JPET #100172

# Pharmacological Evidence for a Functional Serotonin-2B (5-HT$_{2B}$ )

# Receptor in a Human Uterine Smooth Muscle Cell Line

**Curtis R. Kelly and Najam A. Sharif**

**Molecular Pharmacology Unit**

**Alcon Research, Ltd.**

**Fort Worth, TX 76134**

1

JPET #100172

a)      Running Title: Functional 5-HT$_{2B}$ receptors in human uterine cells

b)

**Correspondence:**
Curtis R. Kelly, Ph.D.
Molecular Pharmacology (R2-43)
Alcon Research, Ltd.
6201 South Freeway
Fort Worth, TX  76134

Tel:  (817) 568-6175
Fax: (817) 615-3400
email:  curtis.kelly@alconlabs.com

c)

Text pages: 19
Tables: 3
Figures: 5
References: 33
Abstract Words:  238
Introduction Words: 560
Discussion Words:  1398

d)

**Nonstandard abbreviations:** 5-HT, 5-hydroxytryptamine; $[Ca^{2+}]_i$, intracellular calcium; DMEM, Dulbecco's modified Eagle's medium; FLIPR, fluorometric imaging plate reader; HUSMC, human uterine smooth muscle cells; PI, phosphoinositide

e)

**Section assignment:**  Cellular & Molecular

2

## Abstract

The present study investigated the serotonin-induced increase in phosphoinositide hydrolysis and mobilization of intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) in human uterine smooth muscle cells (HUSMC) in order to identify the serotonergic receptor positively coupled to phospholipase C in these cells. In PI assays, 5-HT and $\alpha$-methyl-5-HT were potent, full agonists ($EC_{50}$ = 20 and 4.1 nM, respectively) while the phenylethylamine, R-DOI was less active ($EC_{50}$ = 63 nM). Proposed 5-HT$_{2B}$ selective agonists, BW-723C86 and (+)-norfenfluramine exhibited strong agonist potency and efficacy comparable to 5-HT ($EC_{50}$ = 18 and 33 nM, respectively) and ~15-fold more potent than (–)-norfenfluramine ($EC_{50}$ = 500 nM). 5-HT$_{2C}$ receptor agonists mCPP and MK-212, were weak agonists in these cells with potencies of 110 and 880 nM, respectively. A similar rank order of potency was observed in $[Ca^{2+}]_i$ mobilization assays (r = 0.9, p < 0.005) in the HUSMC and with contraction of rat stomach fundus strips that contain a 5-HT$_{2B}$ receptor (r = 0.9, p< 0.001). Antagonist studies revealed that a 5-HT$_{2B}$-selective antagonist, RS-127445 ($K_i$ = 0.13 nM), was significantly more effective at inhibiting 5-HT-induced activity than a 5-HT$_{2A}$ antagonist, M-100907 ($K_i$ = 914 nM) and the 5-HT$_{2C}$ antagonists RS-102221 ($K_i$ = 2.5μM) and SB-242084 ($K_i$ = 42.4 nM) in the HUSMC PI turnover assays. Taken together, these studies strongly suggest the presence of a functionally active 5-HT$_{2B}$ receptor subtype in HUSMC. The physiological role of this receptor in these cells remains to be defined.

3

JPET #100172

## Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is known to exert a wide variety of physiological actions through its interaction with an extensive family of 5-HT cell-surface receptors (Hoyer et al., 1994; 2002). In particular, the 5-HT$_2$ receptor family comprises three subtypes, 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$, which have been reported to play a major role in a host of central nervous system functions including anxiety, depression, migraine, obesity, and schizophrenia. Due to the involvement of 5-HT$_2$ receptor signaling in these disorders, there has been an increased interest in the therapeutic potential of selective 5-HT$_2$ ligands as antidepressants, anti-obesity drugs, and anxiolytic agents (Hoyer et al., 1994; 2002).

Further studies have identified serotonin in human aqueous humor (Veglio et al., 1998) and functionally coupled 5-HT$_2$ receptors in rat retinal pigment epithelial cells (Osborne et al., 1993) and bovine ciliary epithelium (Inoue-Matsuhisa et al., 2003). These findings suggested a possible role of serotonin and its receptors in aqueous humor dynamics and as a possible target for ocular pathologies. Indeed, it has recently been shown that topical ocular administration of 5-HT$_2$ receptor agonists effectively lowered intraocular pressure in a nonhuman primate model of laser-induced ocular hypertension (May et al., 2003) suggesting a potential utility of 5-HT$_2$ agonists as anti-glaucoma therapeutics. However, the current lack of subtype selective ligands, especially agonists, has made it difficult to ascribe the hypotensive action of these molecules to an individual 5-HT$_2$ receptor subtype.

