-2), and sphingomyelinase (*21*, *53*−*60*). Additionally, some $sPLA_2$s have antimicrobial activity against bacteria, parasites, and viruses (*9*, *61*−*65*). The role of $sPLA_2$ catalytic activity in the various biological effects is still in large part an unresolved issue. While some biological effects appear to be dependent on $sPLA_2$ catalytic activity (*49*, *54*, *66*), others appear to be associated with the binding of $sPLA_2$ to a specific target at the cell plasma membrane (*53*, *55*−*57*, *59*, *67*). The different conclusions given above were supported by using either hGIIA $sPLA_2$ competitive inhibitors, catalytically inactive $sPLA_2$ mutants or $sPLA_2$ derivatives alkylated at the active site, or transfection of the proposed membrane cellular targets of $sPLA_2$s. In a few cases, it was found that while $sPLA_2$ competitive inhibitors prevented the $sPLA_2$ biological effect, the catalytically inactive $sPLA_2$ mutants were as active as the wild-type (WT) enzyme (*53*, *57*, *59*). In most cases, the true nature of the $sPLA_2$ targets at the plasma membrane which would be unambiguously responsible for the biological effects of the various $sPLA_2$s remains to be determined (*53*, *56*, *57*, *59*, *67*−*69*).

The best known protein target which has been proposed to play a role in $sPLA_2$ biological effects is the M-type $sPLA_2$ receptor, which was initially characterized using the snake venom $sPLA_2$s OS_1 and OS_2 and then using pancreatic group IB $sPLA_2$ (*22*, *70*). This receptor is a member of the superfamily of C-type lectins, and several of its molecular properties have now been addressed. This receptor has been first proposed to play a major role in transducing the various biological effects of pancreatic group IB $sPLA_2$, including cell proliferation, cell migration, cell contraction, hormone release, and activation of intracellular signaling pathways (*21*, *22*, *51*). Gene targeting of the M-type receptor has also suggested a role for this protein in promoting inflammation in a mouse model of endotoxic shock (*71*). On the other hand, the M-type receptor has been suggested to counteract the enzymatic action of $sPLA_2$ by inhibiting catalytic activity upon $sPLA_2$ binding and by internalizing and degrading the enzyme (*22*, *70*).

At the molecular level, we and others have previously shown that group IB, IIA, and X $sPLA_2$s can act as endogenous ligands for the M-type receptor in a very strict manner that depends on both receptor species and $sPLA_2$ subtypes (*22*, *72*). Furthermore, it was shown that the $sPLA_2$ inhibitor Indoxam inhibits the binding of group IB and X $sPLA_2$s to this receptor (*40*, *73*), leading to the proposal that the therapeutic effect of Indoxam in a mouse model of endotoxic shock may be due to blockade of $sPLA_2$ binding rather than inhibition of catalytic activity (*40*). More recently, we have shown that up to seven mouse $sPLA_2$s can bind to the mouse M-type receptor and thus behave as endogenous ligands of this receptor, at least in the mouse species (M. Rouault et al., manuscript submitted for publication). This finding in addition to the fact that several mouse and human $sPLA_2$s are targeted by various competitive inhibitors of hGIIA $sPLA_2$, including Me-Indoxam (*46*), prompted us to analyze in more detail how Me-Indoxam and other competitive inhibitors affect the binding of various $sPLA_2$s to the M-type receptor. To clearly evaluate the effect of Me-Indoxam on the binding of the different mammalian $sPLA_2$s, we produced a D49K mutant of the snake venom $sPLA_2$ OS_2, which has no affinity for Me-Indoxam, but still binds tightly to the M-type receptor. Our results show that Me-Indoxam and various $sPLA_2$ competitive inhibitors are also potent inhibitors of binding of $sPLA_2$ to the M-type receptor. The inhibitors do not fully prevent binding of $sPLA_2$ to the receptor but rather form a $sPLA_2$ complex that binds to the receptor with a much weaker affinity. The shift in overall affinity depends on both the type of inhibitor and $sPLA_2$ and is most likely due to a steric hindrance effect of the inhibitor on the $sPLA_2$−receptor interaction. The inhibitory effect of Me-Indoxam led us to also analyze the binding properties of several radiolabeled mouse $sPLA_2$s on cellular membranes and live cells in the presence of Me-Indoxam. The potential impact of these findings with regard to binding of $sPLA_2$ to live cells and the mechanism of action and future development of $sPLA_2$ inhibitors is discussed.

EXPERIMENTAL PROCEDURES

Preparation of Native sPLA₂s and the Mouse Soluble M-Type Receptor. All of the venom and mammalian recombinant $sPLA_2$s used in this study were prepared and purified as previously described (*46*, *74*; M. Rouault et al., manuscript submitted for publication). The D49K OS_2 mutant was produced in *Escherichia coli* as inclusion bodies and refolded as described previously (*74*). The mouse soluble recombinant M-type $sPLA_2$ receptor was prepared and purified as described (M. Rouault et al., manuscript submitted for publication).

sPLA₂ Inhibitors and Reagents. Me-Indoxam, Indoxam, Pyrazole-1, compounds A, B, 42, 44, and 46, and triazepandione molecule 19 were obtained as described previously (*33*, *40*, *46*, *48*, *75*). LY311727 was a generous gift from E. Mihelich (Eli Lilly Laboratories). Quinacrine, polymyxin B, poly-L-lysine, and polyethyleneimine were from Sigma.

Inhibition of sPLA₂ Enzymatic Activity. Inhibition of $sPLA_2$s by the various inhibitors was analyzed using radio-

labeled *E. coli* membranes as a substrate (*74*). Briefly, the different $sPLA_2$s were preincubated for 15 min with inhibitors in 150 μL of enzymatic activity buffer consisting of 20 mM Tris (pH 7.4), 140 mM NaCl, 1 mM $CaCl_2$, and 0.1% bovine serum albumin (BSA), after which an additional 150 μL of buffer containing 100 000 dpm of [^{3}H]oleate *E. coli* membranes was added. The enzymatic activity was measured at room temperature for 40 min. Reactions were stopped by addition of 600 μL of stop buffer consisting of 0.1 M EDTA (pH 8.0) and 0.1% fatty acid-free BSA and the mixtures centrifuged at 10000*g* for 5 min, and supernatants containing released [^{3}H]oleate were analyzed using liquid scintillation counting. $sPLA_2$ concentrations were chosen to ensure 5−10% hydrolysis of substrate.

Preparation of Cell Membranes Containing Rabbit and Mouse M-Type Receptors. Stably transfected cells expressing rabbit or mouse recombinant M-type receptors were obtained as follows. The cDNA encoding the rabbit M-type receptor (*76*) was subcloned into the expression vector pRc/CMV (InvitroGen) and transfected into HEK-293 cells (ATCC) by the calcium phosphate procedure. Stably transfected cells were selected for 4 weeks in the presence of 2 mg/mL G-418 neomycin. The cDNA encoding the mouse membrane-bound M-type receptor (*72*) was subcloned into the expression vector pCi/neo (Promega) and transfected into COS cells (ATCC) by the DEAE/dextran procedure. G-418 resistant cells were selected as described above. Cell membranes containing endogenously expressed mouse M-type receptor were obtained by growing the osteoblastic-like MC3T3-E_1 cells (Riken cell bank). Cell membranes were prepared from transfected cells or MC3T3-E_1 cells grown to confluency in 140 mm plates. Cells were washed twice with phosphate-buffered saline and then scraped with a rubber policeman at 4 °C in a buffer consisting of 140 mM NaCl, 20 mM Tris (pH 7.4), 2 mM EDTA, and 0.1 mM phenylmethanesulfonyl fluoride. The cell suspension was homogenized with a small cell disrupter, and the resulting homogenate was centrifuged for 5 min at 1000*g* and 4 °C. The supernatant was centrifuged at 100000*g* for 45 min. The pellet was resuspended in lysis buffer at a protein concentration of 10 mg/mL and stored in aliquots at − 80 °C. Protein concentrations were determined using the Bradford method after treatment of membranes with 0.1 N NaOH and using bovine serum albumin as a standard.

Receptor Binding Studies. The different binding experiments were performed under equilibrium conditions using the various iodinated $sPLA_2$s ([^{125}I]OS_1, [^{125}I]OS_2, [^{125}I]OS_2 D49K, [^{125}I]mGIIA, [^{125}I]mGIIF, and [^{125}I]mGX) labeled to a specific activity of 3000−3500 cpm/fmol and purified by reverse-phase HPLC as described previously (*74*). Briefly, the indicated ^{125}I-labeled $sPLA_2$, unlabeled $sPLA_2$, and/or $sPLA_2$ inhibitors were preincubated at room temperature in 0.25 mL of binding buffer [20 mM Tris-HCl (pH 7.4), 140 mM NaCl, 2 mM $CaCl_2$, and 0.1% bovine serum albumin], after which the binding assay was started by addition of receptor (membrane-bound or soluble receptor) diluted in 0.25 mL of binding buffer. After incubation for 1 h, binding assays with membrane-bound receptors were terminated by being filtered through GF/C glass fiber filters (Millipore) presoaked in 0.5% polyethyleneimine (for iodinated OS_1, OS_2, and OS_2 D49K) or presoaked in binding buffer containing 10% BSA (for iodinated mGIIF), or through 0.45 μM acetate filters (Sartorius) presoaked in binding buffer containing 10% BSA (for iodinated mGIIA and mGX). Binding assays with the mouse soluble receptor were stopped when the samples were filtered through GF/F glass fiber filters (Millipore) presoaked in 5% polyethyleneimine (for iodinated OS_1, OS_2, and OS_2 D49K). For assays of binding to live MC3T3-E_1 cells, cells were grown to confluency in six-well plates in αMEM supplemented with 10% FBS and antibiotics. Cells were washed twice with phosphate-buffered saline and incubated for 1 h with 1 mL of each iodinated $sPLA_2$ (100 pM) diluted in αMEM containing 0.1% BSA. Cells were then washed three times with αMEM and 0.1% BSA and scraped in 0.1 N NaOH, and the cell-associated radioactivity was counted on a gamma counter.

Cross-Linking Experiments and Western Blot of the M-Type Receptor. Cross-linking experiments were performed as described previously (*74*). Briefly, the soluble mouse M-type receptor (1 nM) was incubated with iodinated radiolabeled $sPLA_2$ (300 pM) in 150 μL of cross-linking buffer [20 mM Hepes (pH 7.4), 140 mM NaCl, and 1 mM $CaCl_2$] in the presence or absence of Me-Indoxam (20 μM) or unlabeled homologous competitor (100 nM). After incubation for 1 h, 200 μM suberic acid bis-*N*-hydroxysuccinimide ester (DSS, Sigma, dissolved at 10 mM in DMSO) was added for 15 min at room temperature. The cross-linking reaction was quenched by addition of SDS−PAGE sample buffer, and proteins were separated by gel electrophoresis. Gels were stained with Coomassie Brillant blue, dried, and exposed for 3 days at −80 °C using Kodak X-Omat AR film.

For Western blot analysis with the anti-mouse M-type receptor antibodies, cells were sonicated in 20 mM Tris (pH 7.4), 2 mM EDTA, and 2 mM PMSF, and proteins were solubilized in Laemmli buffer but neither boiled nor reduced. Proteins were separated on a 7% SDS−PAGE gel and transferred to an Immobilon-P^{sq} polyvinylidene fluoride membrane (Millipore). The transfer membrane was blocked with 5% blocking agent (Amersham Biosciences) dissolved in TBS-Tween [25 mM Tris (pH 7.8), 150 mM NaCl, and 0.15% Tween 20] for 30 min and then incubated with rabbit polyclonal anti-M-type receptor antibodies (1/5000 in TBS-Tween) for 1 h at room temperature. Rabbit polyclonal antibodies raised against the cloned mouse soluble M-type receptor were obtained as described previously (*59*). These antibodies recognize the mouse receptor only under non-reducing conditions. The membrane was washed six times for 5 min each and incubated with a secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (1/20000 in TBS-Tween) and washed six times for 5 min each. Immunodetection was performed by chemifluorescence using the ECF reagent (Amersham Biosciences).

RESULTS

Dual Effect of Me-Indoxam on the Catalytic Activity and M-Type Receptor Binding of the Snake Venom $sPLA_2$s OS_1 and OS_2. The snake venom $sPLA_2$s OS_1 and OS_2 from the Australian Taipan snake *Oxyuranus scutellatus scutellatus* have been very useful tools for the characterization of the M-type receptor from rabbit, rat, mouse, and human species (*70*, *72*). These $sPLA_2$s have also been important in the study of the binding properties of the full set of mouse $sPLA_2$s by competition binding assays (*72*; M. Rouault et al., manuscript

Table 1: Inhibition of the Catalytic Activity of Venom and Mouse $sPLA_2$s by Me-Indoxam Using [^{3}H]Oleate Radiolabeled *E. coli* Membranes as a Substrate

$sPLA_2$	IC_{50} (nM)	$sPLA_2$	IC_{50} (nM)
OS_1	25	mGIIE	15
OS_2	210	mGIIF	10000
mGIB	300	mGX	300
mGIIA	1.5		

submitted for publication). We therefore first addressed the effect of Me-Indoxam (Figure 8) on the enzymatic and binding properties of these two $sPLA_2$s. Enzymatic activities of both OS_1 and OS_2 were sensitive to Me-Indoxam with IC_{50} values of 25 and 210 nM, respectively (Table 1). It is interesting to note that although OS_1 and the mammalian pancreatic $sPLA_2$s are both pancreatic-type group IB $sPLA_2$s (*77*), Me-Indoxam binds much tighter to OS_1 than to mouse or human pancreatic enzymes (*46*). The affinity of Me-Indoxam for OS_1 is close to those measured for human and mouse group IIE and V $sPLA_2$s, confirming that there is no obvious relationship between the $sPLA_2$ classification into structural groups and their relative affinities for $sPLA_2$ inhibitors (*46*).

