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Cross-interactions of Two p38 Mitogen-activated Protein (MAP) Kinase Inhibitors and Two Cholecystokinin (CCK) Receptor Antagonists with the CCK1 Receptor and P38 MAP Kinase*

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Although SB202190 and SB203580 are described as specific p38 MAP kinase inhibitors, several reports have indicated that other enzymes are also sensitive to SB203580. Using a pharmacological approach, we report for the first time that compounds SB202190 and SB203580 were able to directly and selectively interact with a G-protein-coupled receptor, namely the cholecystokinin receptor subtype CCK1, but not with the CCK2 receptor. We demonstrated that these compounds were non-competitive antagonists of the CCK1 receptor at concentrations typically used to inhibit protein kinases. By chimeric construction of the CCK2 receptor, we determined the involvement of two CCK1 receptor intracellular loops in the binding of SB202190 and SB203580. We also showed that two CCK antagonists, L364,718 and L365,260, were able to regulate p38 mitogen-activated protein (MAP) kinase activity. Using a reporter gene strategy and immunoblotting experiments, we demonstrated that both CCK antagonists inhibited selectively the enzymatic activity of p38 MAP kinase. Kinase assays suggested that this inhibition resulted from a direct interaction with both CCK antagonists. Molecular modeling simulations suggested that this interaction occurs in the ATP binding pocket of p38 MAP kinase. These results suggest that SB202190 and SB203580 bind to the CCK1 receptor and, as such, these compounds should be used with caution in models that express this receptor. We also found that L364,718 and L365,260, two CCK receptor antagonists, directly interacted with p38 MAP kinase and inhibited its activity. These findings suggest that the CCK1 receptor shares structural analogies with the p38 MAP kinase ATP binding site. They open the way to potential design of either a new family of MAP kinase inhibitors from CCK1 receptor ligand structures or new CCK1 receptor ligands based on p38 MAP kinase inhibitor structures.

Cholecystokinin (CCK)¹ is a peptide involved in a wide range of biological functions. It acts as a hormone in the gastrointes-

tinal tract as well as a neurotransmitter in the central nervous system. This hormone was first isolated as a 33-amino acid peptide; the major fully active form is the C-terminal sulfated octapeptide termed CCK8s (1). To induce its biological functions, CCK8s binds to two specific receptors, the CCK1 and CCK2 receptors. They have been classified according to their pharmacological profile for endogenous CCK and gastrin peptide agonists (2). The CCK1 receptor is highly selective for sulfated analogues of CCK and for the nonpeptide antagonist L364,718 (2, 3), whereas the CCK2 receptor also has high affinity for sulfated or non-sulfated peptide analogues of CCK and gastrin and for the nonpeptide antagonist L365,260 (2, 4). The CCK1 receptor is mainly localized in the gastrointestinal tract where it regulates pancreatic enzyme secretions, gallbladder contraction, gastric emptying, and satiety (5). In pancreatic acinar cells, CCK1 receptor is coupled to a G_{q/11} protein that is activated upon binding of CCK8s and then induces the breakdown of phosphatidylinositol 4,5-bisphosphate by phospholipase C, producing both diacylglycerol and inositol 1,4,5-triphosphate (IP₃) (6). Subsequent IP₃-induced intracellular calcium mobilization and diacylglycerol production both activate protein kinase C-mediated digestive enzyme secretions (7). It was further demonstrated that CCK8s activates several members of the MAPK signaling cascades like ERK1/2 (8), stress-activated protein kinase/JNK (9), p38 MAP kinase (10), and other upstream components such as MEK and Ras (11). ERKs, JNKs, and p38 MAP kinases belong to the MAPK family. They mediate cellular growth and differentiation following activation by environmental stress (12). p38 MAP kinase is involved in inflammatory responses through the production of pro-inflammatory cytokines like interleukin-1 β and tumor necrosis factor α (13). Drugs called cytokine synthesis anti-inflammatory drugs (CSAIDs) have been used to understand the physiological roles of p38 MAP kinase. These pyridinyl imidazole-derived compounds are able to bind p38 MAP kinase (13). The development of p38 MAP kinase inhibitors is regarded as the main therapeutic strategy for stopping the production of pro-inflammatory cytokines involved in inflammatory and immunoresponsive diseases (14, 15). Among such compounds, SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-imidazole) and SB202190 (4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-imidazole) were described as potent and selective inhibitors of p38 MAP kinase, having no effect on ERKs and JNKs (16, 17). Interestingly, it has been reported that several other enzymes and a tyrosine kinase receptor were sensitive to SB203580, including cyclo-oxygenase 2 (18), the

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¹ The abbreviations used are: CCK, cholecystokinin; CCK8s, sulfated octapeptide of cholecystokinin; MAPK, mitogen-activated protein kinase; ATF2, activating transcription factor 2; BH, Bolton-Hunter; NBMPR, nitrobenzylmercaptapurine ribonucleoside (6-[(4-nitrobenzyl)thiol]-9- β -D-ribofuranosyl purine; CDTA, trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid; DTT, dithiothreitol; BSA, bovine serum albumin; FCS, fetal calf serum; PBS, phosphate-buffered saline;

ERK, extracellular signal-regulated kinase; JNK, c-Jun NH₂-terminal kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MAPKAPK, MAPK-activated protein kinase.

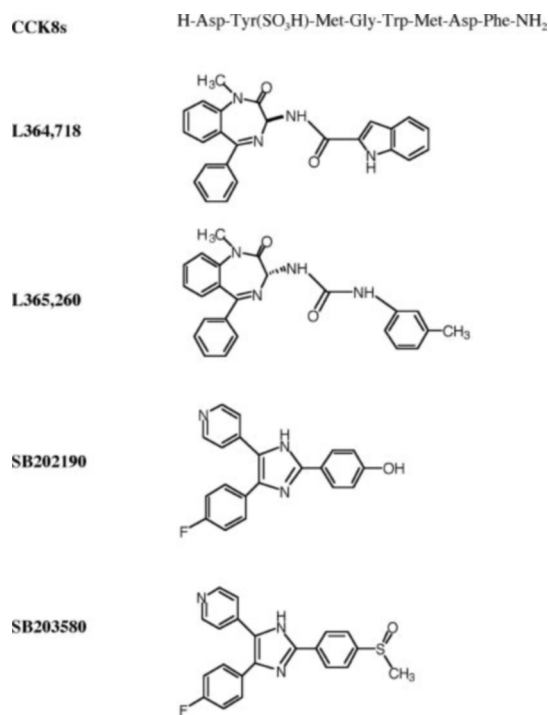


FIG. 1. Chemical structures of CCK8s, nonpeptide CCK receptor antagonists L364,718 and L365,260, and p38 MAP kinase inhibitors SB202190 and SB203580.

protein kinase Raf (19, 20), the protein tyrosine kinase Ick, and the Type-II transforming growth factor β receptor (21). More recently, SB compounds were described as preventing nucleoside transport by interacting with the NBMPR-sensitive equilibrative nucleoside transporter ENT1 (22, 23). This emphasizes the need to ensure the specificity of SB203580 when this compound is used in studies involving the p38 MAP kinase signaling pathway.

While studying the involvement of the CCK1 receptor in the p38 MAP kinase signaling pathway, we found that SB203580 and SB202190 (Fig. 1) were able to interact with this G-protein-coupled receptor. In addition, we showed that the two nonpeptide CCK antagonists, L364,718 and L365,260, respectively selective for the CCK1 and the CCK2 receptors (Fig. 1) (3, 4), were able to directly interact with p38 MAP kinase to inhibit its activity.

EXPERIMENTAL PROCEDURES

Reagents—SB202190 and SB203580 were purchased from VWR International. L364,718 and L365,260 were obtained from Merck. NaCl, KCl, MgCl₂, MgSO₄, CaCl₂, KH₂PO₄, K₂CO₃, LiCl, EGTA, CDTA, ammonium formate, D-glucose, HEPES, phenylmethylsulfonyl fluoride, 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSPF), soybean trypsin inhibitor, bacitracin, ATP, dithiothreitol (DTT), glutathione, and coenzyme A were from Sigma. Bovine serum albumin (BSA) fraction V, luciferin sodium salt, and sodium dodecyl sulfate (SDS) were from Euromedex. Tris was purchased from ICN. Protein concentrations were determined by the Bradford method, using the Bio-Rad protein assay reagent. Dowex AG1-X8 anion exchange resin (100–200 mesh, formate form) was also from Bio-Rad. *m*yo-[2-³H]inositol (16.5 Ci/mmol), ¹²⁵I-Bolton-Hunter reagent (1800 Ci/mmol) were purchased from Amersham Biosciences. Glutamine, streptomycin-penicillin, trypsin-EDTA 1X, RPMI 1640 medium, and fetal calf serum (FCS) were obtained from Invitrogen. Dulbecco's modified Eagles' medium and Medium 199 were from Cambrex (Verviers, Belgium). The rabbit polyclonal anti-p38 MAP kinase, anti-phospho-p38 MAP kinase, anti-phospho-MAPKAPK-2 (Thr-334) and anti-MAPKAPK-2, the mouse monoclonal immobilized anti-phospho-p38 MAP kinase (Thr-180/Tyr-182), anti-phospho-ATF2 (Thr-71), immobilized anti-phospho-p44/p42 MAP kinase (Thr-202/Tyr-204), anti-phospho-Elk1 (Ser-383), anti-phospho-c-Jun (Ser-63), and anti-rabbit secondary antibodies were from New England Biolabs (Beverly, MA). [³H]-SB202190 was purchased from

Fischer Scientific (Illkirch, France). CCK8s was synthesized in our laboratory and radiolabeled with Bolton-Hunter reagent (¹²⁵I-BH) as previously described (24).