Recently, the appetite suppressant fenfluramine was withdrawn from the US market due to its association with valvular heart disease (Connolly et al., 1997).

4

Norfenfluramine, the active metabolite of fenfluramine, has been implicated as the

causative agent in the observed valvular hyperplasia due to its apparent activity at the 5-

$HT_{2B}$ receptor (Fitzgerald et al., 2000) though other biological activities of

norfenfluramine have not been ruled out.  Because of the potential for unwanted side

effects using nonselective agonists, there is great interest in developing 5-$HT_2$ receptor

subtype specific therapeutics that are devoid of 5-$HT_{2B}$ receptor agonist activity.

We sought to identify cell systems endogenously expressing human 5-$HT_2$

receptor subtypes in order to characterize compounds of interest at these therapeutically

important receptors.  Although compound characterization using cell types with

endogenous receptors can be confounded by competing activities at other receptor types,

the resulting pharmacology at physiological expression levels and with the native G-

protein coupling system may give a more relevant profile than the use of cell lines over-

expressing receptors and/or promiscuous G-protein systems.  The 5-$HT_2$ receptor

subtypes are implicated in serotonin-induced smooth muscle contraction and resulting

constriction presumably through $G_q$-coupled activation of phospholipase C,

phosphoinositide (PI) hydrolysis and intracellular calcium mobilization.  For example, 5-

$HT_{2A}$ receptors have been identified in vascular smooth muscle tissue and cells where

activation of 2A receptors induces tissue contraction and vasoconstriction.  5-$HT_{2B}$

receptors are highly expressed in rat stomach fundus and contraction of rat fundic strips is

a prototypic assay used to characterize functional agonist activity of compounds at the

$5HT_{2B}$ receptor (Baxter et al., 1994).  We tested commercially available cell lines for PI

metabolism in response to 5-HT and other prototypic serotonergic compounds.  Through

our search, we discovered that 5-HT and other agonists induced a reproducible PI

5

turnover response and mobilized intracellular calcium in a human uterine smooth muscle

cell type (HUSMC). In this study, we describe the pharmacological profile of the 5-HT$_2$

receptor present in HUSMC using prototypic serotonergic agonists and reported subtype-

selective 5-HT$_2$ receptor antagonists.

## Methods

### Materials / Chemicals

5-hydroxytryptamine hydrochloride; α-methyl-5-hydroxytryptamine maleate; R-(−)-1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane hydrochloride (R-DOI) and other reagents were purchased from Sigma/RBI (Natick, MA).   6-chloro-2-(1-piperaxinyl)pyrazine (MK212); m-chlorophenylpiperazine (m-CPP); α-methyl-5-(2-thienylmethoxy)-1H-indole-3-ethanamine hydrochloride (BW-723C86) and 8-[5-(2,4-dimethoxy-5-(4-trifluoromethylsulfo-amido)phenyl-5-oxopentyl]-1,3,8-triazaspiro[4.5]decane-2,4-dione hydrochloride (RS-102221) were obtained from Tocris Cookson Inc. (Ellisville, MO) R-(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperidinemethanol] (M100907);  6-chloro-5-methyl-1-[6-92-methylpyridin-3-yloxy) pyridine-3-ylcarbamoyl] indoline (SB-242084); 2-amino-4-(4-fluoronaphth-1-yl)-6-isopropylpyrimidine (RS-127445); R-norfenfluramine and S-norfenfluramine were synthesized by the Medicinal Chemistry department of Alcon Research, Ltd or external contract.  [$^3$H]-myoinositol (18.5 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA).  Formic acid, ammonium formate, and LiCl were obtained from Sigma Chemical (St. Louis, MO); AG-1X8 anion-exchange resin and columns were from Bio-Rad (Hercules, CA). Ecolume scintillation cocktail was obtained from ICN Biomedicals (Costa Mesa, CA). Fluorometric imaging plate reader (FLIPR) and calcium assay kit dyes were from Molecular Devices Corp. (Sunnyvale, CA).