We next analyzed the effect of Me-Indoxam on binding of OS_1 and OS_2 to membrane-bound and soluble forms of the mouse recombinant M-type receptor. The binding of radiolabeled OS_1 to the two receptor forms was dose-dependently inhibited by Me-Indoxam with IC_{50} values of ~0.2 μM (Figure 1). The effect of Me-Indoxam on the binding of radiolabeled OS_2 could not be easily analyzed (not shown) because Me-Indoxam leads to a high level of nonspecific binding of [^{125}I]OS_2 to binding filters (see Experimental Procedures) and also increases the level of binding of [^{125}I]OS_2 to membranes from COS cells (Figure 5C) or *E. coli* (not shown). This binding probably occurs on membrane phospholipids (see below). Since the binding of OS_1 to the M-type receptor is Ca^{2+}-independent (*77*), it was possible to address whether the effect of Me-Indoxam on OS_1 binding requires Ca^{2+}. As shown in Figure 1, Me-Indoxam could no longer inhibit [^{125}I]OS_1 binding in the presence of 2 mM EDTA. The absence of inhibition was expected since Me-Indoxam and several other indole analogues are coordinated to the Ca^{2+} ion bound to $sPLA_2$ (Figure 7 and refs *32*, *48*, and *78*).

Preparation of an OS_2 Mutant with High Affinity for the M-Type Receptor but No Affinity for Me-Indoxam. On the basis of the results given above, we reasoned that the most straightforward way to address the effect of Me-Indoxam on the binding properties of different mammalian $sPLA_2$s would be to perform competition binding assays with $sPLA_2$s with and without saturating concentrations of Me-Indoxam and against a radiolabeled $sPLA_2$ ligand that binds to the M-type receptor but does not bind the inhibitor. Since the binding of both OS_1 (Figure 1) and OS_2 (not shown) appeared to be highly sensitive to Me-Indoxam in a Ca^{2+}-dependent manner while binding of $sPLA_2$ to the M-type receptor is Ca^{2+}-independent, we postulated that an OS_1 or OS_2 mutant that cannot bind Ca^{2+} should have altered binding properties toward Me-Indoxam, yet it should still bind to the receptor. In fact, the D49K OS_2 mutant which was recently produced in our laboratory was found to fulfill these criteria. Indeed, this mutant was found to have no enzymatic activity because

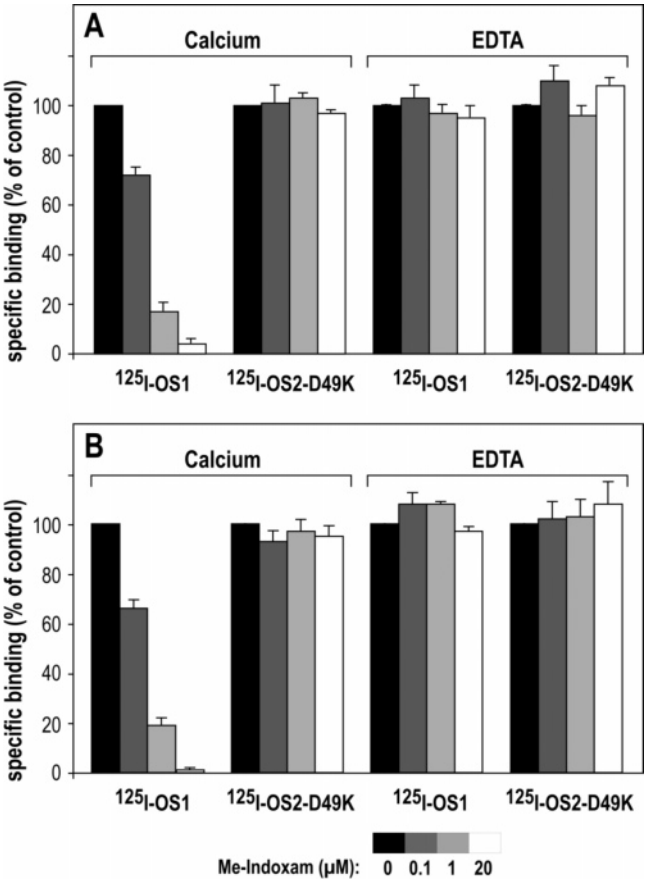

FIGURE 1: Effect of Me-Indoxam on the binding of [^{125}I]OS_1 and [^{125}I]OS_2 D49K to membrane-bound (A) and soluble (B) mouse M-type receptors. The binding of [^{125}I]OS_1 (80 pM) and [^{125}I]OS_2 D49K (80 pM) to recombinant soluble and membrane-bound mouse M-type receptors was assessed in the presence of various concentrations of Me-Indoxam in binding buffer with 1 mM calcium or 2 mM EDTA. Nonspecific binding was assessed by addition of 50 nM homologous unlabeled $sPLA_2$. The data are mean values ± the standard error of the mean of four independent experiments.

it cannot bind Ca^{2+}, yet it can still bind to the M-type receptor (*74*). Assaying the binding of Me-Indoxam to the D49K OS_2 mutant by enzymatic activity was not possible as the mutant is completely devoid of catalytic activity, even using the sensitive *E. coli* assay (not shown). We thus radiolabeled the D49K OS_2 mutant and analyzed whether its binding to the mouse M-type receptor is sensitive to Me-Indoxam. As shown in Figure 1, its binding to membrane-bound and soluble mouse M-type receptors was completely insensitive to Me-Indoxam in the presence or absence of Ca^{2+}. The absence of an effect of Me-Indoxam on the binding of [^{125}I]OS_2 D49K was confirmed by direct binding experiments with the purified mouse soluble M-type receptor preparation with various concentrations of radiolabeled ligand (Figure 2). In the absence or presence of a saturating concentration of Me-Indoxam that was found to impair binding of [^{125}I]OS_1 (Figure 1) or of unlabeled OS_2 (Figure 3), the binding of [^{125}I]OS_2 D49K was unchanged. Scatchard plot analysis shows that [^{125}I]OS_2 D49K has the same affinity of 0.05 nM with and without Me-Indoxam and binds to the same number of binding sites (Figure 2). Together, the results described above indicated that the D49K OS_2 mutant is a potent ligand of the M-type receptor that cannot bind Me-Indoxam. It is thus a good tool for easily monitoring the effect of Me-

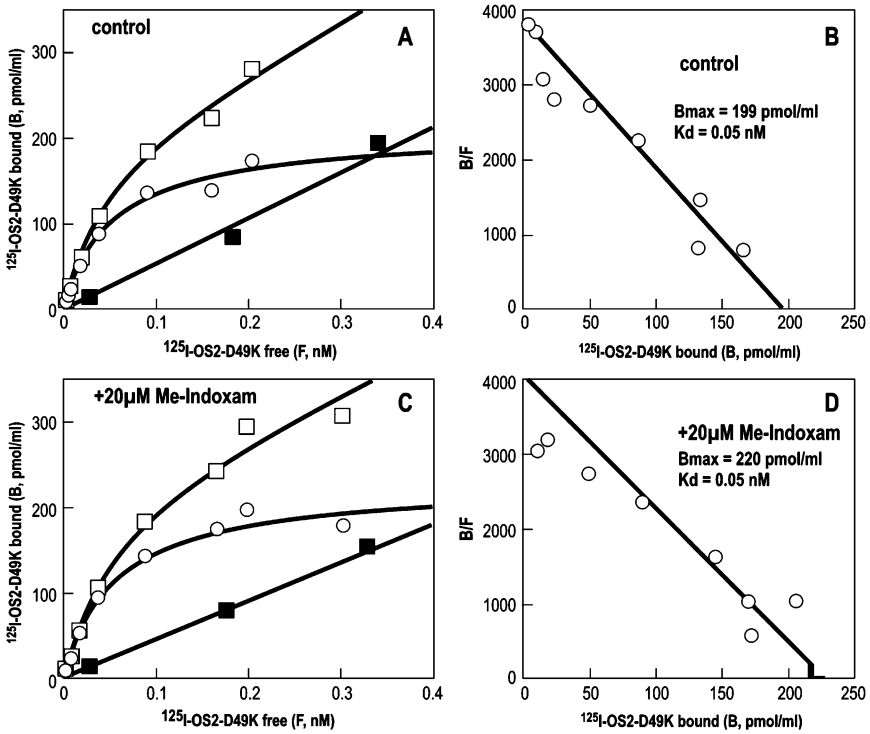

FIGURE 2: Binding of the [^{125}I]OS_2 D49K mutant to the mouse soluble M-type receptor in the presence or absence of Me-Indoxam. Equilibrium binding assays were performed in the absence (A and B) or presence of 20 μM Me-Indoxam (C and D). The mouse soluble M-type receptor was incubated with various concentrations of the [^{125}I]OS_2 D49K mutant in the absence (□) or presence (■) of 50 nM unlabeled OS_2 D49K mutant. Specific binding (○) represents the difference between total binding (□) and nonspecific binding (■). K_d and B_{max} values were determined by Scatchard plot analysis of the specific binding. The data are representative of three independent experiments.

Indoxam on the binding of various $sPLA_2$s to the M-type receptor.

Me-Indoxam Forms a $sPLA_2$−Inhibitor Complex that Has a Dramatically Reduced Affinity for the M-Type Receptor. We first analyzed the effect of Me-Indoxam on the binding of OS_1 and OS_2 to the mouse soluble M-type receptor by competition binding assays between [^{125}I]OS_2 D49K and these $sPLA_2$s in the absence or presence of various concentrations of Me-Indoxam. Performing the binding assays at various Me-Indoxam concentrations allowed us to experimentally determine the concentration of Me-Indoxam that is sufficiently high to saturate the added competing $sPLA_2$. On the basis of the IC_{50} value of Me-Indoxam for OS_1 and OS_2 measured by enzymatic activity (Table 1), we can also calculate by the mass action law that at least 99% of the added $sPLA_2$ is complexed with Me-Indoxam at 20 μM inhibitor. At this latter inhibitor concentration, the only competing ligand is thus the $sPLA_2$−Me-Indoxam complex. As shown in Figure 3, OS_1 and OS_2 competed with [^{125}I]OS_2 D49K for the M-type receptor with high affinities of 0.09 and 0.05 nM, respectively, in the absence of Me-Indoxam. The presence of various concentrations of Me-Indoxam dose-dependently shifted their affinities up to lower limits of 27 and 8 nM for OS_1 and OS_2, respectively. The fact that adding various concentrations of Me-Indoxam leads to maximal shifts in affinities for OS_1 and OS_2 clearly indicates that at concentrations of Me-Indoxam above 10 μM for OS_1 and 15 μM for OS_2, all the competing $sPLA_2$ ligand is not free $sPLA_2$, but $sPLA_2$ bound to Me-Indoxam. These results indicate that the OS_1−Me-Indoxam and OS_2−Me-Indoxam complexes can still bind to the M-type receptor, yet with affinities which are 300- and 138-fold lower than those of free enzymes, respectively (Table 2).

We next monitored the effect of Me-Indoxam on the affinities of the different mouse $sPLA_2$s that were found to be high-affinity ligands of the mouse M-type receptor (M. Rouault et al., manuscript submitted for publication; Table 2) and that are sensitive to Me-Indoxam (Table 1). mGIIA and mGIIE $sPLA_2$s have very high affinities for Me-Indoxam, higher than or similar to that of OS_1. mGIB and mGX have affinities for Me-Indoxam which are about the same as that of OS_2. Only mGIIF $sPLA_2$ has a weak affinity of 10 μM. Thus, as for OS_1 and OS_2, it is expected that addition of 20 μM Me-Indoxam will complex all but one mouse $sPLA_2$ added in the [^{125}I]OS_2 D49K competition binding assays. The only exception will be mGIIF, for which 20 μM Me-Indoxam will complex approximately two-thirds of the total amount of added enzyme, as it can be calculated by the mass action law using the IC_{50} value determined by enzymatic assays (Table 1). Figure 4 and Table 2 show that addition of 20 μM Me-Indoxam shifts the affinity of all mouse $sPLA_2$s for the mouse soluble M-type receptor. To further confirm that 20 μM Me-Indoxam was a sufficient concentration for saturating all of the $sPLA_2$ enzyme added as a competitor, we performed competition binding assays with mGX at different concentrations of Me-Indoxam. mGX $sPLA_2$ was chosen because it has the lowest affinity for Me-Indoxam among the various enzymes that were assayed (except for mGIIF). Interestingly, the shift in affinity (ΔIC_{50}) was dependent on the mouse $sPLA_2$, indicating that Me-Indoxam differentially affects the interaction of each $sPLA_2$ with the M-type receptor. Importantly, the ΔIC_{50} value was not correlated with the IC_{50} value of $sPLA_2$ for Me-Indoxam (Table 1). For example, affinity shifts for mGIIA and mGIIE were 31- and 300-fold, respectively, although mGIIA binds Me-Indoxam 10-fold more tightly than mGIIE. Furthermore,