Construction of CCK2 Chimeric Receptors—Chimeric CCK2 receptors were constructed by the two-step procedure (25) using purified oligonucleotides synthesized by Invitrogen. Construction of plasmids encoding the two mutants of the human CCK2 receptor was performed according to classical methods (26). Mutations were confirmed by sequencing performed at Genome Express.

Cell Culture and Transfection—COS7 cells were cultured in Dulbecco's modified Eagle's medium containing 10% FCS, 0.25 mM L-glutamine, and streptomycin-penicillin (250 μ g/ml-250 units/ml) at 37 °C in a 5% CO₂ atmosphere. The Jurkat T cell line was maintained in RPMI 1640 containing 10% FCS and streptomycin-penicillin (250 μ g/ml-250 units/ml) at 37 °C in a humidified atmosphere of 5% CO₂. Transfection of COS7 cells was performed by electroporation. Ten million cells were harvested by trypsin-EDTA 1X. Cells were rinsed once with electroporation buffer (120 mM KCl, 0.15 mM CaCl₂, 5 mM MgCl₂, 10 mM K₂HPO₄/KH₂PO₄, pH 7.6, 2 mM EGTA) and resuspended with electroporation buffer supplemented with 5 μ M glutathione, 2 μ M ATP in a final volume of 500 μ l. Cells to be used either for binding experiments, plasma membrane preparation, or analysis of inositol phosphate production were transfected with 1 μ g of plasmid encoding the rat CCK1 receptor (27) or 10 μ g of the mutant receptor. Cells to be used for the luciferase assay were transfected with 5 μ g of plasmid encoding chimeric transcription factors Gal₄-ATF2 (pFA2-ATF2), Gal₄-Elk1 (pFA2-Elk1), or Gal₄-c-Jun (pFA2-c-Jun) and 5 μ g of the reporter plasmid (pFR-Luc) in addition to 5 μ g of plasmid encoding MKK3b-E (pcDNA3-MKK3b-E), a gift from Dr. R. Hipskind (Montpellier, France) or 100 ng of plasmid encoding an active MEKK1. Cells to be used in the immunoblotting experiments were transfected with 5 μ g of plasmid encoding MKK3b-E only. Electroporations were performed at 250 V, 1500 μ F using an Easyject Optima electroporator (Equibio). Upon transfection, cells were diluted by addition of Dulbecco's modified Eagle's medium devoid of phenol red, supplemented with 10% FCS, L-glutamine, and streptomycin-penicillin. 2×10^5 cells/well in 24-well plates were seeded for receptor binding assays and 1×10^5 cells/well for inositol phosphate assays. For plasma membrane preparation and immunoblotting experiments, electroporated cells were seeded in 100-mm dishes at a density of 3×10^6 cells/dish. For luciferase activity measurements, 2×10^5 cells/well were seeded in 12-well plates.

Binding Assays—For ¹²⁵I-BH-CCK8s binding experiments on COS7 cells expressing the rat CCK1 receptor or one of the mutant receptors, cells were washed once with phosphate-buffered saline (PBS, pH 7.4), 0.1% BSA ~2 days following transfection. They were then incubated for 1 h at 37 °C in 0.5 ml of Dulbecco's modified Eagle's medium, 0.1% BSA with 10 pM ¹²⁵I-BH-CCK8s in the presence or absence of either CCK8s, SB202190, or SB203580 at various concentrations. Cells were washed once with PBS (pH 7.4), 2% BSA and lysed by addition of 0.1 N NaOH. Samples were collected in tubes and their radioactivity content determined in a γ counter. For binding experiments on plasma membranes of COS7 cells transiently transfected with the rat CCK1 receptor, 2 days after transfection plasma membranes were prepared as previously described (28). Membrane proteins (8 μ g) were incubated for 90 min at 25 °C in a final volume of 0.5 ml of binding buffer containing 50 mM HEPES (pH 7), 115 mM NaCl, 5 mM MgCl₂, 0.01% soybean trypsin inhibitor, 0.1% bacitracin, 1 mM EGTA, and 0.1 mM phenylmethylsulfonyl fluoride, supplemented with 0.05% BSA, in the presence of 10 pM ¹²⁵I-BH-CCK8s and with or without competitive ligands. To stop the reaction, 0.5 ml of ice-cold binding buffer were added. To pellet the bound fraction, samples were centrifuged at $10,000 \times g$, 4 °C. The pellets were washed once with binding buffer, and their radioactivity was counted in a γ counter. The binding experiments with Jurkat T cells were performed using 4×10^6 cells/ml incubated in PBS containing 10 pM ¹²⁵I-BH-CCK8s and either unlabeled peptide, SB202190, or SB203580 at increasing concentrations for 1 h at 37 °C. Incubations were stopped by addition of 1 ml of ice-cold PBS containing 2% BSA. Cells were centrifuged for 10 min at $3,000 \times g$, 4 °C. The supernatants were removed and pellets measured for their radioactivity content in a γ counter. For [³H]-SB202190 binding experiments, COS7 cells expressing the rat CCK1 receptor were washed once with PBS, 0.1% BSA. They were then incubated for 1 h at 37 °C in the presence of 10 nM [³H]-SB202190 and increasing concentrations of either SB202190, SB203580, or CCK8s in a final volume of 0.5 ml of binding buffer containing 20 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, and 0.1% BSA. Following the incubation, cells were washed once with 1 ml of PBS, 2% BSA to remove unbound ligand and then lysed with 0.1 N NaOH. The samples were neutralized by addition of 0.1 N HCl. 10 ml of Complete

Phase combining system were added to each vial for determination of the radioactivity content using a liquid scintillation β counter. Each experiment was performed in triplicate, and mean values were used for calculations.

Inositol Phosphate Assay in COS7 Cells Transiently Transfected with the Rat CCK1 Receptor—Inositol phosphate assays were determined according to Qian *et al.* (29) with some modifications. The day before the experiment, transfected cells were incubated overnight with 2 μ Ci/ml of *myo*-[2- 3 H]inositol in Medium 199 at 37 °C. Cells were washed with Medium 199 (1 ml/well) and incubated for 30 min at 37 °C with Medium 199 supplemented with 30 mM LiCl. Cells were washed with inositol phosphate buffer containing 20 mM HEPES, 135 mM NaCl, 2 mM CaCl₂, 1.2 mM MgSO₄, 1 mM EGTA, 10 mM LiCl, supplemented with 11.1 mM D-glucose and 0.5% BSA. Incubations were performed in the absence or presence of ligands for 1 h at 37 °C in a final volume of 0.5 ml. The incubation medium was removed, and cells were lysed with 12 N HCl containing ethanol 0.3% (v/v). Supernatants were collected and applied to a column containing Dowex AG1-X8 anion-exchange resin in distilled water (1:4, w/w, 1.6 ml). Columns were successively washed with distilled water and 40 mM ammonium formate. Inositol phosphates were eluted with 1 M ammonium formate. The radioactivity of each eluate was determined in a liquid scintillation β counter upon addition of Complete Phase combining system in each vial.

Luciferase Activity Assay—Approximately 36 h after transfection, COS7 cells were incubated overnight at 37 °C in the presence of each compound to be tested. Cells were then washed once with ice-cold PBS and lysed with lysis buffer containing 125 mM Tris (pH 7.6), 10 mM CDTA, 10 mM DTT, 50% glycerol, and 5% Triton X-100. Lysates were transferred in a 96-well plate, and luciferase activity was measured using a Wallac 1450 Microbeta Jet upon injection of luciferase detection buffer (pH 7.4) containing 15 mM KH₂PO₄/K₂HPO₄, 1.05 mM MgCl₂, 2.7 mM MgSO₄, 0.2 mM EDTA, 0.53 mM ATP, 0.27 mM coenzyme A, and 0.5 mM luciferin.