### Cell Culture

JPET #100172

Human uterine smooth muscle cells (HUSMCs), catalog #CC-2562, were obtained from

Cambrex (Walkersville, MD). Cell culture media, antibiotics, and trypsin-EDTA were

purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was obtained from

Hyclone Laboratories (Logan, UT), heat-inactivated at 56ºC for 30 min and stored at -

20ºC. HUSMCs were cultured in Dulbecco's modified Eagle's medium (DMEM)

containing 4.5 g/l glucose, 110 mg/l sodium pyruvate, pyridoxine hydrochloride, and

GlutaMax I, supplemented with 10% fetal bovine serum and 10µg/ml gentamicin sulfate.

## In Vitro Functional Assays

### Phosphoinositide Hydrolysis Assay

Phosphoinositide hydrolysis (PI turnover) assays of phospholipase C activity were

conducted by measurement of the agonist-stimulated production of $[^3H]$-inositol

phosphates from $[^3H]$-myoinositol as previously described (Sharif and Xu, 1996; Griffin

et al., 1998). Briefly, confluent monolayers of human uterine smooth muscle cells were

exposed for 24 - 30 hours to 1 - 1.5 µCi of $[^3H]$-myoinositol (18.5 Ci/mmol) in 0.5 mL of

serum-free media to label cell membrane phospholipids. Cells were rinsed once with

DMEM/Ham's F12 containing 10 mM LiCl before incubation with agonist for 1 hour at

37ºC.

For antagonist experiments, the selected antagonist was preincubated with cells

for 15 minutes prior to addition of agonist and continued incubation for 1 hour at 37ºC.

The reaction was quenched by aspiration of the medium and addition of 1 mL cold 0.1 M

formic acid to lyse the cells. Cell sample processing and separation of $[^3H]$-inositol

phosphates was achieved with anion-exchange chromatography.  Cell lysates were loaded

onto columns containing AG-1X8 anion-exchange resin.  Unincorporated [$^3$H]-

myoinositol was removed by sequential washing with water and 50 mM ammonium

formate.  Total [$^3$H]-inositol phosphates fraction was eluted from the columns with 1.2 M

ammonium formate containing 0.1 M formic acid.  The eluate was collected and mixed

with 15 mL scintillation cocktail and the total [$^3$H]-inositol phosphates was determined

by liquid scintillation counting on a beta counter at ~50% efficiency (LS6000; Beckman

Instruments, Carlsbad, CA).  Apparent inhibition constants ($K_i$) values for the functional

antagonist experiments using phosphoinositide hydrolysis were calculated from the

determined 50% inhibition concentrations ($IC_{50}$s) as described elsewhere (Cheng and

Prusoff, 1973).


**Intracellular Calcium Mobilization Assay**

Intracellular calcium mobilization [$Ca^{2+}$]$_i$ induced by 5-HT and other serotonergic

compounds in HUSMCs was studied using a Fluorescence Imaging Plate Reader (FLIPR)

instrument (Molecular Devices, Sunnyvale, CA)(Schroeder and Neagle, 1996).

Evaluation of the functional agonist activity of test compounds was performed using a

protocol previously described (Kelly et al., 2003; May et al., 2003).  Briefly, confluent

cell monolayers of human uterine smooth muscle cells were trypsinized, pelleted, and

seeded at a density of 20,000 cells per well in black-walled, 96-well tissue culture plates

and grown to confluency.  The fluorescence response of HUSMCs was enhanced by

growing the cells in medium containing 10% dialyzed FBS for 2 - 3 days followed by

incubation in serum-free medium overnight prior to the experiment.  For the experiment,

cells were loaded with a calcium sensitive fluorescent dye provided in a calcium assay kit

(Molecular Devices).  The dye was reconstituted in FLIPR buffer [Hanks' balanced salt

solution buffered with 20 mM Hepes, pH 7.4, 2.5 mM probenecid] and incubated with

cells at 23 ºC for 1 hr.  Test compounds were diluted in 25% dimethyl sulfoxide / 25%

ethanol and further dilutions were prepared in FLIPR buffer and evaluated in

concentration response formats.  Agonist-stimulated intracellular calcium mobilization

was measured on a FLIPR I system monitoring real-time changes in cellular fluorescence

($\lambda_{ex} = 488$ nm, $\lambda_{em} = 540$ nm) upon agonist additions.  Calibration of the instrument was

performed using manufacturer's standard procedures.