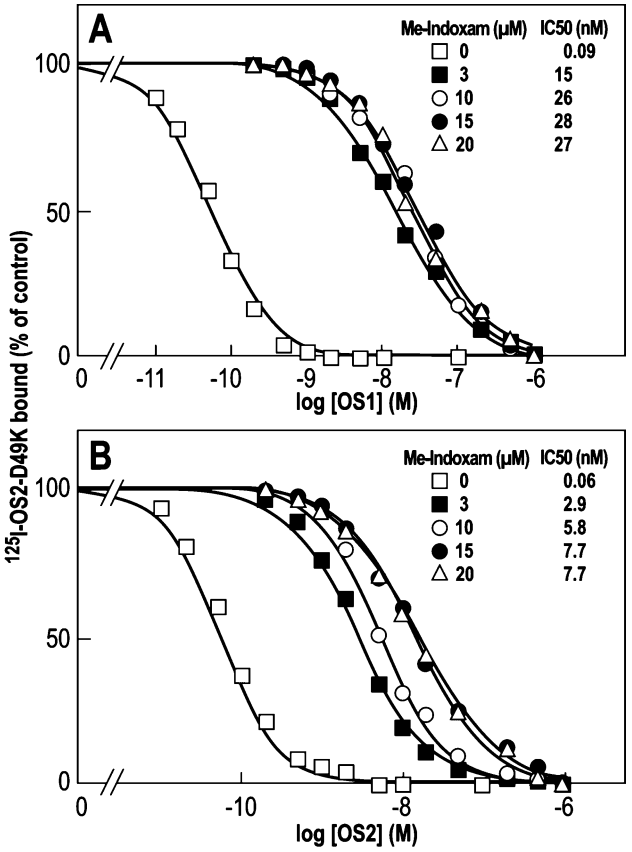

FIGURE 3: Me-Indoxam interferes with the binding of OS_1 and OS_2 to the M-type receptor by shifting their affinities for the receptor. Competition binding assays were performed by incubating the mouse soluble M-type receptor in the presence of [^{125}I]OS_2 D49K (50 pM) and various concentrations of unlabeled OS_1 or OS_2 at the indicated concentrations of Me-Indoxam. Nonspecific binding was assessed by addition of 50 nM unlabeled OS_2 D49K mutant. The IC_{50} values are the concentrations of unlabeled OS_1 or OS_2 that inhibit 50% of [^{125}I]OS_2 D49K mutant specific binding. The data are representative of at least four independent experiments.

Table 2: Properties of Binding of Venom and Mouse $sPLA_2$s to the Mouse M-Type Receptor in the Presence or Absence of 20 μM Me-Indoxam[a]

$sPLA_2$	IC_{50}(control) (nM)	IC_{50}(Me-Indoxam) (nM)	ΔIC_{50}
OS_1	0.090	27	300
OS_2	0.056	7.7	138
OS_2 D49K	0.051	0.049	0.96
mGIB	1.6	7.9	5
mGIIA	1	31	31
mGIIA[b]	0.53	0.74	1.4
mGIIE	0.158	47	300
mGIIF	0.316	0.890	2.8
mGX	2.5	250	100

[a] IC_{50} values were determined by competition binding experiments with [^{125}I]OS_2 D49K and the different unlabeled $sPLA_2$s as shown in Figures 3 and 4. All of the binding assays were performed in the presence of 2 mM $CaCl_2$ except when specified. ΔIC_{50} is the ratio of the IC_{50} values measured in the absence (control) and presence of Me-Indoxam. The values are representative of at least three competition binding assays. [b] Competition binding assays performed with 2 mM EDTA.

the affinity shifts for mGIB and mGX were 5- and 100-fold, respectively, while the two enzymes have identical affinities for Me-Indoxam. These observations indicate that the affinity shift is not due to an incomplete saturation of the competing $sPLA_2$ with Me-Indoxam but rather reflects a differential perturbating effect of Me-Indoxam on the interaction of the various mouse $sPLA_2$s with the M-type receptor. Our binding conditions did not allow a clear evaluation of the effect of Me-Indoxam on mGIIF binding as this $sPLA_2$ binds the inhibitor too poorly. The very modest 3-fold shift in affinity observed in this case most likely reflects inhibition of [^{125}I]OS_2 D49K binding by the remaining mGIIF $sPLA_2$ which is free of Me-Indoxam. Figure 4A also shows that Me-Indoxam has no effect on the binding of mGIIA to the M-type receptor when the binding assay is performed in the absence of Ca^{2+}, further demonstrating that Ca^{2+} is required for binding of Me-Indoxam to the $sPLA_2$. Finally, as expected, competition binding curves with the unlabeled D49K OS_2 mutant were identical in the presence or absence of 20 μM Me-Indoxam.

To further confirm that an $sPLA_2$−Me-Indoxam complex can still bind to the M-type receptor, we performed cross-linking experiments with [^{125}I]OS_1 and [^{125}I]OS_2 in the absence or presence of a saturating concentration of Me-Indoxam relative to the concentration of radiolabeled ligand (Figure 4B). Control cross-linking assays to confirm that [^{125}I]OS_2 D49K and [^{125}I]mGIIF are insensitive to the presence of Me-Indoxam were also performed. Figure 4B shows that the mouse soluble M-type receptor could be efficiently cross-linked to [^{125}I]OS_1 and [^{125}I]OS_2 in the presence of Me-Indoxam. The receptor could also be labeled with [^{125}I]OS_2 D49K and [^{125}I]mGIIF in a manner independent of the presence of Me-Indoxam.

Direct Binding of Mouse and Snake Venom $sPLA_2$s to Cell Membranes and Live Cells Expressing the M-Type Receptor in the Presence of Me-Indoxam. To further illustrate how Me-Indoxam can interfere with the binding of different $sPLA_2$s in a more physiologically relevant context, we performed direct binding experiments with radiolabeled OS_1, OS_2, OS_2 D49K, mGIIA, mGIIF, and mGX on cellular membranes and live cells expressing the M-type receptor (Figures 5 and 6). Assays of direct binding of [^{125}I]mGIIA to membrane preparations from COS cells transfected with the mouse M-type receptor gave a strong binding that could not be inhibited by unlabeled mGIIA (Figure 5A), suggesting that this binding occurs on an abundant population of binding sites. This binding does not occur on the transfected M-type receptor since OS_1, the very specific $sPLA_2$ ligand of the M-type receptor, does not compete with [^{125}I]mGIIA binding. Furthermore, the strong and similar binding of [^{125}I]mGIIA on membranes from mock-transfected COS cells clearly indicates that this binding occurs on a cellular component which is distinct from the M-type receptor (Figure 5C). Because human and mouse group IIA $sPLA_2$s are basic proteins that strongly interact with anionic heparan sulfate proteoglycans (*79*), we analyzed the effect of various concentrations of the basic compound poly-L-lysine as a competitor. Under our binding assay conditions, concentrations of poly-L-lysine higher than 50 μg/mL inhibited the binding to the M-type receptor (not shown). However, at the optimal concentration of 50 μg/mL, we could reduce the level of nonspecific binding of [^{125}I]mGIIA and observe the specific binding to the transfected M-type receptor (Figure 5A). Under these conditions, the binding of [^{125}I]mGIIA could be inhibited by unlabeled mGIIA and OS_1, but not by the bee venom $sPLA_2$ [which does not bind to the M-type

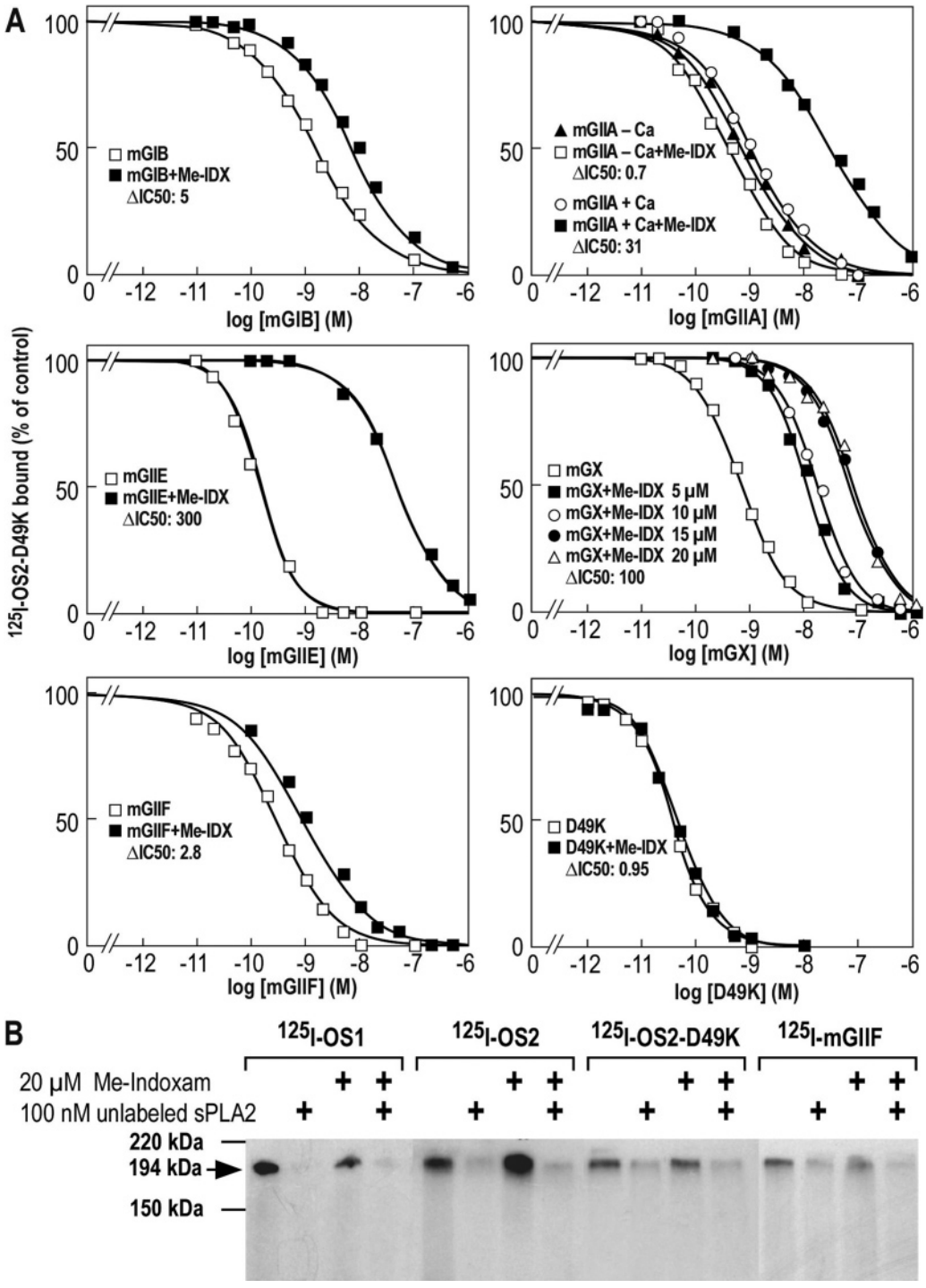

FIGURE 4: Me-Indoxam (Me-IDX) shifts to different extents the affinities of mouse $sPLA_2$s for the M-type receptor. (A) Competition binding assays were performed by incubating the mouse soluble M-type receptor in the presence of [^{125}I]OS_2 D49K (50 pM) and various concentrations of unlabeled mouse $sPLA_2$s or the D49K mutant in the presence or absence of 20 μM Me-Indoxam (except when specified). A buffer containing 1 mM $CaCl_2$ was used in all binding assays except for mGIIA where the effect of Me-Indoxam was also analyzed in the presence of 2 mM EDTA (−Ca). Nonspecific binding was assessed by addition of 50 nM unlabeled OS_2 D49K mutant. The IC_{50} values are the concentrations of unlabeled $sPLA_2$s that inhibit 50% of [^{125}I]OS_2 D49K specific binding. The ΔIC_{50} value is the ratio of the IC_{50} value measured at an inhibitor concentration sufficient to saturate unlabeled $sPLA_2$ to the IC_{50} value measured in the absence of Me-Indoxam. The data are representative of at least four independent experiments. (B) Cross-linking of iodinated OS_1, OS_2, OS_2 D49K, and mGIIF to the mouse soluble M-type receptor in the presence or absence of 20 μM Me-Indoxam. Binding assays and cross-linking were performed as described in Experimental Procedures. The resulting complexes were separated on a 4 to 12% SDS−polyacrylamide gel and visualized by autoradiography. The results are representative of two independent experiments.

receptor (*72*)], confirming that this binding is indeed to the M-type receptor.