Western Blotting, Immunoprecipitation, Kinase and Pulldown Assays—For immunoblotting experiments, after washing twice with ice-cold PBS COS7 cells transiently transfected either with the rat CCK1 receptor or with MKK3b-E and untransfected cells treated by anisomycin (10 μ g/ml) for 30 min were scraped in lysis buffer containing 12.5 mM Tris (pH 6.8), 195 mM DTT, 0.1 M SDS, and 2.2 M glycerol. Lysed cells and membranes were then boiled for 10 min. Each sample contained an equal amount of protein according to the Bradford assay. For immunoprecipitation and the pull-down assay, COS7 cells were incubated for 30 min in the presence of anisomycin (10 μ g/ml) for JNK and p38 MAP kinase assays or for 5 min in the presence of epidermal growth factor (10 ng/ml) at 37 °C for ERK assays. Following incubation, cells containing active p38 MAP kinase, JNK, or ERK were lysed with non-denaturing lysis buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin completed with 1 mM AEBF. Proteins (200 μ g) were incubated overnight at 4 °C with 15 μ l of either phospho-p38 MAP kinase, phospho-ERK monoclonal antibodies immobilized on beads for the kinase assays, or GST-c-Jun immobilized on beads for the pull-down assay. *In vitro* kinase assays were performed at 30 °C for 30 min in the presence of 2 μ g of GST-ATF2 or GST-Elk1, 200 μ M ATP in a kinase buffer containing 25 mM Tris (pH 6.8), 5 mM β -glycerophosphate, 2 mM DTT, 0.1 mM Na₃VO₄, and 10 mM MgCl₂ according to the manufacturer's instructions. The reaction was stopped by addition of 3 \times SDS sample buffer containing 187.5 mM Tris, pH 6.8, 6% SDS, 30% glycerol, 150 mM DTT, and 0.03% bromphenol blue. Samples for Western immunoblotting were subjected to a 10% polyacrylamide gel (SDS-PAGE) and then transferred to nitrocellulose ECL membranes by electrophoresis. Membranes were blocked and then incubated overnight with the appropriate primary antibody at 4 °C. Anti-p38 MAP kinase, anti-phospho-p38 MAP kinase, anti-MAPKAPK-2, and anti-phospho-MAPKAPK-2 antibodies were used for Western immunoblotting, and anti-phospho-ATF2, anti-phospho-Elk1, and anti-phospho-c-Jun antibodies were used for kinase and pull-down assays (all were diluted at 1/1000). Membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody. Bound antibody was detected by reaction with the LumiGLO[®] chemiluminescent reagent and exposed to Biomax ML scientific imaging film.

Dynamic Docking Studies in the ATP Binding Site of p38 MAP Kinase—The molecular model of the ATP binding site of p38 MAP kinase was constructed on the basis of the co-crystallized structure of p38 MAP kinase with the SB203580 compound (30). The docking of L364,718 and L365,260 compounds was improved by intensive annealing calculations. The resulting structure obtained for each nonpeptide

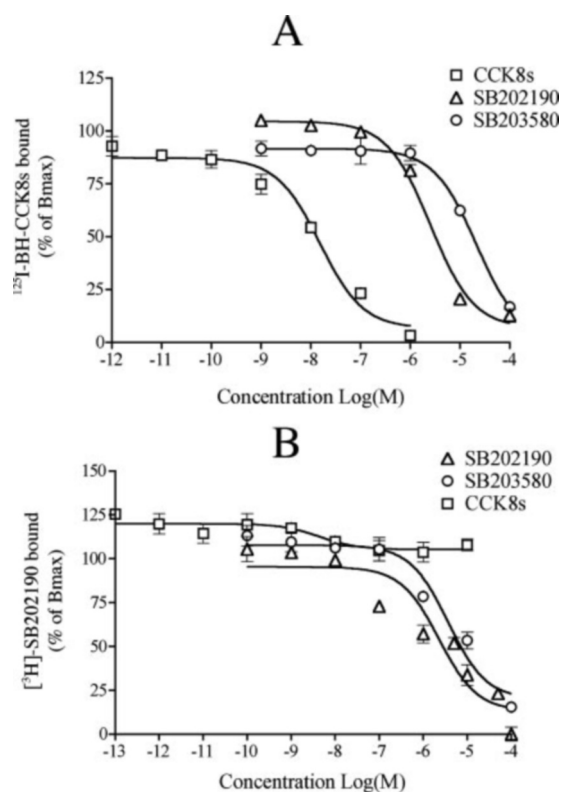


FIG. 2. Binding experiments in COS7 cells expressing the rat CCK1 receptor. COS7 cells transiently expressing the rat CCK1 receptor were incubated with either 10 pM 125 I-BH-CCK8s (A) or 10 nM [3 H]-SB202190 (B) in the absence or the presence of increasing concentrations of CCK8s (□), SB202190 (△), and SB203580 (○). Total specific binding for each of the three compounds has been represented as 100%. Data are expressed as percent of the maximal specific binding measured in the absence of unlabeled ligand. Nonspecific binding was determined in the presence of 10 μ M CCK8s (A) or 100 μ M SB202190 (B). Results are the mean \pm S.D. of at least three independent experiments, each performed in triplicate.

CCK analogue complexed with the ATP binding site of p38 MAP kinase was further refined using molecular dynamics and energy minimization. All the annealing refinement and docking calculations were performed using Biosym software (Accelrys, San Diego, CA).

Data Analysis—Dose response curves and binding data were analyzed using GraphPad Prism nonlinear regression software programs (GraphPad, San Diego, CA). Significance was determined using the Student's unpaired *t* test with $p < 0.05$.

RESULTS

Pharmacological Studies of the Interaction of SB202190 and SB203580 with the Rat CCK1 Receptor—We investigated the effect of the two p38 MAP kinase inhibitors (SB202190 and SB203580) on the binding of 125 I-BH-CCK8s to rat CCK1 transiently transfected in COS7 cells. As shown in Fig. 2A, whereas the natural ligand CCK8s bound with a K_i value of 14 ± 3.5 nM, SB202190 and SB203580 were able to inhibit the specific binding of 125 I-BH-CCK8s with K_i values of 4.2 ± 0.2 and 23 ± 3.8 μ M, respectively ($p < 0.02$, $n = 9$). Interestingly, these K_i values are in the range of inhibitor concentrations classically used to block the p38 MAP kinase activity. We also performed binding experiments using [3 H]-SB202190 on COS7 cells expressing the CCK1 receptor to determine the binding site of both p38 MAP kinase inhibitors. As shown in Fig. 2B, SB202190 and SB203580 displaced the binding of [3 H]-SB202190 with K_i values of 2.3 ± 0.7 and 8.5 ± 1.7 μ M, respectively, whereas CCK8s was without effect, suggesting distinct binding sites on the CCK1 receptor.

To determine the competitive nature of the interaction between SB202190 or SB203580 and the CCK1 receptor, binding

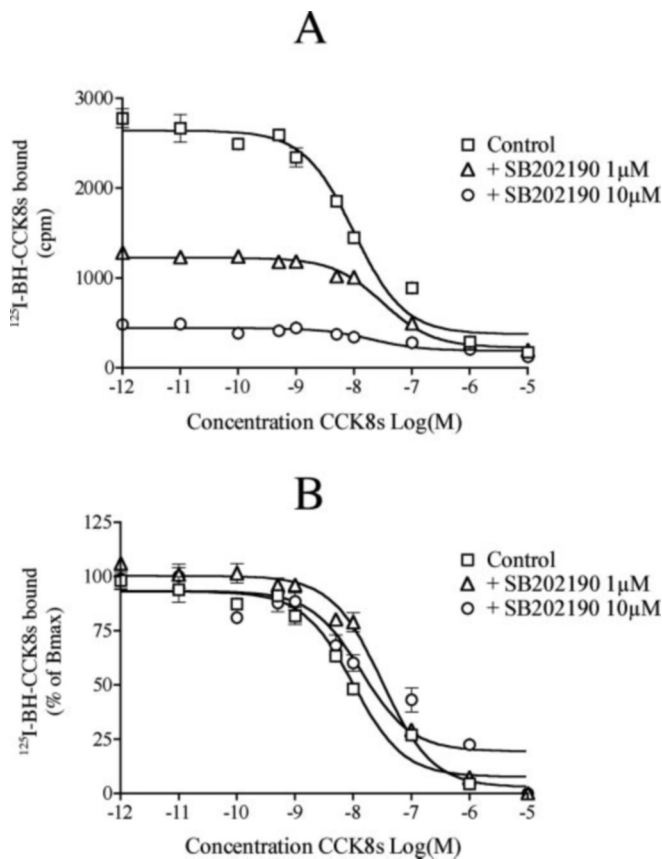


FIG. 3. Determination of the non-competitive behavior of SB202190 on COS7 cells expressing the rat CCK1 receptor. Binding experiments using ^{125}I -BH-CCK8s and CCK8s as competitors were performed in the absence (\square) or presence of SB202190 1 μM (Δ) or 10 μM (\circ). Data are expressed as either cpm of bound ^{125}I -BH-CCK8s (A) or as percent of the maximal specific binding (B). Results are the mean \pm S.D. of at least three independent experiments, each performed in triplicate.