## Results

### Phosphoinositide Hydrolysis Agonist Studies

In phosphoinositide hydrolysis (PI turnover) experiments using [$^3$H]-myoinositol,

treatment of human uterine smooth muscle cell (HUSMC) monolayers with 5-HT and

other prototypic serotonergic compounds consistently resulted in a production of [$^3$H]-

inositol phosphates.  This agonist-stimulated generation of inositol phosphates suggested

the presence of $G_q$-coupled 5-HT$_2$ receptors in the HUSMC.  In order to further

characterize the 5-HT$_2$ receptor subtype(s) responsible for the observed PI turnover,

prototypic serotonergic agonists with some reported 5-HT$_2$ receptor subtype selectivity

were analyzed in concentration-response format using the PI turnover assay in HUSMC.

5-HT, nonselective, and reportedly subtype selective 5-HT$_2$ agonists induced the

activation of phospholipase C and subsequent inositol phosphate generation in a

concentration - dependent manner (Figure 1 A and B).  Tested compounds were then

compared and ranked based on the functional potencies determined from the

concentration-response curves (Table 1).  The nonselective natural ligand, 5-HT, was a

potent, full agonist (EC$_{50}$ = 20 ± 2.8 nM, E$_{max}$ = 105 ± 6%) whereas α-methyl-5-HT

exhibited approximately 5-fold stronger potency than 5-HT (EC$_{50}$ = 4.1 ± 2.1 nM, E$_{max}$ =

93 ± 12 %) in the assay.  The tryptamine analog BW-723C86, described as a 5-HT$_{2B}$

selective agonist, exhibited strong agonist potency and efficacy comparable to 5-HT

(EC$_{50}$ = 18 ± 2.5 nM, E$_{max}$ = 104 ± 4 %).  The phenylethylamine ligand R-DOI,

considered to be a relatively selective and potent 5-HT$_{2A}$ receptor agonist, was a full

agonist with a modest potency weaker than 5-HT, α-methyl-5-HT and BW723C86.  The

piperazines mCPP and MK-212 exhibited functional potencies of 110 ± 32 nM and 880 ±

11

JPET #100172

320 nM respectively, with mCPP consistently displaying partial agonist efficacy ($E_{max}$ = 37 ± 5 %).  MK-212 had the weakest potency of the compounds tested.  The (+)- and (–)-isomers of norfenfluramine, the active metabolite of fenfluramine, were tested for agonist activity in PI turnover dose response.  (+)-norfenfluramine displayed very good potency comparable to 5-HT and ~15-fold better than (–)-norfenfluramine (Figure 1B and Table 1).

**Intracellular Calcium Mobilization**

The human uterine smooth muscle cells were tested for mobilization of intracellular calcium in response to 5-HT and selected serotonergic agonists.   As expected, 5-HT at 10μM elicited a rapid increase in relative fluorescence compared to basal fluorescence in HUSMCs indicative of increased intracellular calcium ($[Ca^{2+}]_i$) as monitored in real-time using the FLIPR instrument.  Surprisingly, the relative fluorescence change measuring calcium mobilization in response to 5-HT and other serotonergic compounds was lower than anticipated based on the robust, consistent phosphoinositide metabolism response in these cells.  It was also significantly lower than the 5-HT-induced fluorescence change observed with endogenous 5-HT$_2$ receptors of rat aortic smooth muscle (A7r5) cells (Doyle et al., 1986; May et al., 2003).  The HUSMCs exhibited good viability and adequate loading of the calcium-sensitive dye based on a very robust fluorescence increase in response to 10μM histamine observed in these cells.  The consistency and magnitude of the 5-HT-induced $[Ca^{2+}]_i$ mobilization was improved by growing the cells in media with 10% dialyzed serum for 2 -3 days and then overnight in serum-free medium prior to the experiment.  All serotonergics tested induced increases in relative

12

fluorescence in HUSMC in a concentration-dependent manner (Figure 2; Figures 3A and

3B) and potency and efficacy values were determined from the generated curve fits

(Table 2).  The rank order potencies of the tested compounds were similar to those

observed using PI hydrolysis as the functional readout.  5-HT and α-methyl 5-HT were

the most potent agonists in the calcium assay.  The potencies of BW-723C86 and (+)-

norfenfluramine were weaker than observed in the PI experiments but comparable to the

activity of R-DOI.  MK212, mCPP, and (–)-norfenfluramine were weak agonists for

calcium mobilization as they were for PI hydrolysis ($EC_{50} > 1$ µM).  mCPP showed

partial agonist efficacy in the assay ($E_{max} = 54\%$).