As shown in panels B and C of Figure 5, Me-Indoxam produces different effects on the binding of the various radiolabeled $sPLA_2$s to cell membranes containing or lacking the M-type receptor. As expected, Me-Indoxam inhibited the binding of [^{125}I]OS_1 to membranes containing the M-type receptor (Figure 5B). On the other hand, Me-Indoxam had no effect on the very weak binding of [^{125}I]OS_1 to mock-transfected cells (Figure 5C). Me-Indoxam also inhibits the

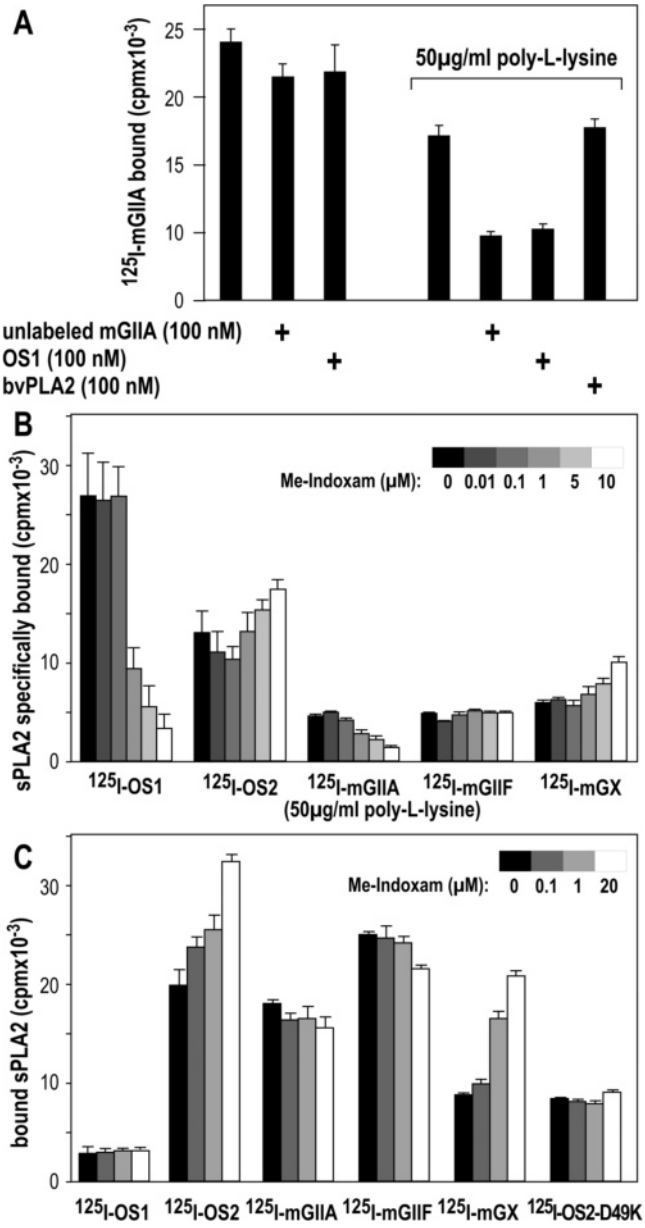

FIGURE 5: Effect of Me-Indoxam on the binding of iodinated mouse $sPLA_2$s to COS cell membranes containing the M-type receptor. The indicated $sPLA_2$s were iodinated, and their binding properties were analyzed with COS cell membranes expressing the mouse M-type receptor (A and B) or not (C). (A) Direct binding properties of [^{125}I]mGIIA in the absence or presence of 50 μg/mL poly-L-lysine. Addition of poly-L-lysine to the binding buffer allows detection of the specific binding of [^{125}I]mGIIA to the M-type receptor, as shown by inhibition with unlabeled OS_1 and mGIIA, but not with unlabeled bee venom $sPLA_2$. (B and C) Effect of Me-Indoxam on the binding of iodinated OS_1, OS_2, mGIIA, mGIIF, and mGX $sPLA_2$s to membranes from COS cells transfected with the M-type receptor (B) or mock-transfected (C). COS cell membranes were incubated at room temperature for 1 h with each iodinated $sPLA_2$ at 100 pM in the presence of various concentrations of Me-Indoxam. Binding of [^{125}I]mGIIA was performed in the presence of 50 μg/mL poly-L-lysine. The specific binding to the mouse M-type receptor and the total binding are given in panels B and C, respectively. The level of specific binding was calculated by substracting the level of nonspecific binding (measured with 50 nM homologous unlabeled $sPLA_2$) from the level of total binding (no unlabeled homologous $sPLA_2$ competitor). These results are representative of at least four independent experiments.

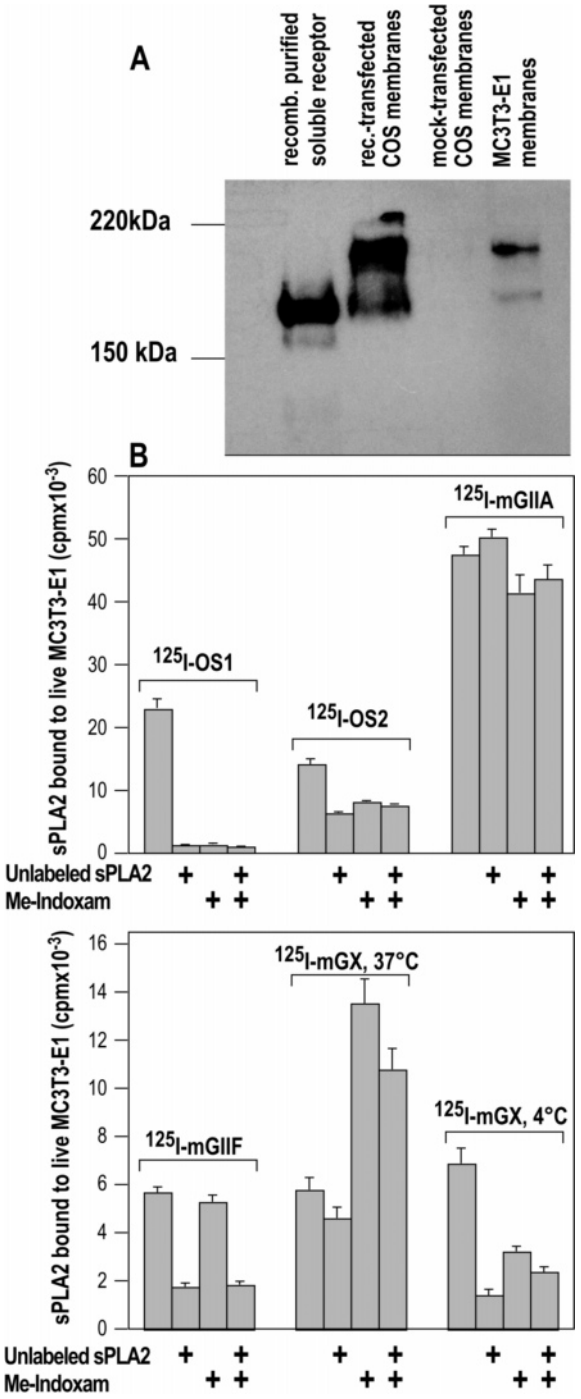

FIGURE 6: Effect of Me-Indoxam on the binding of iodinated mouse $sPLA_2$s to mouse osteoblastic MC3T3-E_1 cells endogenously expressing the M-type receptor. (A) Western blot showing the endogenous expression of the M-type receptor in MC3T3-E_1 cells. The two bands observed for both endogenous and transfected receptor preparations are due to incomplete denaturation of the receptor under nonreducing conditions (see Experimental Procedures). (B) Binding of the different iodinated $sPLA_2$s to live MC3T3-E_1 cells. sPLA2 binding was assessed at 37 °C with 100 pM iodinated $sPLA_2$ in the presence or absence of 20 μM Me-Indoxam and 100 nM unlabeled homologous $sPLA_2$ competitor in a binding buffer containing 1 mM $CaCl_2$ (see Experimental Procedures). Binding was also performed at 4 °C for iodinated mGX. After being incubated for 60 min, cells were washed three times with binding buffer, and the cell-associated radioactivity was measured. The data are representative of three independent experiments.

binding of [^{125}I]mGIIA when the binding assays were run in the presence of poly-L-lysine (Figure 5B). On the other hand, Me-Indoxam has no effect on the high-capacity binding of [^{125}I]mGIIA to mock-transfected cells (Figure 5C). Although Me-Indoxam was expected to inhibit the binding of [^{125}I]OS_2 and [^{125}I]mGX to membranes containing the M-type receptor (Figure 3), the binding of both ligands was strengthened. Since Me-Indoxam also strengthens the binding of the two ligands to mock-transfected cell membranes (Figure 5C) and *E. coli* membranes (not shown), it is likely that Me-Indoxam has two effects on these two $sPLA_2$ ligands; i.e., it strengthens their binding to membrane phospholipids, while it weakens their binding to the M-type receptor. This probably explains why the increase in level of binding produced by Me-Indoxam is smaller for membranes containing the receptor (Figure 5B) than for membranes lacking the receptor. Finally, Me-Indoxam did not strengthen the binding of [^{125}I]OS_2 D49K to COS cell membranes (Figure 5C), and as expected from the weak affinity of mGIIF for the inhibitor, the binding of [^{125}I]mGIIF to both types of cell membranes was not very sensitive to the inhibitor (Figure 5B,C).

We finally analyzed the effect of Me-Indoxam on the binding of the various iodinated $sPLA_2$s at 37 °C to live osteoblastic MC3T3-E_1 cells which constitutively express the mouse M-type receptor (*73*). Western blot analysis confirmed that our MC3T3-E_1 subclone does express a receptor similar in size to the transfected membrane-bound mouse M-type receptor (Figure 6A). As expected, a specific binding of [^{125}I]OS_1 to the cell surface M-type receptor was detected, and this binding was efficiently inhibited by Me-Indoxam (Figure 6B). A similar situation was found for [^{125}I]OS_2 (Figure 6B). As observed above with cellular membranes, the binding of [^{125}I]mGIIA was very strong on live MC3T3-E_1 cells. This binding was not inhibited by unlabeled mGIIA, and the level of binding was neither decreased nor increased by Me-Indoxam, indicating that mGIIA binds to a cellular component which is insensitive to the inhibitor. We could not use poly-L-lysine on cells to decrease the level of mGIIA binding because the compound was toxic to cells and they become detached from the culture dish. The binding of [^{125}I]mGIIF was specific. On the basis of inhibition by unlabeled OS_1 (not shown), this binding was found to occur on the M-type receptor. As expected (Table 2), it was almost insensitive to Me-Indoxam. Finally, at 37 °C, the binding of [^{125}I]mGX was found to occur on a major cellular component which is different from the M-type receptor, as checked by the poor inhibition with unlabeled mGX (Figure 6B) and OS_1 (not shown). The binding to this cellular component was dramatically strengthened by Me-Indoxam (Figure 6B). Since mGX binds to phosphatidylcholine which is present in large amounts at the cell surface, the most likely explanation is that Me-Indoxam strengthens the binding of [^{125}I]mGX to this phospholipid surface. To decrease the level of binding of [^{125}I]mGX to this phospholipid, we performed binding assays at 4 °C. At this temperature, the binding of mGX to the M-type receptor could be revealed since [^{125}I]mGX binding was inhibited by unlabeled mGX, OS_1 (not shown), and Me-Indoxam (Figure 6B).

Structurally Distinct Competitive $sPLA_2$ Inhibitors of Different Sizes Differentially Modulate the Binding of $sPLA_2$s to the M-Type Receptor. Since Me-Indoxam binds to the catalytic site of $sPLA_2$, we postulated that the perturbating

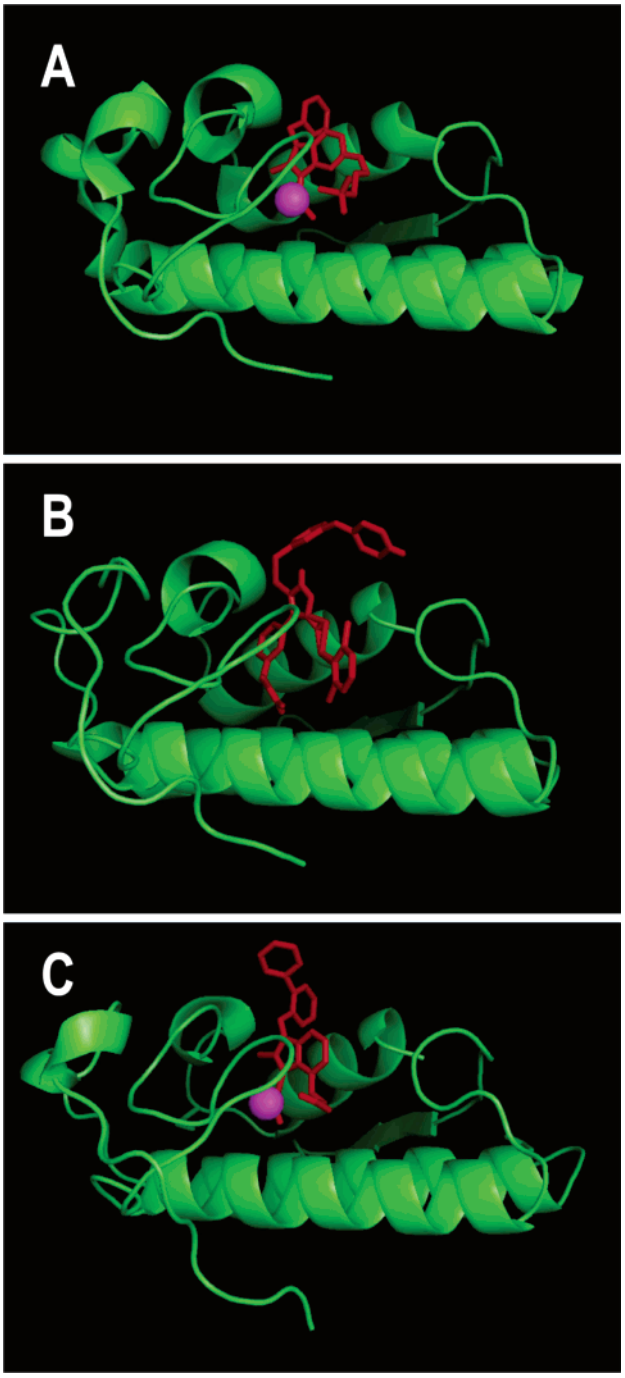

FIGURE 7: (A) Structure of hGIIA $sPLA_2$ complexed with LY311727 in the presence of Ca^{2+} (*32*). (B) Structural model of hGIIA complexed with Pyrazole-1. The model was generated by docking using FLO/QXP (*88*). (C) Structure of hGX $sPLA_2$ complexed with Me-Indoxam in the presence of Ca^{2+} (*48*). The ribbon views were generated from the PDB files with Molscript and show the different protrudings of inhibitors at the interfacial binding surface of $sPLA_2$.