experiments using ^{125}I -BH-CCK8s in competition with CCK8s were undertaken in the absence or in the presence of 1 or 10 μM SB202190 or SB203580 (Fig. 3A). The specific binding of CCK8s decreased by 2.4-fold in the presence of SB202190 1 μM and 7.2-fold in the presence of SB202190 10 μM . Moreover, the affinity of CCK8s was not affected by the presence of SB202190, because close K_i values were measured under each situation: 8.9 ± 0.9 nM in the absence of SB202190, 28 ± 4 and 12 ± 2 nM in the presence of SB202190, 1 μM and 10 μM , respectively (Fig. 3B). The same behavior was observed with SB203580 (data not shown). These data suggested that both p38 MAP kinase inhibitors were non-competitive ligands for the CCK binding site. To determine whether these observations were or were not related to the inhibitory property of SB202190 and SB203580 on p38 MAP kinase, we first controlled for the absence of p38 MAP kinase on COS7 cell plasma membranes in comparison with whole cells (Fig. 4A). As shown in Fig. 4A, immunoblotting experiments with a p38 MAP kinase antibody confirmed the absence of p38 MAP kinase in membrane fractions compared with whole cells. We performed additional binding experiments using plasma membranes and observed that both compounds maintained their ability to displace ^{125}I -BH-CCK8s with K_i values of 0.9 ± 0.3 μM for SB202190 and 14.7 ± 5 μM for SB203580 (Fig. 4B), suggesting that they interact with the CCK1 receptor independently of the presence of p38 MAP kinase. Additionally, we performed the same binding experiments on rat pancreatic acini, which endogenously express CCK1 receptor. We measured affinity values

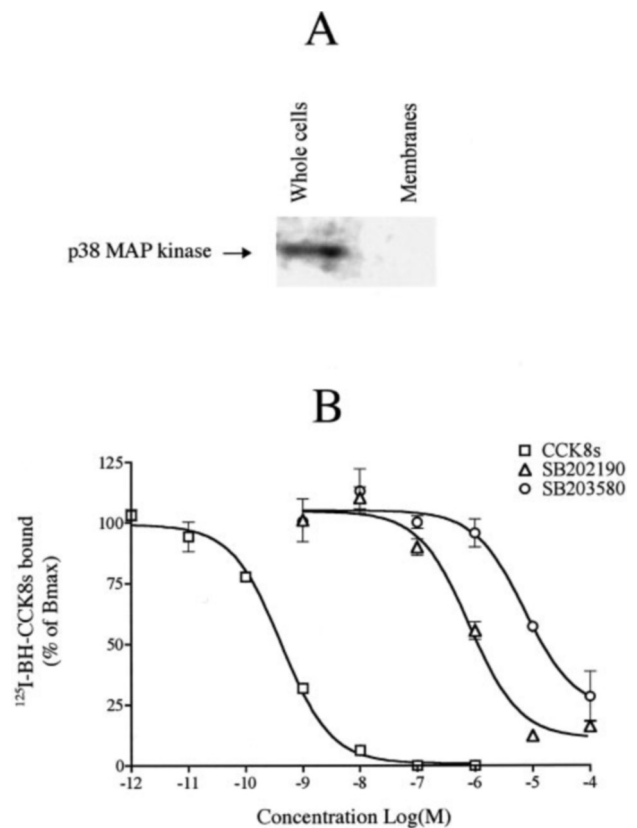


FIG. 4. Binding properties of SB202190 and SB203580 on plasma membranes not expressing p38 MAP kinase. A, comparative immunoblotting of either whole cell samples taken from COS7 cells expressing the rat CCK1 receptor or plasma membrane samples. Detection was performed using a specific antibody against the unphosphorylated form of p38 MAP kinase. B, binding experiments using plasma membranes prepared from COS7 cells transiently expressing the rat CCK1 receptor as described under "Experimental Procedures." Membranes (8 μg proteins) were incubated with 10 pM ^{125}I -BH-CCK8s and with various concentrations either of CCK8s (\square), SB202190 (Δ), or SB203580 (\circ). Data are expressed as the percentage of the maximal specific binding measured in the absence of unlabeled CCK8s. Results are the mean \pm S.D. of at least three independent experiments, each performed in triplicate.

for the two p38 MAP kinase inhibitors identical to those previously found (data not shown).

Specificity of SB202190 and SB203580 for the CCK1 Receptor—To test the selectivity of the two p38 MAP kinase inhibitors for the CCK1 receptor, we measured the ability of SB202190 and SB203580 to displace the specific binding of ^{125}I -BH-CCK8s on the CCK2 receptor, endogenously expressed in Jurkat T cells (31). Whereas CCK8s displaced the specific binding of ^{125}I -BH-CCK8s with an expected affinity of 0.4 ± 0.05 nM, SB202190 and SB203580 were unable to compete with the radiolabeled ligand (Fig. 5), indicating a specificity for the CCK1 receptor. We further confirmed these observations using two other G-protein-coupled receptors, the gastrin-releasing peptide receptor (GRP-R) expressed in Swiss 3T3 cells (32) and the α -MSH type 1 receptor (MC1-R) expressed in Dorier cells (33), to which both p38 MAP kinase inhibitors were unable to bind although the corresponding natural ligands bound with the expected affinity (data not shown).

Regulation of the Rat CCK1 Receptor-mediated Inositol Phosphate Production by SB202190 and SB203580—Previous studies have demonstrated that the binding of CCK8s to the CCK1 receptor type induced inositol phosphate production via activation of a G_q protein (7). Therefore, we measured inositol phos-

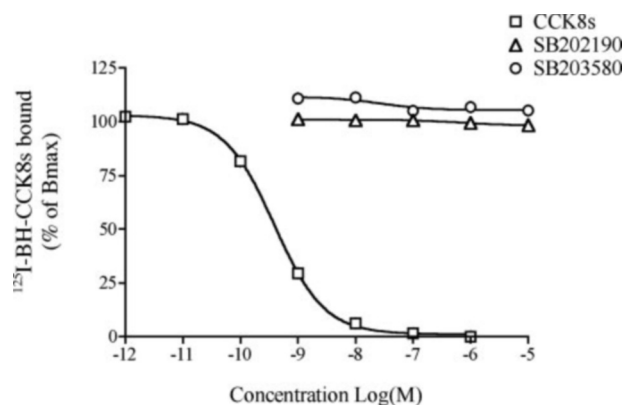


FIG. 5. Selectivity of SB202190 and SB203580 for the CCK1 receptor. Binding experiments were performed using the Jurkat T cell line endogenously expressing the CCK2 receptor. Cells were incubated with 10 pM ^{125}I -BH-CCK8s in the presence of either CCK8s (\square), SB202190 (\triangle), or SB203580 (\circ) at increasing concentrations. Data are expressed as the percentage of the maximal ^{125}I -BH-CCK8s bound, measured in the absence of unlabeled CCK8s. Results are the mean \pm S.D. of at least three independent experiments, each performed in triplicate.

phate production in COS7 cells transiently transfected with the rat CCK1 receptor to determine the behavior of SB202190 and SB203580. We found that both pyridinyl imidazole compounds were unable to induce inositol phosphate production even at high concentration, unlike CCK8s which induced a 12-fold maximal increase of inositol phosphate accumulation at 0.1 μM compared with the basal value with an effective concentration (EC_{50}) of 4 ± 0.5 nM (Fig. 6A). We determined the antagonist characteristics of both p38 MAP kinase inhibitors on the CCK8s-induced inositol phosphate production. As shown in Fig. 6B, SB202190 and SB203580 were able to inhibit inositol phosphate production with respective K_{inact} (constant of inactivation) values of 3.4 ± 2 and 26 ± 2 μM ($p < 0.05$, $n = 5$). We further confirmed the antagonist potency of both compounds using the rat pancreatic acini model where SB202190 and SB203580 inhibited CCK8s-induced inositol phosphate production as well as CCK8s-induced amylase release with similar K_{inact} values (data not shown).