**Phosphoinositide Hydrolysis Antagonist Studies**

To better characterize the 5-HT$_2$ receptor subtype responsible for PI hydrolysis in human

uterine smooth muscle cells, we tested subtype-selective antagonists for the ability to

inhibit the 5-HT-mediated PI metabolism.  The agonist activity of a submaximal

concentration of 5-HT (30 nM) was challenged using increasing concentrations of the

selective 5-HT$_{2A}$ antagonist, M-100907, the 5-HT$_{2C}$ selective antagonists, SB-242084 and

RS-102221, and the 5-HT$_{2B}$ selective antagonist, RS-127445.  All 5-HT$_2$ receptor

antagonists tested inhibited the 5-HT-induced PI hydrolysis in HUSMCs in a

concentration-dependent fashion (Figure 4).  However, there was greater than two orders

of magnitude separation in potency between RS-127445 and the other antagonists tested.

The 5-HT$_{2B}$ antagonist was by far the most potent inhibitor of the 5-HT agonist response,

exhibiting a subnanomolar IC$_{50}$ (Figure 4).  The IC$_{50}$ values derived from the antagonist

concentration response studies were converted into apparent inhibition constant (K$_i$)

values using the Cheng-Prusoff equation (Cheng and Prusoff, 1973) (Table 3).  The 5-$HT_{2C}$ receptor selective antagonists SB-242084 and RS-102221, were much less effective inhibitors in these cells producing apparent $K_i$ values approximately 400-fold and >10,000-fold weaker than the potency of RS-127445, respectively (Table 3).  The potent 5-$HT_{2A}$ receptor antagonist, M-100907, was also a very weak inhibitor of the 5-HT response in this system, $K_i$ = 914 nM, which was nearly 10,000-fold weaker than the potency of the 5-$HT_{2B}$ antagonist.

As mentioned above, the rank order potencies of serotonergic agonists were comparable using either PI hydrolysis or mobilization of intracellular calcium as the functional readout.  A correlation plot of $pEC_{50}$ data for PI turnover (Table 1) against $pEC_{50}$ data for functional $Ca^{2+}$ mobilization (Table 2) shows a high level of agreement (r = 0.9, p< 0.005) between these two sets of data (Figure 5A).  The $[Ca^{2+}]_i$ mobilization $pEC_{50}$ data from the current study in HUSMCs also correlated with previous FLIPR functional data using cloned human 5-$HT_{2B}$ receptors expressed in CHO-K1 cells, r =0.84, p<0.01 (Table 2 and Porter et al., 1999).  The functional PI data ($pEC_{50}$; $pK_i$) obtained with selected agonists in HUSMCs in the present study correlated most strongly with the reported functional data ($pEC_{50}$; $pA_2$) for these same compounds inducing the 5-$HT_{2B}$-mediated contraction of rat stomach fundus tissues obtained by Baxter and coworkers, r = 0.91, p<0.001 (Table 1 and Figure 5B) (Baxter et al., 1994 and Baxter, 1996).

## Discussion

Exposure of human uterine smooth muscle cells to 5-HT and other prototypic serotonergic compounds elicited production of $[^3H]$-inositol phosphates in PI hydrolysis and triggered mobilization of intracellular calcium consistent with the presence of functional $G_q$-coupled 5-HT$_2$ receptors in these cells.

A panel of prototypic serotonergic agonists with reported selectivity at the various 5-HT$_2$ receptor subtypes was profiled using PI turnover and $[Ca^{2+}]_i$ mobilization assays in HUSMC. There is a paucity of available 5-HT$_2$ subtype-selective agonists and the compounds reported in the literature to exhibit selectivity give conflicting results in diverse assay systems. Selectivity is further complicated by observed species-dependent differences in agonist activity (Wainscott et al., 1996). However, the strong potency of reported 2B selective agonists and the rank order profile of agonists from our results suggested a 5-HT$_{2B}$ receptor was present in human uterine smooth muscle cells.

5-HT and $\alpha$-methyl-5-HT were potent, full agonists as measured by PI turnover and $[Ca^{2+}]_i$ mobilization in the uterine cells whilst R-DOI, a prototypic 2A agonist, was less active. Although both 5-HT and $\alpha$-methyl-5-HT are considered nonselective 5-HT$_2$ agonists, previous studies comparing cloned human 5-HT$_2$ receptor subtypes have shown $\alpha$-methyl-5-HT, and to a lesser extent 5-HT, to have a propensity to selectively activate 5-HT$_{2B}$ receptors (Porter et al., 1999 and Jerman et al., 2001). R-DOI exhibited less activity than might be expected if the receptor was a 5-HT$_{2A}$ receptor subtype.