effect of Me-Indoxam on the $sPLA_2$−receptor interaction could be due in large part to its protrusion out of the catalytic groove, as indicated by analysis of the cocrystal structure of hGX sPLA2 complexed with Me-Indoxam (Figure 7). The fact that $sPLA_2$ residues located at the interfacial binding surface are important for interaction with the receptor also fits with this view (*77*). We thus hypothesized that the impact of various $sPLA_2$ inhibitors on the $sPLA_2$−receptor interaction will be different depending on the inhibitor size and

Table 3: Effect of Various $sPLA_2$ Inhibitors on the Catalytic Activity and Binding Properties to the M-Type Receptor of hGIIA and hGV $sPLA_2$s[a]

$sPLA_2$	inhibitor	inhibitor IC_{50} on enzymatic activity (μM)	inhibitor concentration in the binding assay (μM)	$sPLA_2$ IC_{50} for M-type receptor binding (nM)	ΔIC_{50} for M-type receptor binding
hGIIA	–	–	–	14	1
	Me-Indoxam	0.010	20	180	13
	Indoxam	0.006	20	350	25
	LY311727	0.030	20	105	8
	compound A	0.007	20	370	26
	compound B	0.007	20	480	34
	compound 42	0.025	10	66	5
	compound 44	0.025	10	44	3
	compound 46	0.020	10	83	6
	Pyrazole-1	0.100	20	430	31
hGV	–	–	–	18	1
	molecule 19	10	100	900	50

[a] IC_{50} values of inhibitors with $sPLA_2$ enzymatic activity were determined using *E. coli* membranes as a substrate. IC_{50} values of hGIIA or hGV $sPLA_2$s for the M-type receptor were measured by competition binding assays with unlabeled $sPLA_2$s and [^{125}I]OS_2 D49K for binding to the rabbit M-type receptor in the absence or presence of a saturating concentration of inhibitor. The ΔIC_{50} value for M-type receptor binding is the ratio of the IC_{50} value measured with inhibitor to the IC_{50} value measured without inhibitor.

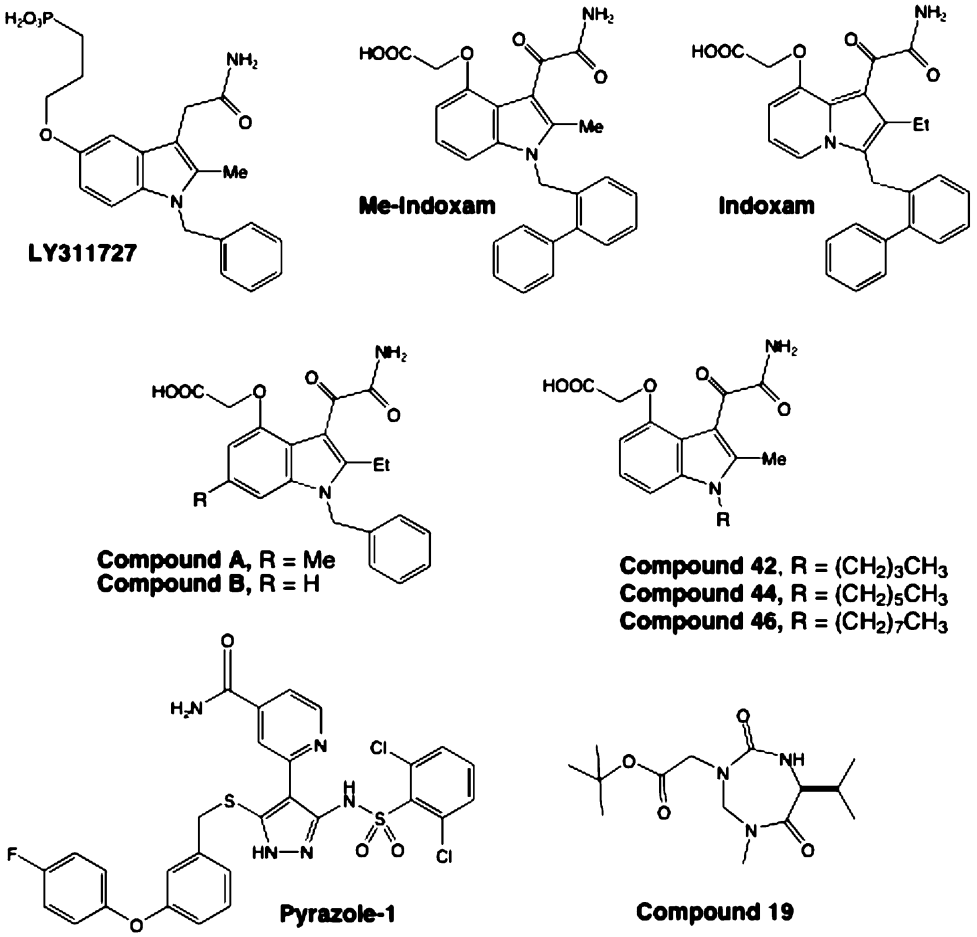

FIGURE 8: Structures of the sPLA2 inhibitors used in this study: LY311727 (*32*), Indoxam (*40*), Me-Indoxam and Pyrazole-1 (*46*), compounds A and B (*33*), compounds 42, 44, and 46 (*48*), and compound 19 (*75*, *80*). Note that Me-Indoxam is an indole analogue with a methyl group at the 2-position, whereas Indoxam is an indolizine analogue with an ethyl group at the 2-position.

extent of protrusion from the surface of the $sPLA_2$. To evaluate this, we analyzed the effect of various hGIIA $sPLA_2$ inhibitors (Table 3 and Figure 8) on the binding of this $sPLA_2$ to the M-type receptor. These inhibitors include LY311727 and Pyrazole-1 which differentially protrude from the hGIIA active site (Figure 7). We also assayed Indoxam (*40*), compounds A and B (*33*), and indole compounds 42, 44, and 46 which bear small alkyl groups at the N1 position (*48*). Importantly, compound B is identical to S-5920/LY315920Na, which has been used in several animal models and clinical trials (*31*). As expected, the $sPLA_2$ inhibitors that protrude more substantially from the catalytic site produced larger affinity shifts for the binding of hGIIA to the M-type receptor (Table 3). Interestingly, compounds 42, 44, and 46, which are predicted to be buried in the catalytic site (*48*), produce only marginal effects on the affinity of

hGIIA, although they are quite potent inhibitors of the enzyme (Table 3 and ref *48*).

$sPLA_2$ Binding Assay with the M-Type Receptor Can Be Used To Screen for True Competitive $sPLA_2$ Inhibitors. $sPLA_2$ inhibitors can be divided into true competitive inhibitors that bind tightly to the catalytic site and nonspecific inhibitors that bind to the phospholipid interface and promote desorption of $sPLA_2$ (*1*). We reasoned that our $sPLA_2$ receptor binding assay could be a sensitive and rapid method for identifying novel compounds or characterizing putative $sPLA_2$ inhibitors as true competitive $sPLA_2$ inhibitors. As shown above, we showed that many $sPLA_2$ inhibitors that are true competitive inhibitors of $sPLA_2$ induce a shift in the affinity of $sPLA_2$ for the M-type receptor. To verify the specificity of our $sPLA_2$ binding assay for true competitive inhibitors, we analyzed the effect of the nonspecific inhibitors quinacrine (1 mM) and polymyxin B (5 μg/mL). None of the two compounds were able to affect the binding of OS_1, OS_2, hGIIA, or hGX under conditions similar to those described in the legend of Figure 4 (not shown). To demonstrate the utility of this approach, we analyzed the effect of compound 19 (Figure 8) on the $sPLA_2$−M-type receptor interaction. This compound is a triazepandione derivative that was recently identified as a novel inhibitor of hGV $sPLA_2$ by using an in silico-guided method of screening (*75*, *80*). As indicated in Table 3, compound 19 was found to inhibit the binding of hGV $sPLA_2$ to the M-type receptor, indicating that this compound indeed binds to hGV $sPLA_2$. A shift in affinity of 50-fold was observed.

DISCUSSION

We have shown here that several active site-directed, competitive inhibitors of $sPLA_2$s are also potent inhibitors of the binding of various venom and mammalian $sPLA_2$s to the M-type receptor. Our results extend the original observation showing that the indole analogue Indoxam blocks the binding of group IB and X $sPLA_2$s to the mouse M-type receptor (also called PLA_2R) (*40*, *73*). The mechanism of inhibition of $sPLA_2$ binding by Indoxam was however not addressed in these former studies. Using the Indoxam analogue Me-Indoxam as a prototypic and potent $sPLA_2$ inhibitor (*46*), a D49K OS_2 mutant that cannot bind this inhibitor (and all other inhibitors used in this study), and a purified recombinant soluble M-type receptor preparation, we now clearly demonstrate that Me-Indoxam weakens the binding of various $sPLA_2$s to the receptor by inducing a dramatic shift in $sPLA_2$ affinity for the receptor. Our results also indicate that the Me-Indoxam−$sPLA_2$ complex can still bind to the receptor, yet with a much weaker affinity. This latter view is clearly supported by the fact that the shift in sPLA2−receptor affinity reaches a limiting value as the concentration of Me-Indoxam is increased. For example, only a limiting value of a 30-fold shift in the affinity of mGIIA for the receptor was observed. If the Me-Indoxam−mGIIA complex were unable to bind to the receptor, an approximative 10000-fold shift in affinity would have been expected on the basis of the apparent affinity of Me-Indoxam for mGIIA [IC_{50} value of 1.5 nM (Table 1)] and the maximal concentration of 20 μM Me-Indoxam used in binding assays (Figure 4).

The effect of Me-Indoxam on the binding of OS_1, OS_2, mGIB, mGIIA, hGIIA, mGIIE, and mGX to the M-type receptor indicates that the shift in affinity induced by the inhibitor depends on the $sPLA_2$ subtype. We also found that $sPLA_2$ inhibitors of different sizes and modes of binding to the $sPLA_2$ active site induce different shifts in affinity for the binding of the same $sPLA_2$ (Table 3). This finding prompted us to search for $sPLA_2$ inhibitors that would be able to inhibit enzymatic activity but that would produce minimal effects on receptor binding. Although future work is required to identify such inhibitors for the different $sPLA_2$s, our current data show that compounds 42, 44, and 46 which are Me-Indoxam analogues bearing small alkyl groups at the N1 position (*48*) are attractive candidates. Indeed, these inhibitors are potent inhibitors of the catalytic activity of hGIIA but produce minimal effects on binding of hGIIA to the M-type receptor (Table 3). Such inhibitors would be interesting tools for discriminating between enzymatic activity and receptor binding in $sPLA_2$ biological effects.

More detailed studies, including determination of the $sPLA_2$−receptor crystal structure in the absence and presence of an inhibitor bound to the $sPLA_2$ active site, are required to determine the exact mechanism by which Me-Indoxam and other inhibitors weaken the binding of $sPLA_2$ to the M-type receptor. Our current findings suggest that the $sPLA_2$ inhibitor acts by a steric hindrance effect that may be primarily due to the protrusion of the inhibitor from the active site. Cocrystal structures of LY311727 with hGIIA $sPLA_2$ and of Me-Indoxam with hGX $sPLA_2$ as well as docking of Pyrazole-1 with hGIIA clearly indicate different protrusions of these inhibitors that fit fairly well with their effects on receptor binding (Figure 7 and Table 3). We cannot exclude the possibility that the inhibitors may also induce subtle allosteric changes in the $sPLA_2$ structure that would affect the affinity for the receptor. We thus propose that the presence of the inhibitor in the catalytic site induces allosteric changes to the $sPLA_2$, and along with the protrusion from the catalytic groove, this combination affects the binding of the enzyme to the M-type receptor in a manner that depends on both the nature of the enzyme and the inhibitor. Our results agree with the finding that $sPLA_2$ residues from the interfacial binding surface and Ca^{2+} loop make molecular contacts with the receptor (*77*). The fact that compounds 42, 44, and 46 have minimal effects on the binding of hGIIA to the M-type receptor at 10 μM while they fully inhibit hGIIA $sPLA_2$ activity at this concentration (Table 3) suggests that the amino acid side chains of the receptor do not make direct molecular interactions with $sPLA_2$ residues from the hydrophobic channel or the active site. The mechanism by which $sPLA_2$ inhibitors modulate the $sPLA_2$−receptor interaction is analogous to those of the small drugs antimycin A or nutlin-2 that prevent protein−protein interactions by binding to the hydrophobic groove of one protein partner (*81*, *82*). However, nutlin-2 and antimycin A act in a strict competitive manner. Indeed, nutlin-2 binds to the hydrophobic pocket of MDM-2 and directly competes with the three amino acid residues of P53 that normally bind to the pocket (*82*). Similarly, antimycin A binds to the hydrophobic groove of Bcl-X_L and prevents the binding of BH3 domain peptides of Bak, Bax, and Bik (*81*). Finally, we found that Me-Indoxam requires Ca^{2+} to inhibit binding of $sPLA_2$ to the receptor, providing direct proof that Me-Indoxam and many other indole inhibitors indeed require Ca^{2+} for a potent interaction with $sPLA_2$ (*32*, *48*).