Structural Characterization of the Binding Site of SB202190 and SB203580 on the Human CCK1 Receptor—To identify the binding site of the p38 MAP kinase inhibitors on the human CCK1 receptor, we constructed mutants incorporating regions of the CCK1 receptor in place of the corresponding regions of the CCK2 receptor. Following sequence alignment studies, we determined sequence similarities between the ATP binding site of p38 MAP kinase and the second and third intracellular loops of the CCK1 receptor, which are absent from the CCK2 receptor (Fig. 7). To determine whether these sequence identities were involved in the interaction of SB202190 and SB203580 with the CCK1 receptor, we alternately substituted the second or third intracellular loop of the CCK2 receptor with its CCK1 counterpart. As shown in Fig. 8A, the CCK2 receptor mutant containing the second intracellular loop i2, the extracellular loop e1, and the transmembrane domains TM2, TM3, and TM4 of the CCK1 receptor was called mutant “i2”. The second mutant containing the third intracellular loop i3 and the transmembrane domain TM6 of the CCK1 receptor was named mutant “i3”. Binding properties of CCK8s and SB202190 for each mutant were determined. Whereas CCK8s displayed a high affinity for the wild-type human CCK2 receptor with an IC_{50} of 8.5 ± 2.2 nM (Fig. 8B), 8- and 15.4-fold decreases were observed in the affinity of CCK8s for mutants i2 and i3 with IC_{50} values of 68 ± 3.6 nM and 131 ± 25 nM, respectively (Fig. 8, C and D). Interestingly, although SB202190 was unable to displace the

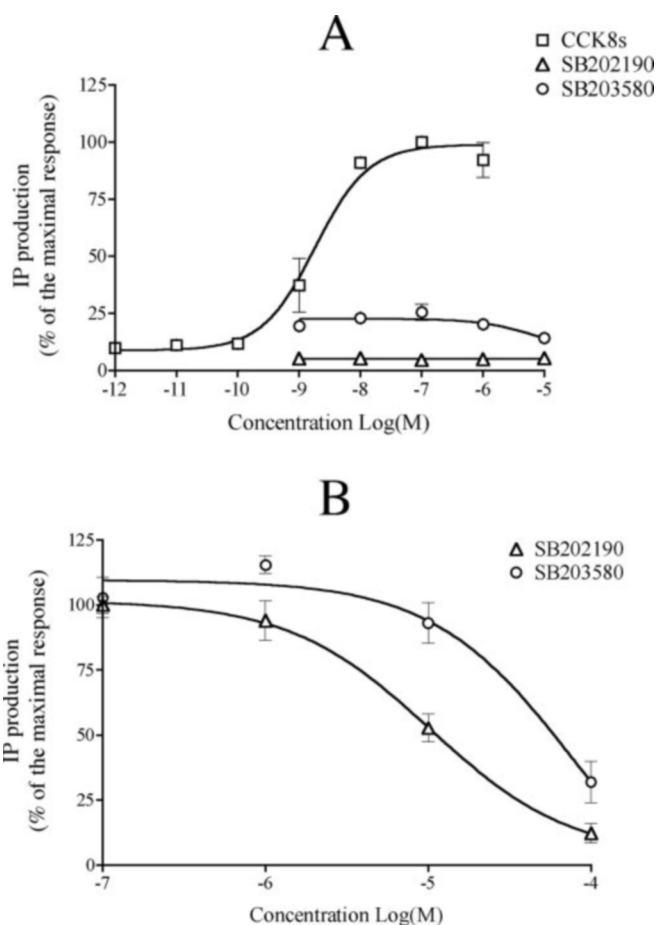


FIG. 6. Determination of the antagonist properties of SB202190 and SB203580 on the CCK1 receptor. A, COS7 cells transiently expressing the CCK1 receptor were incubated with various concentrations either of CCK8s (\square), SB202190 (\triangle), or SB203580 (\circ). Inositol phosphate production was expressed as the percentage of the maximal response obtained with 1 μM CCK8s corrected for basal inositol phosphate production. B, concentration-dependent inhibition of CCK8s-induced inositol phosphate production. SB202190 (\triangle) or SB203580 (\circ) were co-incubated at various concentrations in the presence of 1 nM CCK8s. Inositol phosphate production was expressed as the percentage of the maximal response, determined in the absence of pyridinyl imidazole compounds, corrected for the basal activity. Results are the mean \pm S.D. of at least three independent experiments, each performed in triplicate.

binding of radiolabeled CCK8s to the wild-type human CCK2 receptor and to the mutated receptor i3 (Fig. 8, B and D), it was able to displace ^{125}I -BH-CCK8s binding to the mutated receptor i2 (Fig. 8C) with an IC_{50} value of 27 ± 3.2 μM . This result suggests that the second intracellular loop of the CCK1 receptor is involved in the interaction with the two p38 MAP kinase inhibitors.

Effect of L364,718 and L365,260 on p38 MAP Kinase Activity—We studied the interaction of the two nonpeptide CCK receptor antagonists (Fig. 1) with p38 MAP kinase and the modulation of its enzymatic activity. In addition we studied the *in vitro* effect of the CCK receptor antagonists L364,718 and L365,260 on the transcriptional activity of Gal₄-ATF2, a target of p38 MAP kinase (34, 35). P38 MAP kinase was specifically activated by MKK3b-E, a constitutively active form of MKK3 in which the amino acid residues of the activating phosphorylation sites were replaced with glutamic acid residues and which contains an additional 29 amino acids fused to the N terminus end (36, 37).

As expected, SB202190 dose-dependently inhibited the transcriptional activity of Gal₄-ATF2 induced by MKK3b-E, caus-

30

40

P38 MAP kinase active site regionNLSPVGS**G**AYGSVCAAFDTK**2nd Intra-cellular loop of CCK1 receptor**NLVAISLER YGA**I**CKP LQSRV

140

150

FIG. 7. **Sequence identities between the active site of p38 MAP kinase and the second intracellular loop of the CCK1 receptor.** Residues Val-30-Val-38 of the p38 MAP kinase are located in the ATP binding site. The second intracellular loop of the CCK1 receptor includes Asn-131-Arg-150. The homologies between residues in the two proteins are indicated in *bold*.

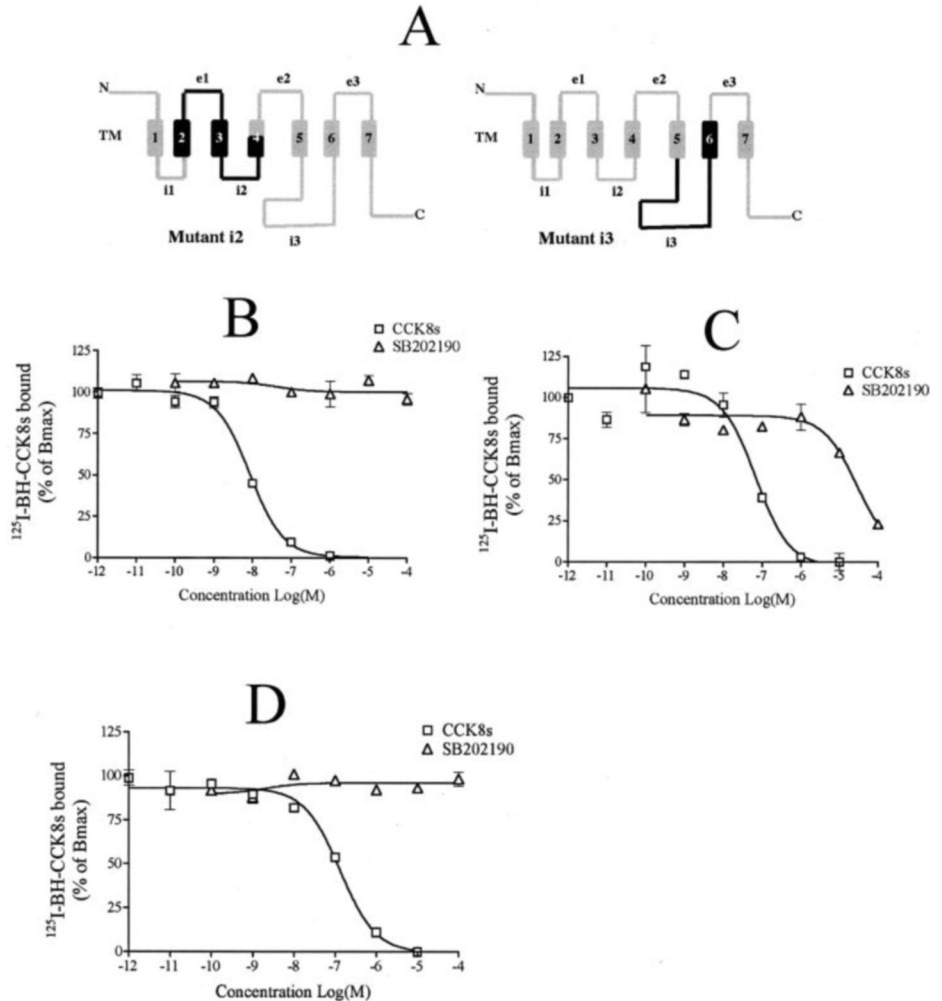


FIG. 8. **Involvement of the CCK1 receptor intracellular domains in the binding of SB202190.** A, selected domains of the wild-type CCK2 receptor (gray) were substituted with the corresponding domains of the CCK1 receptor (black). The mutant i2 contains the second intracellular loop of the human CCK1 receptor, whereas the mutant i3 contains the third intracellular loop of the CCK1 receptor. COS7 cells transiently transfected with the wild-type CCK2 receptor (B) or either mutant i2 (C) or mutant i3 (D) were incubated with 10 pM ¹²⁵I-BH-CCK8s in the presence of various concentrations of either CCK8s (□) or SB202190 (△) for 1 h at 37 °C. Data are expressed as the percentage of the maximal ¹²⁵I-BH-CCK8s bound, measured in the absence of unlabeled CCK8s. Results are the mean \pm S.D. of at least three independent experiments, each performed in triplicate.

ing $87 \pm 0.7\%$ inhibition at $10 \mu\text{M}$ (Fig. 9A). Compounds L364,718 and L365,260 were also able to dose-dependently inhibit the transcriptional activity of Gal₄-ATF2, causing $61.2 \pm 6.7\%$ inhibition for L364,718 ($10 \mu\text{M}$) and $47.3 \pm 2.6\%$ for L365,260 ($10 \mu\text{M}$) ($p < 0.001$, $n = 6$ in both cases). We measured IC₅₀ values of $49.8 \pm 7.6 \text{ nM}$ for SB202190, $5.2 \pm 1.1 \mu\text{M}$ for L364,718, and $19.9 \pm 2.6 \mu\text{M}$ for L365,260 ($p < 0.05$, $n = 6$). These results suggest that both CCK receptor antagonists were able to cross the plasma membrane and inhibit the transcriptional activity of Gal₄-ATF2.

Considering that ATF-2 was not described as a physiological substrate of p38 MAP kinase (38), we tested by immunoblotting experiments the two CCK receptor antagonists for their ability to inhibit the phosphorylation of MAPKAPK-2, which is a very specific target of p38 MAP kinase. As shown in Fig. 9B, the presence of anisomycin induced a very strong activation of MAPKAPK-2. The addition of 5-, 10-, and 50- μM concentrations of L364,718 or L365,260 led to partial and dose-dependent inhibition of the MAPKAPK-2 phosphorylation. This result

confirmed that the two nonpeptide CCK antagonists were able to inhibit the p38 MAP kinase pathway.

It remained to be determined at which stage of the p38 MAP kinase pathway both CCK antagonists had an inhibitory effect. To assess whether their effect was due to the inhibition of p38 MAP kinase activation or to a direct inhibition of its kinase activity, we tested both CCK receptor antagonists for their ability to modulate p38 MAP kinase activation. We performed Western blotting experiments on COS7 cells transiently transfected with MKK3b-E and examined the level of phosphorylated p38 MAP kinase in the presence or absence of $10 \mu\text{M}$ SB202190, L364,718, or L365,260. As shown in Fig. 9C, both L364,718 and L365,260, as well as SB202190, were unable to inhibit p38 MAP kinase phosphorylation. These data strongly suggest that inhibition of Gal₄-ATF2 transcriptional activity and of the MAPKAPK-2 phosphorylation by L364,718 and L365,260 was due to a direct inhibition of p38 MAP kinase activity as described for SB202190.

To confirm the inhibitory effect of both nonpeptide CCK

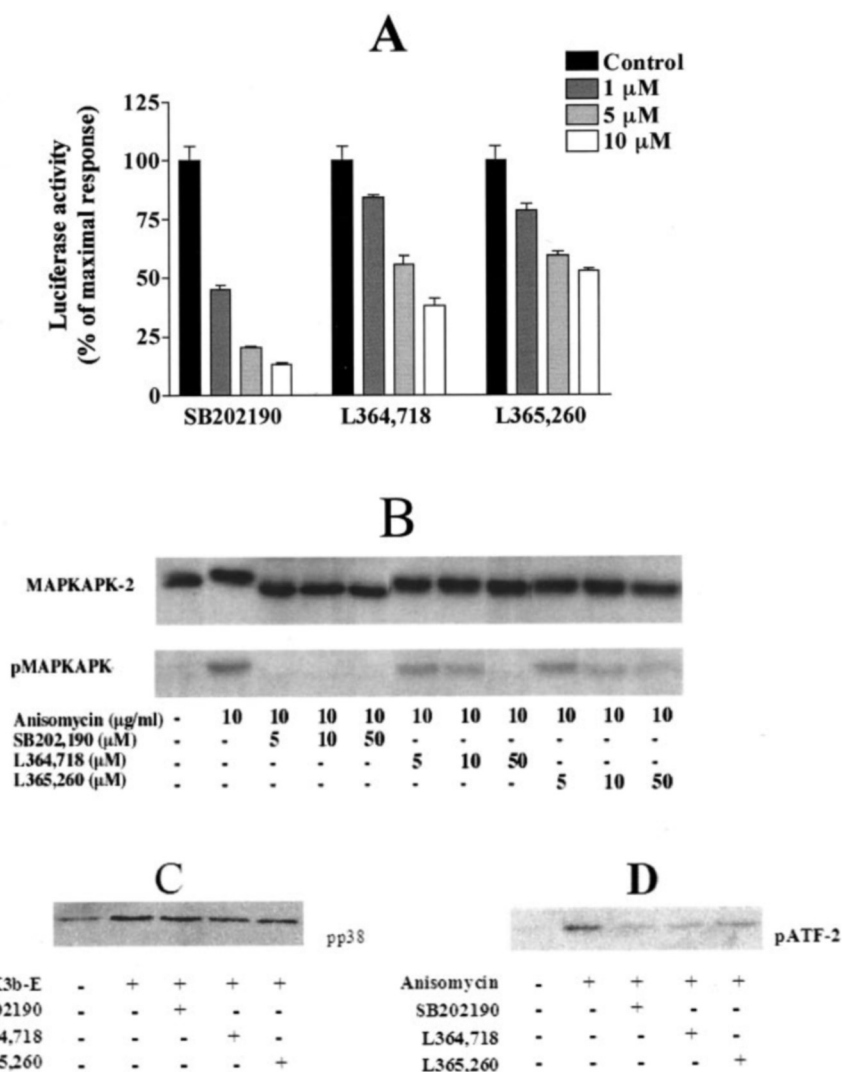


FIG. 9. Effect of the nonpeptide CCK antagonists L364,718 and L365,260 on p38 MAP kinase activity. *A*, inhibition of Gal₄-ATF2 transcriptional activity. COS7 cells were transiently co-transfected with a plasmid encoding MKK3b-E, a plasmid encoding the chimeric Gal₄-ATF2 transcriptional factor, and the pFR-Luc reporter plasmid. The transcriptional activity of Gal₄-ATF2 was determined upon incubation of cells in the presence of either SB202190, L364,718, or L365,260 at several concentrations (1, 5, and 10 μM) as described under "Experimental Procedures." This activity was expressed as the percentage of the maximal responses determined in the absence of compounds (*Control*). Each value is the mean ± S.D. of at least three independent experiments, each performed in triplicate. *B*, detection of phospho-MAPKAPK-2 and its unphosphorylated form by immunoblotting. COS7 cells were treated by anisomycin (see "Experimental Procedures") and by SB202190, L364,718, or L365,260 at the indicated concentrations. Phospho-MAPKAPK-2 and MAPKAPK-2 were detected using the appropriate specific primary antibody. The experiments were performed independently six times with similar results. The autoradiographs shown are from one such experiment. *C*, detection of phospho-p38 MAP kinase by immunoblotting. COS7 cells transiently expressing MKK3b-E were incubated in the absence (untreated cells) or in the presence of SB202190 (10 μM), L364,718 (10 μM), or L365,260 (10 μM) and then lysed, and the intracellular content was subjected to immunoblotting (see "Experimental Procedures"). The basal level of phospho-p38 MAP kinase was controlled using the empty expression vector pcDNA3. Phospho-p38 MAP kinase was detected using a specific anti-phospho p38 MAP kinase primary antibody. *D*, detection of phospho-ATF2 by immunoblotting. Phospho-p38 MAP kinase was immunoprecipitated after incubation of COS7 cells with anisomycin (10 μg/ml), and kinase assays were performed using GST-ATF-2 as a substrate. Inhibition of the p38 MAP kinase activity was measured in the presence of SB202190 (50 μM), L364,718 (50 μM), or L365,260 (50 μM). Immunodetection was performed using an anti-phospho-ATF2 primary antibody.

receptor antagonists on the enzymatic activity of p38 MAP kinase, we performed kinase assays to detect phospho-ATF2. As shown in Fig. 9D, anisomycin strongly stimulated the p38 MAP kinase-induced phosphorylation of ATF2 in COS7 cells. As expected, 50 μM SB202190 strongly inhibited the kinase activity of p38 MAP kinase. We also showed the inhibitory effect of L364,718 and L365,260 on the p38 MAP kinase-induced phosphorylation of ATF2. These data indicated that L364,718 and L365,260 behaved *in vitro* as inhibitors of p38 MAP kinase activity.