We have also analyzed the direct binding of various iodinated $sPLA_2$s to COS cell membranes transfected with the M-type receptor or not transfected and to live MC3T3-E_1 cells endogenously expressing the M-type receptor to demonstrate that Me-Indoxam can inhibit the binding of these $sPLA_2$s to the M-type receptor in a more relevant cell biological context (Figures 5 and 6). These binding studies on membranes and live cells revealed additional binding features for mGIIA and mGX $sPLA_2$s over their specific binding to the M-type receptor and inhibition by Me-Indoxam. When exogenously added to live cells or membranes, mGIIA can bind to a very abundant plasma membrane component which consists most likely of heparan sulfate proteoglycans, and this binding is insensitive to Me-Indoxam (Figures 5 and 6). This view is supported by many studies documenting the interaction of group IIA $sPLA_2$ with various heparan sulfate proteoglycans (*7*, *69*), by the absence of inhibition by OS_1 which does not bind to heparin (unpublished data), and by inhibition with the basic compound poly-L-lysine, which suggest that mGIIA binds to anionic structures at the plasma membrane. The tight binding of mGIIA probably does not occur on phospholipids, since mGIIA binds very poorly to phosphatidylcholine and cannot release arachidonic acid from the cell surface (*16*, *46*). Since both unlabeled mGIIA and unlabeled OS_1 could not significantly decrease the total level of binding of iodinated mGIIA, these heparan sulfate proteoglycans appeared as major low-affinity binding sites for mGIIA which are much more abundant than the M-type receptor (Figures 5 and 6). The exact role of this tight binding is still unclear and is in remarkable contrast with the inability of group IIA $sPLA_2$ to bind to phosphatidylcholine and hydrolyze phospholipids on the outer leaflet of the plasma membrane when exogenously added to cells (*7*, *12*, *16*, *46*). How this binding is related to regulation of enzymatic activity, cross-talk with $cPLA_2$ IVA and COX-2, or $sPLA_2$ internalization remains to be determined (*13*, *16*, *53*, *57*, *79*). In addition to the M-type receptor, exogenously added mGX can bind to an abundant binding component which is most likely plasma membrane phospholipids, especially phosphatidylcholine (Figures 5 and 6). In this case, the binding was strengthened by Me-Indoxam (Figures 5 and 6) and by Indoxam or compound A (not shown). In support of this view, a similar binding of iodinated mGX and an enhancing effect by Me-Indoxam were observed with *E. coli* membranes (not shown). Me-Indoxam could also enhance the binding to cellular membranes or live cells of OS_2 but not of OS_1, D49K OS_2, mGIIA, or mGIIF (Figures 5 and 6), in good agreement with their phospholipid binding properties (*46*, *74*). Together, these results suggest that the enhancing effect of Me-Indoxam on $sPLA_2$ binding is due to a higher affinity of the $sPLA_2$ for phospholipids when Me-Indoxam is bound to the active site. Me-Indoxam probably acts as an anchor that increases the hydrophobicity of the $sPLA_2$ interfacial binding surface and partially contributes to adsorption of $sPLA_2$ to the phospholipid interface. This view fits well with the crystal structure of the hGX−Me-Indoxam complex in which the 2-phenylbenzyl group of Me-Indoxam protrudes from the enzyme catalytic groove (Figure 7) and the suggestion that the phenyl group could penetrate into the membrane interface to more tightly anchor the $sPLA_2$ to the membrane (*48*). It is also possible that Me-Indoxam could partition into membrane phospholipids and increase the negative charge of the membrane since it bears a carboxylate functional group, and this could promote $sPLA_2$ binding for anionic binding pockets of $sPLA_2$ (*83*). In the case of live cells, it is most likely that the enhanced binding occurs on phosphatidylcholine in the outer leaflet of the plasma membrane onto which group X $sPLA_2$ and OS_2 bind tightly (*12*, *74*). In accordance with the very poor binding of mGIIA to phosphatidylcholine (*46*), the contribution of Me-Indoxam in interfacial binding of mGIIA to plasma membrane phospholipids is not strong enough by itself to promote binding. Finally, since the M-type receptor has the ability to internalize various $sPLA_2$s, including mGX (*22*, *70*), the total level of binding of iodinated mGX to live cells measured at 37 °C is complex and is likely to be the sum of $sPLA_2$ bound to the receptor at the cell surface, $sPLA_2$ internalized by the receptor, and $sPLA_2$ bound to phospholipids. The fact that the level of binding of iodinated mGX is weakly reduced by unlabeled mGX (and OS_1, not shown) at 37 °C indicates that most of this binding does not result from binding to the M-type receptor and is not due to subsequent internalization but occurs on an abundant target that cannot be saturated by the excess of unlabeled mGX. This is in contrast with the binding data obtained with iodinated OS_1 and mGIIF, which both bind very specifically to the M-type receptor. Shifting the binding experiments to 4 °C, which is known to decrease both internalization and lipid membrane fluidity, was expected to lead to a more specific binding of mGX to the cell surface M-type receptor. The effect of Me-Indoxam at 4 °C fits with this view, since the inhibitor no longer strengthens mGX binding but rather blocks mGX binding, as shown previously for Indoxam on the same $sPLA_2$ (*73*). Importantly, it should be noted that even though Me-Indoxam strengthens the binding of group X $sPLA_2$ to phospholipids, a high concentration of Me-Indoxam fully inhibited arachidonic acid release induced by this $sPLA_2$ when added exogenously (*16*).

These findings and those previously obtained with Indoxam (*40*) raise the possibility that the mechanism of action of $sPLA_2$ inhibitors, including indole analogues, which have been largely used in vitro and in vivo (*5*, *6*, *30*, *31*), is more complex than "simple" blockade of catalytic activity. Our findings first indicate that under conditions where the M-type receptor would play a role in $sPLA_2$ biological actions (see the introductory section), Me-Indoxam or other inhibitors would prevent $sPLA_2$ binding and, thus, $sPLA_2$ biological effects of not only group IIA $sPLA_2$ but also other $sPLA_2$s targeted by these inhibitors. This hypothesis has been proposed for the M-type receptor to explain the therapeutic effects of Indoxam in a mouse model of endotoxic shock (*40*). This was supported by the fact that on one hand mice deficient for the M-type receptor (*71*) and on the other hand naive mice treated with Indoxam (*40*) exhibited higher resistance to lipopolysaccharide-induced lethality with reduced plasma levels of TNF-α and IL-1β. If this hypothesis is correct, then Indoxam or Me-Indoxam would be expected to have more limited or no therapeutic effects on M-type receptor-deficient mice. Furthermore, $sPLA_2$ inhibitors that would not produce a shift in the affinity of $sPLA_2$ for the M-type receptor while blocking catalytic activity would have no therapeutic benefits. Such experiments remain to be performed to evaluate these possibilities. On the other hand, although group IB $sPLA_2$ was suggested to be important in

the mouse model of endotoxic shock described above where the mice used are naturally deficient for mGIIA (*40*), we now know that other $sPLA_2$s bind to the mouse M-type receptor (M. Rouault et al., manuscript submitted for publication) and are sensitive to Indoxam (B. P. Smart et al., unpublished data) and related indole analogues (*46*). Thus, several mouse $sPLA_2$s other than group IB $sPLA_2$ may play a role in this model. Mouse group IIE $sPLA_2$ would be an attractive candidate as it has a very low catalytic activity, binds Me-Indoxam and other inhibitors with high affinity (*46*), and binds very avidly to the M-type receptor and its level of gene expression is increased in lipopolysaccharide-treated mice (*47*). By extension, one can speculate that for $sPLA_2$s which have very low enzymatic activity and may primarily exert their biological effects by binding to proteins with or without the M-type receptor, an inhibitory effect of Me-Indoxam or related inhibitors would be due to inhibition of $sPLA_2$ binding rather than enzymatic activity. Finally, since the M-type receptor can internalize and degrade $sPLA_2$s (*22*, *70*), $sPLA_2$ inhibitors affecting receptor binding may increase the half-life of circulating extracellular $sPLA_2$s, while blocking their enzymatic activity. Whether the use of these inhibitors will have detrimental or beneficial therapeutic efects will thus depend on their balanced action on enzymatic activity and receptor binding.

A few other $sPLA_2$ binding proteins that include surfactant protein A, glypican-1, factor Xa, vimentin, and the VEGF receptor KDR2 have been identified over the past decade for mammalian $sPLA_2$s, including the human group IIA form (*2*, *50*, *79*, *84*, *85*). Whether $sPLA_2$ inhibitors modulate the binding of mammalian $sPLA_2$s to these protein targets remains an open question, except for the vimentin−hGIIA interaction which was found to be prevented by addition of the inhibitor LY311727 (*50*). This was in accordance with the fact that basic amino acids from the interfacial binding site, thus near the catalytic groove of hGIIA, are important for interaction with vimentin (*50*).

Our findings may also explain the results of a few studies in which $sPLA_2$ inhibitors were preventing $sPLA_2$ biological actions while catalytically inactive mutants were as active as the wild-type enzyme (*53*, *55*, *57*, *59*). For example, the induction of COX-2 by group IIA $sPLA_2$ was blocked by LY311727 in human synovial cells (*55*), but COX-2 was induced by two catalytically inactive mutants of this $sPLA_2$ in rat mast cells (*53*). In another study in mesangial cells, we found that the H48Q catalytically inactive mutants of porcine group IB and human group IIA $sPLA_2$s potentiated the expression of rat group IIA $sPLA_2$ induced by TNF-α, while LY311727 prevented the action of both the wild type and H48Q mutants (*57*). The latter finding indicated that the H48Q mutant should still bind LY311727. In agreement with this view, we found that the H48Q porcine group IB mutant and the OS_2 H48Q mutant (*74*) can still bind Me-Indoxam, as indicated by inhibition of their residual catalytic activities and their binding to the M-type receptor by Me-Indoxam (data not shown). Last, the inhibitory effects of Me-Indoxam on the secretion of IL-6 and TNF-α triggered by porcine group IB and its mutant H48Q on human lung macrophages (*59*) are also in agreement with the findings described above. It should be noted that in all of these studies, the contribution of the M-type receptor is still unclear and that the proteins to which the $sPLA_2$s bind to exert their effects remain to be identified.

Our findings further indicate that it is critical to develop potent and selective inhibitors of the different $sPLA_2$ enzymes and/or other tools like receptor antagonists to accurately probe the physiological and physiopathological functions of $sPLA_2$s (*5*, *6*, *30*, *31*, *33*). Ideally, well-designed $sPLA_2$ inhibitors that prevent catalytic activity but not interaction of $sPLA_2$ with their protein targets would help to resolve the key issue of the contribution of enzymatic activity versus receptor binding in sPLA2 biological effects. Initially, most of the current $sPLA_2$ inhibitors were developed with the aim of blocking the enzymatic activity of hGIIA $sPLA_2$. The interpretation of the data obtained with these inhibitors in vitro and in vivo under pathophysiological conditions (*34*, *36*, *37*, *39*−*44*, *47*) need to be reevaluated on the basis of the fact that they bind to several $sPLA_2$s and may exert dual effects on $sPLA_2$ catalytic activity and interaction of $sPLA_2$ with binding proteins. The fact that $sPLA_2$ inhibitors are expected to have detrimental effects in situations where it would be important to maintain $sPLA_2$s as active enzymes should also be highlighted. This would be the case when $sPLA_2$ functions as a host defense factor against infection by bacteria like *Staphylococcus aureus* or *Bacillus anthracis*, viruses like HIV or adenovirus, or parasites like *Plasmodium falciparum* (*9*, *62*−*65*). For example, the fact that injection of LY311727 led to an earlier mortality in a murine toxoplasmosis experimental model is in line with this view and suggests a protective role for at least one mouse $sPLA_2$ sensitive to this inhibitor (*86*). This view may also explain the absence or even adverse effects of the hGIIA $sPLA_2$ inhibitor S-5920/LY315920Na observed on a cohort of patients with severe sepsis and organ failure (*41*, *43*).

Finally, the receptor binding assay with the D49K OS_2 mutant developed in this work could be used to screen for new $sPLA_2$ inhibitors. Technically, the binding assay can be performed using various formats, including surface plasmon resonance technology, fluorescence resonance energy transfer analysis, or ELISA. This screening has an advantage in that it may lead to the identification of compounds that bind to the $sPLA_2$ and would behave as $sPLA_2$ inhibitors or that bind to the M-type receptor or other $sPLA_2$ protein targets and would behave as receptor antagonists. Since binding of $sPLA_2$ to the M-type receptor can be performed in the total absence of a phospholipid substrate, another advantage is that this screen will identify $sPLA_2$ inhibitors that would not act nonspecifically by promoting desorption of the $sPLA_2$ from the lipid interface but that would act in a specific and competitive mode by binding to the $sPLA_2$ catalytic site (*87*). In the past, numerous investigators have used phospholipid analogues with very short fatty acyl chains, i.e., dihexadecanoylphosphatidylcholine, to measure the activity of $sPLA_2$s in the absence of a membrane interface in an effort to characterize competitive inhibitors. Even though the concentration of the short chain substrate was below the critical micelle concentration, careful analysis shows that $sPLA_2$s tend to form protein−phospholipid microaggregates with these substrates presumably due to collection of multiple short chain substrates on the interfacial binding surface of the enzyme (*1*). In this context, the $sPLA_2$ inhibition assay using the soluble M-type receptor described in this study to monitor direct binding of the inhibitor to the

active site of the $sPLA_2$, or near the active site, provides a more reliable way of monitoring $sPLA_2$−inhibitor interaction in the absence of a phospholipid interface. The receptor binding assay may also serve to demonstrate whether an identified yet not fully characterized $sPLA_2$ inhibitor acts in a competitive mode or via nonspecific modulation of binding of $sPLA_2$ to the lipid−water interface (*1*).