Specificity of L364,718 and L365,260 Compounds for p38 MAP Kinase—Following these results, we determined whether the two CCK receptor antagonists were able to inhibit the two other main MAP kinase pathways, ERK and JNK. We first

tested compounds L364,718 and L365,260 for their ability to inhibit the transcriptional activity of Gal₄-Elk1 induced by serum (indicating ERK activation) and of Gal₄-c-Jun induced by a constitutively active form of MEKK1 (indicating JNK activation). As shown in Fig. 10A, the MEK1/2 inhibitor U0126 totally inhibited the transcriptional activity of Gal₄-Elk1 at 10 μM, suggesting that ERK was directly involved in the activation of this transcription factor. We also observed that SB202190 and L364,718 partially decreased the activity of Gal₄-Elk1 with 57 ± 1.8% and 47 ± 3.4% inhibition, respectively, whereas L365,260 was without effect. Concerning the transcriptional activity of Gal₄-c-Jun, we observed that the JNK inhibitor SP600125 caused a strong inhibition at 10 μM (Fig. 10B). Interestingly, L364,718 and L365,260 had no inhib-

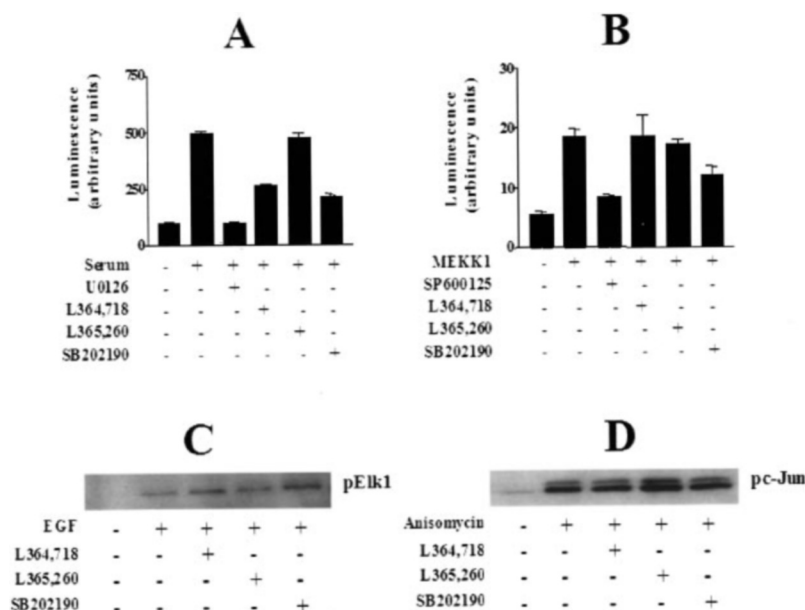


FIG. 10. **Determination of L364,718 and L365,260 specificity for p38 MAP kinase.** COS7 cells were co-transfected with Gal₄-Elk1 (A) or Gal₄-c-Jun (B) and with pFR-Luc (A and B). The transcriptional activity of Gal₄-Elk1 and Gal₄-c-Jun was measured after stimulation with 10% FCS (A) or by expression of MEKK1 (B) and was expressed as arbitrary units of luminescence. A, the effects of 10 μ M U0126 (a MEK1/2 inhibitor), 10 μ M L364,718, 10 μ M L365,260, and 10 μ M SB202190 are shown. B, the effects of 10 μ M SP600125 (a JNK inhibitor), 10 μ M L364,718, 10 μ M L365,260 and 10 μ M SB202190 are shown. Each value is the mean \pm S.D. of at least three independent experiments, each performed in triplicate. After treatment of COS7 cells with epidermal growth factor (10 ng/ml), phospho-p44/p42 MAP kinase was immunoprecipitated and the kinase assay was performed with GST-Elk1 (see "Experimental Procedures") in the presence or absence of 10 μ M SB202190, 10 μ M L364,718, or 10 μ M L365,260. C, phospho-Elk1 was detected by immunoblotting using anti-phospho-Elk1 primary antibody. D, phospho-c-Jun was detected by immunoblotting after pulldown experiment on COS7 cells treated with anisomycin (see "Experimental Procedures"). The kinase assay was performed in the presence or the absence of 10 μ M SB202190, 10 μ M L364,718, or 10 μ M L365,260.

itory effect on the transcriptional activity of Gal₄-c-Jun, whereas SB202190 caused a weak inhibition of $35 \pm 6.2\%$. To confirm these observations, we performed kinase assays and pulldown experiments on ERK and JNK using GST-Elk1 and GST-c-Jun as respective substrates. As shown in Fig. 10C, the presence of SB202190 or of each CCK receptor antagonist (10 μ M) was without effect on the ERK-induced phosphorylation of GST-Elk1. We also showed that these three compounds had no inhibitory effect on the JNK-induced phosphorylation of GST-c-Jun (Fig. 10D). Taken together, these results suggest that the inhibitory effect of L364,718 and L365,260 was specific to p38 MAP kinase.

Molecular Modeling Studies of L364,718 and L365,260 Interactions in the ATP Binding Site of p38 MAP Kinase—We have used the data of Wang *et al.* (30) on the co-crystal structure of SB203580 in the ATP binding site of p38 MAP kinase to perform dynamic docking studies with the two nonpeptide CCK receptor antagonists (Fig. 11A). These studies showed that L364,718 and L365,260 could fit into the ATP binding site of p38 MAP kinase close to the position of SB203580. Each compound can interact with Asp-168 through two hydrogen bonds established through the amide and urea motifs of L364,718 and L365,260, respectively (Fig. 11, B and C). In addition, the urea motif of L365,260 can interact with Lys-53 through an additional hydrogen bond. The two nonpeptide CCK receptor antagonists can also make hydrophobic interactions with Tyr-35, as in the SB203580/p38 MAP kinase co-crystals. These data suggest that L364,718 and L365,260 can possibly bind in the ATP binding site of p38 MAP kinase.

DISCUSSION

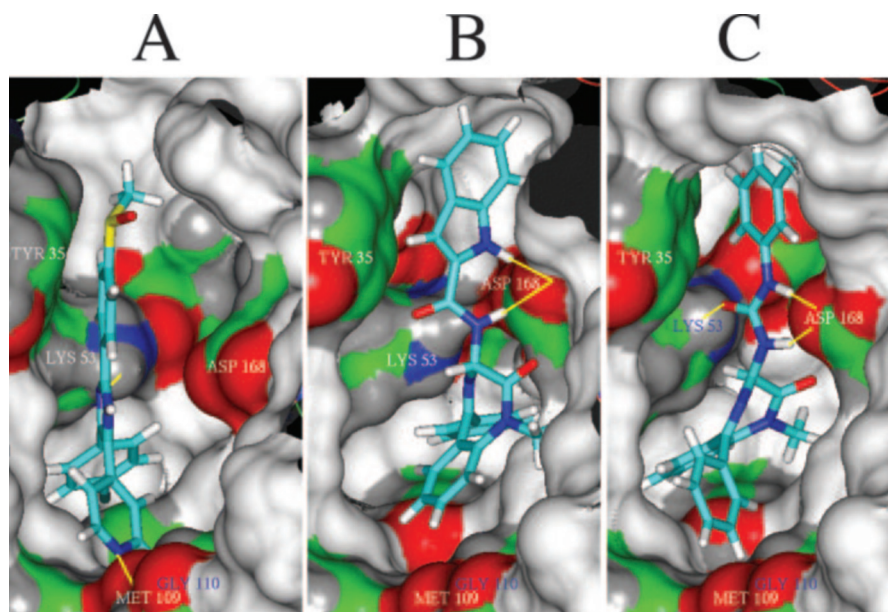
Several reports have demonstrated that the p38 MAP kinase inhibitors SB202190 and SB203580 were highly selective for p38 α and p38 β MAP kinases with IC₅₀ values in the tenth nanomolar range (21, 17). Recent studies have also suggested

that other protein kinases were sensitive to SB203580, because of the presence of a Ser or Thr at a position equivalent to Thr-106 of p38 MAP kinase. Indeed, types I and II transforming growth factor β receptors, Ick, and c-Raf, are also inhibited by SB203580 with IC₅₀ values of 20, 40, 25, and 2 μ M, respectively (21, 19). In this study, we showed for the first time that SB202190 and SB203580 behaved not only as selective p38 MAP kinase inhibitors but also as non-competitive receptor antagonists of a G-protein-coupled receptor, namely the CCK1 receptor. We have also pointed out that two nonpeptide CCK receptor antagonists, L364,718 and L365,260, were in turn able to inhibit p38 MAP kinase activity.