ACKNOWLEDGMENT

We thank Dr. Sabine Scarzello and Romain Gauthier for expert assistance in the recombinant expression and structural characterization of sPLA2s and computer modeling of sPLA2−inhibitor complexes. We are grateful to Dr. Gilles Guichard and Didier Rognan for providing triazepandione compound 19 and for helpful discussions.

REFERENCES

1. Berg, O. G., Gelb, M. H., Tsai, M. D., and Jain, M. K. (2001) Interfacial enzymology: The secreted phospholipase A2 paradigm, *Chem. Rev. 101*, 2613−2654.
2. Valentin, E., and Lambeau, G. (2000) Increasing molecular diversity of secreted phospholipases A2 and their receptors and binding proteins, *Biochim. Biophys. Acta 1488*, 59−70.
3. Rouault, M., Bollinger, J. G., Lazdunski, M., Gelb, M. H., and Lambeau, G. (2003) Novel mammalian group XII secreted phospholipase A2 lacking enzymatic activity, *Biochemistry 42*, 11494−11503.
4. Diaz, B. L., and Arm, J. P. (2003) Phospholipase A2, *Prostaglandins, Leukotrienes Essent. Fatty Acids 69*, 87−97.
5. Laye, J. P., and Gill, J. H. (2003) Phospholipase A2 expression in tumours: A target for therapeutic intervention? *Drug Discovery Today 8*, 710−716.
6. Scott, K. F., Graham, G. G., and Bryant, K. J. (2003) Secreted phospholipase A2 enzymes as therapeutic targets, *Expert Opin. Ther. Targets 7*, 427−440.
7. Murakami, M., and Kudo, I. (2004) Secretory phospholipase A2, *Biol. Pharm. Bull. 27*, 1158−1164.
8. Webb, N. R. (2005) Secretory phospholipase A2 enzymes in atherogenesis, *Curr. Opin. Lipidol. 16*, 341−344.
9. Piris-Gimenez, A., Paya, M., Lambeau, G., Chignard, M., Mock, M., Touqui, L., and Goossens, P. L. (2005) In vivo protective role of human group IIa phospholipase A2 against experimental anthrax, *J. Immunol. 175*, 6786−6791.
10. Dong, Q., Patel, M., Scott, K. F., Graham, G. G., Russell, P. J., and Sved, P. (2006) Oncogenic action of phospholipase A2 in prostate cancer, *Cancer Lett. 240*, 9−16.
11. Triggiani, M., Granata, F., Giannattasio, G., and Marone, G. (2005) Secretory phospholipases A2 in inflammatory and allergic diseases: Not just enzymes, *J. Allergy Clin. Immunol. 116*, 1000−1006.
12. Bezzine, S., Koduri, R. S., Valentin, E., Murakami, M., Kudo, I., Ghomashchi, F., Sadilek, M., Lambeau, G., and Gelb, M. H. (2000) Exogenously added human group X secreted phospholipase A2 but not the group IB, IIA, and V enzymes efficiently release arachidonic acid from adherent mammalian cells, *J. Biol. Chem. 275*, 3179−3191.
13. Kim, Y. J., Kim, K. P., Rhee, H. J., Das, S., Rafter, J. D., Oh, Y. S., and Cho, W. (2002) Internalized group V secretory phospholipase A2 acts on the perinuclear membranes, *J. Biol. Chem. 277*, 9358−9365.
14. Hanasaki, K., and Arita, H. (2003) Biological functions of group X secretory PLA2, *Adv. Exp. Med. Biol. 525*, 93−96.
15. Murakami, M. (2004) Hot topics in phospholipase A2 field, *Biol. Pharm. Bull. 27*, 1179−1182.
16. Mounier, C. M., Ghomashchi, F., Lindsay, M. R., James, S., Singer, A. G., Parton, R. G., and Gelb, M. H. (2004) Arachidonic acid release from mammalian cells transfected with human groups IIA and X secreted phospholipase A_2 occurs predominantly during the secretory process and with the involvement of cytosolic phospholipase A_2-α, *J. Biol. Chem. 279*, 25024−25038.
17. Ni, Z., Okeley, N. M., Smart, B. P., and Gelb, M. H. (2006) Intracellular Actions of Group IIA Secreted Phospholipase A2 and Group IVA Cytosolic Phospholipase A2 Contribute to Arachidonic Acid Release and Prostaglandin Production in Rat Gastric Mucosal Cells and Transfected Human Embryonic Kidney Cells, *J. Biol. Chem. 281*, 16245−16255.
18. Satake, Y., Diaz, B. L., Balestrieri, B., Lam, B. K., Kanaoka, Y., Grusby, M. J., and Arm, J. P. (2004) Role of group V phospholipase A2 in zymosan-induced eicosanoid generation and vascular permeability revealed by targeted gene disruption, *J. Biol. Chem. 279*, 16488−16494.
19. Saiga, A., Uozumi, N., Ono, T., Seno, K., Ishimoto, Y., Arita, H., Shimizu, T., and Hanasaki, K. (2005) Group X secretory phospholipase A2 can induce arachidonic acid release and eicosanoid production without activation of cytosolic phospholipase A2 α, *Prostaglandins Other Lipid Mediators 75*, 79−89.
20. Balsinde, J., and Balboa, M. A. (2005) Cellular regulation and proposed biological functions of group VIA calcium-independent phospholipase A2 in activated cells, *Cell. Signalling 17*, 1052−1062.
21. Mandal, A. K., Zhang, Z., Chou, J. Y., and Mukherjee, A. B. (2001) Pancreatic phospholipase A2 via its receptor regulates expression of key enzymes of phospholipid and sphingolipid metabolism, *FASEB J. 15*, 1834−1836.
22. Hanasaki, K., and Arita, H. (2002) Phospholipase A2 receptor: A regulator of biological functions of secretory phospholipase A2, *Prostaglandins Other Lipid Mediators 68−69*, 71−82.
23. Murakami, M., Masuda, S., Shimbara, S., Ishikawa, Y., Ishii, T., and Kudo, I. (2005) Cellular distribution, post-translational modification, and tumorigenic potential of human group III secreted phospholipase A_2, *J. Biol. Chem. 280*, 24987−24998.
24. Vadas, P., Wasi, S., Movat, H. Z., and Hay, J. B. (1981) Extracellular phospholipase A2 mediates inflammatory hyperaemia, *Nature 293*, 583−585.
25. Vadas, P., Browning, J., Edelson, J., and Pruzanski, W. (1993) Extracellular phospholipase A2 expression and inflammation: The relationship with associated disease states, *J. Lipid Mediators 8*, 1−30.
26. Andreani, M., Olivier, J. L., Berenbaum, F., Raymondjean, M., and Bereziat, G. (2000) Transcriptional regulation of inflammatory secreted phospholipases A_2, *Biochim. Biophys. Acta 1488*, 149−158.
27. Nevalainen, T. J., Haapamaki, M. M., and Gronroos, J. M. (2000) Roles of secretory phospholipases A_2 in inflammatory diseases and trauma, *Biochim. Biophys. Acta 1488*, 83−90.
28. Pruzanski, W., and Vadas, P. (1991) Phospholipase A2: A mediator between proximal and distal effectors of inflammation, *Immunol. Today 12*, 143−146.
29. Wery, J. P., Schevitz, R. W., Clawson, D. K., Bobbitt, E. R., Dow, E. R., Gamboa, G., Goodson, T., Hermann, R. B., Jr., Kramer, R. M., McClure, D. B., Michelich, E. D., Putnam, J. E., Sharp, J. D., Stark, D. H., Teater, C., Warrick, M. W., and Jones, N. D. (1991) Structure of recombinant human rheumatoid arthritic synovial fluid phospholipase A2 at 2.2 Å resolution, *Nature 352*, 79−82.
30. Springer, D. M. (2001) An update on inhibitors of human 14 kDa Type II s-PLA2 in development, *Curr. Pharm. Des. 7*, 181−198.
31. Reid, R. C. (2005) Inhibitors of secretory phospholipase A2 group IIA, *Curr. Med. Chem. 12*, 3011−3026.
32. Schevitz, R. W., Bach, N. J., Carlson, D. G., Chirgadze, N. Y., Clawson, D. K., Dillard, R. D., Draheim, S. E., Hartley, L. W., Jones, N. D., Mihelich, E. D., et al. (1995) Structure-based design of the first potent and selective inhibitor of human non-pancreatic secretory phospholipase A2, *Nat. Struct. Biol. 2*, 458−465.
33. Smart, B. P., Oslund, R. C., Walsh, L. A., and Gelb, M. H. (2006) The first potent inhibitor of mammalian group X secreted phospholipase A2: Elucidation of sites for enhanced binding, *J. Med. Chem. 49*, 2858−2860.
34. Arbibe, L., Koumanov, K., Vial, D., Rougeot, C., Faure, G., Havet, N., Longacre, S., Vargaftig, B. B., Bereziat, G., Voelker, D. R., Wolf, C., and Touqui, L. (1998) Generation of lyso-phospholipids from surfactant in acute lung injury is mediated by type-II phospholipase A2 and inhibited by a direct surfactant protein A-phospholipase A2 protein interaction, *J. Clin. Invest. 102*, 1152−1160.
35. Furue, S., Mikawa, K., Nishina, K., Shiga, M., Ueno, M., Tomita, Y., Kuwabara, K., Teshirogi, I., Ono, T., Hori, Y., Matsukawa, A., Yoshinaga, M., and Obara, H. (2001) Therapeutic time-window of a group IIA phospholipase A2 inhibitor in rabbit acute lung injury: Correlation with lung surfactant protection, *Crit. Care Med. 29*, 719−727.

36. Woodruff, T. M., Arumugam, T. V., Shiels, I. A., Newman, M. L., Ross, P. A., Reid, R. C., Fairlie, D. P., and Taylor, S. M. (2005) A potent and selective inhibitor of group IIa secretory phospholipase A2 protects rats from TNBS-induced colitis, *Int. Immunopharmacol. 5*, 883−892.
37. Svensson, C. I., Lucas, K. K., Hua, X. Y., Powell, H. C., Dennis, E. A., and Yaksh, T. L. (2005) Spinal phospholipase A2 in inflammatory hyperalgesia: Role of the small, secretory phospholipase A2, *Neuroscience 133*, 543−553.
38. Shoseyov, D., Bibi, H., Offer, S., Schwob, O., Krimsky, M., Kleiman, M., and Yedgar, S. (2005) Treatment of ovalbumin-induced experimental allergic bronchitis in rats by inhaled inhibitor of secretory phospholipase A2, *Thorax 60*, 747−753.
39. Gregory, L. S., Kelly, W. L., Reid, R. C., Fairlie, D. P., and Forwood, M. R. (2006) Inhibitors of cyclo-oxygenase-2 and secretory phospholipase A2 preserve bone architecture following ovariectomy in adult rats, *Bone 39*, 134−142.
40. Yokota, Y., Hanasaki, K., Ono, T., Nakazato, H., Kobayashi, T., and Arita, H. (1999) Suppression of murine endotoxic shock by sPLA2 inhibitor, indoxam, through group IIA sPLA2-independent mechanisms, *Biochim. Biophys. Acta 1438*, 213−222.
41. Abraham, E., Naum, C., Bandi, V., Gervich, D., Lowry, S. F., Wunderink, R., Schein, R. M., Macias, W., Skerjanec, S., Dmitrienko, A., Farid, N., Forgue, S. T., and Jiang, F. (2003) Efficacy and safety of LY315920Na/S-5920, a selective inhibitor of 14-kDa group IIA secretory phospholipase A2, in patients with suspected sepsis and organ failure, *Crit. Care Med. 31*, 718−728.
42. Zeiher, B. G., Steingrub, J., Laterre, P. F., Dmitrienko, A., Fukiishi, Y., and Abraham, E. (2005) LY315920NA/S-5920, a selective inhibitor of group IIA secretory phospholipase A2, fails to improve clinical outcome for patients with severe sepsis, *Crit. Care Med. 33*, 1741−1748.
43. Bowton, D. L., Dmitrienko, A. A., Israel, E., Zeiher, B. G., and Sides, G. D. (2005) Impact of a soluble phospholipase A2 inhibitor on inhaled allergen challenge in subjects with asthma, *J. Asthma 42*, 65−71.
44. Bradley, J. D., Dmitrienko, A. A., Kivitz, A. J., Gluck, O. S., Weaver, A. L., Wiesenhutter, C., Myers, S. L., and Sides, G. D. (2005) A randomized, double-blinded, placebo-controlled clinical trial of LY333013, a selective inhibitor of group II secretory phospholipase A2, in the treatment of rheumatoid arthritis, *J. Rheumatol. 32*, 417−423.
45. Suzuki, N., Ishizaki, J., Yokota, Y., Higashino, K., Ono, T., Ikeda, M., Fujii, N., Kawamoto, K., and Hanasaki, K. (2000) Structures, enzymatic properties, and expression of novel human and mouse secretory phospholipases A2, *J. Biol. Chem. 275*, 5785−5793.
46. Singer, A. G., Ghomashchi, F., Le Calvez, C., Bollinger, J., Bezzine, S., Rouault, M., Sadilek, M., Nguyen, E., Lazdunski, M., Lambeau, G., and Gelb, M. H. (2002) Interfacial kinetic and binding properties of the complete set of human and mouse groups I, II, V, X, and XII secreted phospholipases A2, *J. Biol. Chem. 277*, 48535−48549.
47. Hamaguchi, K., Kuwata, H., Yoshihara, K., Masuda, S., Shimbara, S., Oh-ishi, S., Murakami, M., and Kudo, I. (2003) Induction of distinct sets of secretory phospholipase A_2 in rodents during inflammation, *Biochim. Biophys. Acta 1635*, 37−47.
48. Smart, B. P., Pan, Y. H., Weeks, A. K., Bollinger, J. G., Bahnson, B. J., and Gelb, M. H. (2004) Inhibition of the complete set of mammalian secreted phospholipases A_2 by indole analogues: A structure-guided study, *Bioorg. Med. Chem. Lett. 12*, 1737−1749.
49. Rizzo, M. T., Nguyen, E., Aldo-Benson, M., and Lambeau, G. (2000) Secreted phospholipase A2 induces vascular endothelial cell migration, *Blood 96*, 3809−3815.
50. Boilard, E., Bourgoin, S. G., Bernatchez, C., and Surette, M. E. (2003) Identification of an autoantigen on the surface of apoptotic human T cells as a new protein interacting with inflammatory group IIA phospholipase A2, *Blood 102*, 2901−2909.
51. Jo, E. J., Lee, H. Y., Lee, Y. N., Kim, J. I., Kang, H. K., Park, D. W., Baek, S. H., Kwak, J. Y., and Bae, Y. S. (2004) Group IB secretory phospholipase A2 stimulates CXC chemokine ligand 8 production via ERK and NF-κB in human neutrophils, *J. Immunol. 173*, 6433−6439.
52. Lee, C., Park, D. W., Lee, J., Lee, T. I., Kim, Y. J., Lee, Y. S., and Baek, S. H. (2006) Secretory phospholipase A2 induces apoptosis through TNF-α and cytochrome c-mediated caspase cascade in murine macrophage RAW 264.7 cells, *Eur. J. Pharmacol. 536*, 47−53.
53. Tada, K., Murakami, M., Kambe, T., and Kudo, I. (1998) Induction of cyclooxygenase-2 by secretory phospholipases A2 in nerve growth factor-stimulated rat serosal mast cells is facilitated by interaction with fibroblasts and mediated by a mechanism independent of their enzymatic functions, *J. Immunol. 161*, 5008−5015.
54. Balsinde, J., Shinohara, H., Lefkowitz, L. J., Johnson, C. A., Balboa, M. A., and Dennis, E. A. (1999) Group V phospholipase A_2-dependent induction of cyclooxygenase-2 in macrophages, *J. Biol. Chem. 274*, 25967−25970.
55. Bidgood, M. J., Jamal, O. S., Cunningham, A. M., Brooks, P. M., and Scott, K. F. (2000) Type IIA secretory phospholipase A2 up-regulates cyclooxygenase-2 and amplifies cytokine-mediated prostaglandin production in human rheumatoid synoviocytes, *J. Immunol. 165*, 2790−2797.
56. Fuentes, L., Hernandez, M., Nieto, M. L., and Sanchez Crespo, M. (2002) Biological effects of group IIA secreted phosholipase A_2, *FEBS Lett. 531*, 7−11.
57. Beck, S., Lambeau, G., Scholz-Pedretti, K., Gelb, M. H., Janssen, M. J., Edwards, S. H., Wilton, D. C., Pfeilschifter, J., and Kaszkin, M. (2003) Potentiation of TNFα-induced sPLA2-IIA expression in mesangial cells by an autocrine loop involving secreted phospholipase A2 and PPARα activation, *J. Biol. Chem. 278*, 29799−29812.
58. Choi, Y. A., Lim, H. K., Kim, J. R., Lee, C. H., Kim, Y. J., Kang, S. S., and Baek, S. H. (2004) Group IB secretory phospholipase A2 promotes matrix metalloproteinase-2-mediated cell migration via the phosphatidylinositol-3 kinase and Akt pathway, *J. Biol. Chem. 279*, 36579−36585.
59. Granata, F., Petraroli, A., Boilard, E., Bezzine, S., Bollinger, J., Del Vecchio, L., Gelb, M. H., Lambeau, G., Marone, G., and Triggiani, M. (2005) Activation of cytokine production by secreted phospholipase A2 in human lung macrophages expressing the M-type receptor, *J. Immunol. 174*, 464−474.
60. Granata, F., Frattini, A., Loffredo, S., Del Prete, A., Sozzani, S., Marone, G., and Triggiani, M. (2006) Signaling events involved in cytokine and chemokine production induced by secretory phospholipase A_2 in human lung macrophages, *Eur. J. Immunol.* (in press).
61. Laine, V. J., Grass, D. S., and Nevalainen, T. J. (1999) Protection by group II phospholipase A2 against *Staphylococcus aureus*, *J. Immunol. 162*, 7402−7408.
62. Femling, J. K., Nauseef, W. M., and Weiss, J. P. (2005) Synergy between Extracellular Group IIA Phospholipase A2 and Phagocyte NADPH Oxidase in Digestion of Phospholipids of *Staphylococcus aureus* Ingested by Human Neutrophils, *J. Immunol. 175*, 4653−4661.
63. Guillaume, C., Calzada, C., Lagarde, M., Schrevel, J., and Deregnaucourt, C. (2006) Interplay between lipoproteins and bee venom phospholipase A2 in relation to their anti-plasmodium toxicity, *J. Lipid. Res.* (in press).
64. Fenard, D., Lambeau, G., Valentin, E., Lefebvre, J. C., Lazdunski, M., and Doglio, A. (1999) Secreted phospholipases A2, a new class of HIV inhibitors that block virus entry into host cells, *J. Clin. Invest. 104*, 611−618.
65. Mitsuishi, M., Masuda, S., Kudo, I., and Murakami, M. (2006) Group V and X secretory phospholipase A2 prevents adenoviral infection in mammalian cells, *Biochem. J. 393*, 97−106.
66. Koprivnjak, T., Peschel, A., Gelb, M. H., Liang, N. S., and Weiss, J. P. (2002) Role of charge properties of bacterial envelope in bactericidal action of human group IIA phospholipase A2 against *Staphylococcus aureus*, *J. Biol. Chem. 277*, 47636−47644.
67. Jaulmes, A., Janvier, B., Andreani, M., and Raymondjean, M. (2005) Autocrine and Paracrine Transcriptional Regulation of Type IIA Secretory Phospholipase A2 Gene in Vascular Smooth Muscle Cells, *Arterioscler. Thromb. Vasc. Biol. 25*, 1161−1167.
68. Murakami, M., Nakatani, Y., Kuwata, H., and Kudo, I. (2000) Cellular components that functionally interact with signaling phospholipase A2s, *Biochim. Biophys. Acta 1488*, 159−166.
69. Boilard, E., Bourgoin, S. G., Bernatchez, C., Poubelle, P. E., and Surette, M. E. (2003) Interaction of low molecular weight group IIA phospholipase A2 with apoptotic human T cells: Role of heparan sulfate proteoglycans, *FASEB J. 17*, 1068−1080.
70. Lambeau, G., and Lazdunski, M. (1999) Receptors for a growing family of secreted phospholipases A2, *Trends Pharmacol. Sci. 20*, 162−170.
71. Hanasaki, K., Yokota, Y., Ishizaki, J., Itoh, T., and Arita, H. (1997) Resistance to endotoxic shock in phospholipase A2 receptor-deficient mice, *J. Biol. Chem. 272*, 32792−32797.

72. Cupillard, L., Mulherkar, R., Gomez, N., Kadam, S., Valentin, E., Lazdunski, M., and Lambeau, G. (1999) Both group IB and group IIA secreted phospholipases A2 are natural ligands of the mouse M-type receptor, *J. Biol. Chem. 274*, 7043−7051.
73. Yokota, Y., Higashino, K., Nakano, K., Arita, H., and Hanasaki, K. (2000) Identification of group X secretory phospholipase A_2 as a natural ligand for mouse phospholipase A_2 receptor, *FEBS Lett. 478*, 187−191.
74. Rouault, M., Rash, L. D., Escoubas, P., Boilard, E., Bollinger, J., Lomonte, B., Maurin, T., Guillaume, C., Canaan, S., Deregnaucourt, C., Schrevel, J., Doglio, A., Gutierrez, J. M., Lazdunski, M., Gelb, M. H., and Lambeau, G. (2006) Neurotoxicity and Other Pharmacological Activities of the Snake Venom Phospholipase A2 OS2: The N-Terminal Region Is More Important Than Enzymatic Activity, *Biochemistry 45*, 5800−5816.
75. Lena, G., Lallemand, E., Gruner, A. C., Boeglin, J., Roussel, S., Schaffner, A. P., Aubry, A., Franetich, J. F., Mazier, D., Landau, I., Briand, J. P., Didierjean, C., Renia, L., and Guichard, G. (2006) 1,3,5-Triazepan-2,6-diones as Structurally Diverse and Conformationally Constrained Dipeptide Mimetics: Identification of Malaria Liver Stage Inhibitors from a Small Pilot Library, *Chem.—Eur. J.* (in press).
76. Lambeau, G., Ancian, P., Barhanin, J., and Lazdunski, M. (1994) Cloning and expression of a membrane receptor for secretory phospholipases A2, *J. Biol. Chem. 269*, 1575−1578.
77. Lambeau, G., Ancian, P., Nicolas, J. P., Beiboer, S., Moinier, D., Verheij, H., and Lazdunski, M. (1995) Structural elements of secretory phospholipases A2 involved in the binding to M-type receptors, *J. Biol. Chem. 270*, 5534−5540.
78. Kitadokoro, K., Hagishita, S., Sato, T., Ohtani, M., and Miki, K. (1998) Crystal structure of human secretory phospholipase A2-IIA complex with the potent indolizine inhibitor 120-1032, *J. Biochem. 123*, 619−623.
79. Murakami, M., Kambe, T., Shimbara, S., Yamamoto, S., Kuwata, H., and Kudo, I. (1999) Functional association of type IIA secretory phospholipase A2 with the glycosylphosphatidylinositol-anchored heparan sulfate proteoglycan in the cyclooxygenase-2-mediated delayed prostanoid-biosynthetic pathway, *J. Biol. Chem. 274*, 29927−29936.
80. Muller, P., Lena, G., Boilard, E., Bezzine, S., Lambeau, G., Guichard, G., and Rognan, D. (2006) In silico-guided target identification of a scaffold focused library: 1,3,5-Triazepan-2,6-diones as novel phospholipase A2 inhibitors, *J. Med. Chem.* (in press).
81. Tzung, S. P., Kim, K. M., Basanez, G., Giedt, C. D., Simon, J., Zimmerberg, J., Zhang, K. Y., and Hockenbery, D. M. (2001) Antimycin A mimics a cell-death-inducing Bcl-2 homology domain 3, *Nat. Cell Biol. 3*, 183−191.
82. Vassilev, L. T., Vu, B. T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C., Fotouhi, N., and Liu, E. A. (2004) In vivo activation of the p53 pathway by small-molecule antagonists of MDM2, *Science 303*, 844−848.
83. Pan, Y. H., Epstein, T. M., Jain, M. K., and Bahnson, B. J. (2001) Five coplanar anion binding sites on one face of phospholipase A2: Relationship to interface binding, *Biochemistry 40*, 609−617.
84. Chabot, S., Koumanov, K., Lambeau, G., Gelb, M. H., Chignard, M., Whitsett, J. A., and Touqui, L. (2003) Inhibitory effects of surfactant protein A on surfactant phospholipid hydrolysis by secreted phospholipases A2, *J. Immunol. 171*, 995−1000.
85. Yamazaki, Y., Matsunaga, Y., Nakano, Y., and Morita, T. (2005) Identification of vascular endothelial growth factor receptor-binding protein in the venom of eastern cottonmouth. A new role of snake venom myotoxic Lys49-phospholipase A2, *J. Biol. Chem. 280*, 29989−29992.
86. Buitrago-Rey, R., Olarte, J., and Gomez-Marin, J. E. (2002) Evaluation of two inhibitors of invasion: LY311727 [3-(3-acetamide-1-benzyl-2-ethyl-indolyl-5-oxy)propane phosphonic acid] and AEBSF [4-(2-aminoethyl)-benzenesulphonyl fluoride] in acute murine toxoplasmosis, *J. Antimicrob. Chemother. 49*, 871−874.
87. Jain, M. K., Yuan, W., and Gelb, M. H. (1989) Competitive inhibition of phospholipase A2 in vesicles, *Biochemistry 28*, 4135−4139.
88. McMartin, C., and Bohacek, R. S. (1997) QXP: Powerful, rapid computer algorithms for structure-based drug design, *J. Comput.-Aided Mol. Des. 11*, 333−344.

BI061376D