We first observed that the p38 MAP kinase inhibitors SB202190 and SB203580 were able to modify the binding of ¹²⁵I-BH-CCK8s to both rat and human (data not shown) CCK1 receptors with affinity constants in the micromolar range. To eliminate the involvement of p38 MAP kinase itself in the pharmacological effect of SB202190 and SB203580 on the CCK1 receptor, displacement experiments were performed using plasma membranes that do not express the enzyme. Both compounds were able to displace ¹²⁵I-BH-CCK8s from the plasma membranes in the absence of the enzyme (Fig. 4). These results suggest a direct interaction between both of the p38 MAP kinase inhibitors and the CCK1 receptor.

To elucidate by which mechanism SB202190 and SB203580 interacted with the CCK1 receptor, we combined pharmacological and molecular approaches. Binding experiments revealed that both p38 MAP kinase inhibitors displaced binding of [³H]-SB202190 to the rat CCK1 receptor with affinity values similar to those determined with ¹²⁵I-BH-CCK8s, whereas CCK8s had no effect even at high concentrations (Fig. 2B). This suggests that the two p38 MAP kinase inhibitors do not interact at the same binding site. Furthermore, the binding of CCK8s was not modified by the addition of high concentration of either SB202190 or SB203580, strengthening the idea that both in-

FIG. 11. Dynamic docking of two nonpeptide CCK antagonists into the ATP binding site of p38 MAP kinase. Docking studies were performed using x-ray data of co-crystallized SB203580 into the ATP binding site of p38 MAP kinase (Protein Data Bank accession number 1A9U). *A*, *B*, and *C* show the docking of SB203580, L364,718, and L365,260, respectively. Possible hydrogen bonds are shown in yellow.



inhibitors might act as non-competitive ligands (Fig. 3). Interestingly, both SB202190 and SB203580 compounds were selective for the CCK1 receptor, because no binding was observed for the CCK2 receptor type either in the Jurkat T cell line or in transfected COS7 cells.

The non-competitive behavior of SB202190 and SB203580 for the CCK1 receptor involved a specific binding site distinct from the CCK binding site. CCK1 and CCK2 receptors share 48% homology, with the main differences localized in the extracellular and intracellular loops. Although it is known that the ligand binding sites of such G-protein-coupled receptors are different, it is also known that in both cases this binding involves the extracellular and transmembrane domains (39, 40). Taking these results together, we hypothesized that SB202190 and SB203580 interact with the CCK1 receptor at a distinct site involving the intracellular domains of this receptor. Because SB202190 and SB203580 are reported to inhibit p38 MAP kinase upon occupation of its ATP binding site, we performed sequence alignment and determined sequence homologies between the ATP binding site and the second and third intracellular loops of the CCK1 receptor. To determine whether these intracellular loops of the CCK1 receptor could be involved in the binding of SB202190 and SB203580, we alternately introduced these two CCK1 domains into the CCK2 receptor. Interestingly, although SB202190 had no effect on the binding of CCK8s to the wild-type CCK2 receptor, the CCK2 receptor mutant containing the second intracellular loop of CCK1 gained the ability to bind SB202190. This, therefore, suggests that the second intracellular loop of the CCK1 receptor could be directly involved in this interaction. For the CCK2 receptor mutant containing the third intracellular loop of the CCK1 receptor, SB202190 was unable to displace CCK8s binding, indicating that this domain was not crucial for the interaction between the p38 MAP kinase inhibitors and the CCK1 receptor. However, construction of a CCK2 mutant containing both the second and third intracellular domains of the CCK1 receptor resulted in a mutant having the expected affinity for SB202190, indicating that the third intracellular loop could potentiate the binding of such a compound (data not shown).

Compounds SB202190 and SB203580 were not only able to directly bind to the CCK1 receptor but were also able to antagonize the biological effects of the natural ligand. Both abolished CCK8s-induced inositol phosphate production (Fig. 6) and am-

ylase release from rat pancreatic acini (data not shown). Once again, we determined K_i values similar to those measured in binding experiments. Although these affinity values are low, they are in the range of concentrations classically used in experiments dealing with p38 MAP kinase inhibition. These results should alert researchers to the use of these p38 MAP kinase inhibitors in pharmacological models expressing the CCK1 receptor. Several reports have demonstrated that CCK1 receptors peripherally localized in the gastrointestinal tract regulate feeding behavior (41) and that CCK1 receptors are very important targets for the pharmacological treatment of obesity (42). Hence, the two pyridinyl imidazole derivatives might interfere with the pharmacological responses of the CCK1 receptor in food intake disorders and might enable the development of new anti-obesity agents.

Because p38 MAP kinase inhibitors were able to bind to the CCK1 receptor, we wondered whether CCK antagonists could interact with p38 MAP kinase. We therefore selected the non-peptide CCK antagonists L364,718 and L365,260 for further studies (Fig. 1). We showed that these two compounds dose-dependently inhibited the transcriptional activity of Gal₄-ATF2 specifically activated by p38 MAP kinase (Fig. 9A) and the phosphorylation of MAPKAPK-2 (Fig. 9B). These results are supported by the results of Oiry *et al.* (43) suggesting that these two benzodiazepine derivatives are able to cross the plasma membrane and interact with intracellular targets. We have also observed that L365,260 was unable to inhibit the transcriptional activity of Elk1 and c-Jun induced by ERK and JNK, respectively (Fig. 9, A and B) and to inhibit the kinase activity of ERK and JNK (Fig. 10, C and D). In the presence of SB202190 and L364,718, we showed a partial inhibition of the transcriptional activity of Elk1, but these compounds were unable to block the kinase activity of ERK (Fig. 10, A and C), suggesting that ERK was not involved in the inhibitory effect of both compounds on Elk1. It has been demonstrated that the transcription factor Elk1 is a target of p38 MAP kinase (44), strongly suggesting that the inhibitory effect of compounds SB202190 and L364,718 might be because of an interaction with p38 MAP kinase. The data presented here strongly suggest that the two CCK receptor antagonists acted at the p38 MAP kinase pathway. In addition, immunoblotting experiments demonstrated that phosphorylation of p38 MAP kinase was not affected by the presence of high concentration of either nonpeptide CCK receptor antagonists or SB202190, indicating

that their inhibitory effect rose from a direct interaction with p38 MAP kinase (Fig. 9C). The fact that L364,718 and L365,260 inhibited the phosphorylation of ATF2 with the same efficacy as SB202190 (Fig. 9D) confirmed that L364,718 and L365,260 were not only CCK receptor antagonists but also behaved as specific and direct p38 MAP kinase inhibitors. L364,718 is a specific CCK1 receptor antagonist (45), whereas L365,260 is a specific CCK2 receptor antagonist, and both interact with p38 MAP kinase. SB202190 and SB203580 interact specifically with the CCK1 receptor but not with the CCK2 receptor. According to docking experiments, it was suggested that these CCK receptor antagonists could establish hydrogen bonds with Asp-168 and Lys-53, two main amino acids of the ATP binding site of p38 MAP kinase (Fig. 11). These pharmacological studies and molecular modeling experiments suggest that the main interactions of L364,718 and L365,260 reside in the benzodiazepine and amide or urea moieties. Neither the stereochemistry of the chiral carbon nor the indole or aromatic side chains seem to be of great importance for the interaction of these CCK receptor antagonists with p38 MAP kinase.

CONCLUSION

In this study, we have shown that p38 MAP kinase inhibitors such as compounds SB202190 and SB203580 can interact with the CCK1 receptor and act as antagonists at this receptor at doses that are usually used for studying their effect. To our knowledge, this is the first time that these compounds are described as interacting with a G-protein-coupled receptor. These findings might change how these common compounds are used or at the very least how one interprets the results generated when using these compounds. As a future application of these findings, we propose the possible use of SB compounds as "hit compounds" for the design of a new series of CCK receptor antagonists.

Our study has also revealed the possible existence of structural similarities between the CCK1 receptor and p38 MAP kinase. Indeed, CCK receptor antagonists were found to inhibit, although with low potency, p38 MAP kinase activity. These findings suggest that p38 MAP kinase inhibitors could be designed on the model of CCK receptor antagonists.

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