The reportedly 5-HT$_{2C}$ selective piperazines, mCPP and MK-212, exhibited a weak potency in the agonist assays and mCPP consistently produced partial efficacy. Our findings are in good agreement with previous studies showing mCPP to be a weak,

partial agonist at the 2B receptor (Baxter et al., 1994, Porter et al., 1999, Rothman et al., 2000 and Vickers et al., 2001). Others have observed that mCPP displays no intrinsic agonist activity and acts as an antagonist at the cloned human 5-HT$_{2B}$ receptor (Wood et al., 1997 and Thomas et al., 1996). As a weak, partial agonist at the 2B receptor, mCPP may behave as a functional antagonist in some assay systems.

The tryptamine analog, BW-723C86, has been shown to be a selective agonist for rat (Baxter, 1996; Vickers et al., 2001) and recombinant human (Jerman et al., 2001; Porter et al., 1999) 5-HT$_{2B}$ receptors, although others have found BW-723C86 to be non-selective in receptor binding (Knight et al., 2004) and functional (Cussac et al., 2002) assays. We found that BW-723C86 was a potent agonist for PI hydrolysis assay and calcium mobilization in HUSMCs. This result was in contrast to the very weak 5-HT$_2$ agonist activity of BW-723C86 we have observed using the same functional assays in A7r5 rat vascular smooth muscle cells and primary human ocular trabecular meshwork cells (unpublished observations). Similarly, (+)- and (−)-norfenfluramine gave functional profiles consistent with the observed 5HT$_{2B}$-selective potency reported by Porter et al., 1999 at the cloned human 5-HT$_{2B}$ receptor and similar to the activities for the norfenfluramine isomers observed by others in a variety of systems (Fitzgerald et al., 2000, Rothman et al., 2000 and Setola et al., 2003). The high agonist potencies of the BW-723C86 compound and (+)-norfenfluramine exhibited here strongly suggested the presence of a 5-HT$_{2B}$ receptor in the HUSMCs.

A prior study using ketanserin as antagonist suggested 5-HT$_{2A}$ receptors mediate a serotonin-dependent collagenase induction in rat uterine smooth muscle (Rydelek-Fitzgerald et al., 1993) whereas, we find evidence of a 2B receptor in human uterine

16

smooth muscle. It would be interesting to test currently available subtype selective

antagonists for the ability to inhibit 5-HT induction of collagenase in rat uterine cells. It

is unclear if rat and human uterine smooth muscle express different $5-HT_2$ receptor

subtypes or possess both 2A and 2B with distinct functions. Rydelek-Fitzgerald and

coworkers do note that 5-HT induction of collagenase was unique to the uterine smooth

muscle as no such response was observed in rat aortic smooth muscle (Rydelek-

Fitzgerald et al., 1993). In human uterine tissue, $5-HT_{2B}$ receptor mRNA is expressed at

high levels and the receptor was cloned from uterine cDNA libraries (Kursar et al., 1994)

as well as from the human neuroblastoma cell line, SH-SY5Y (Schmuck et al., 1994).

The presence of high levels of $5-HT_{2B}$ receptor mRNA in human uterine tissue supports

our contention that the $5-HT_2$ receptor signal transduction we observe in HUSMCs can be

attributed to the $5-HT_{2B}$ receptor subtype.

5-HT and $\alpha$-methyl-5-HT showed stronger potencies for calcium mobilization

than PI hydrolysis while BW-723C86 and the other agonists tested were stronger in PI

hydrolysis. The reasons for these discrepancies is unclear, though it is tempting to

speculate that the increased potency of the nonselective agonists may involve activity at

other 5-HT receptors or a greater role of these agonists for the influx of extracellular

calcium. Despite these discrepancies, the rank order potencies of agonists derived from

our two functional assays are in close agreement (r = 0.9, p<0.005; Figure 5A). Agonist

functional data from our studies were also compared to existing data in the literature

obtained using rat fundus contraction (Table 1; Baxter et al., 1994) and cloned human 5-

$HT_{2B}$ receptor (Table 2, Porter et al., 1999). For a majority of compounds, the potency

values were increased in the rat tissue contraction assay versus PI hydrolysis (Table 1).

17

This is perhaps not surprising considering the inherent differences in assay conditions,

including readout, tissue vs. cell culture, and potential species differences.  In fact, we

observe differences in absolute potency within calcium mobilization between cloned and

endogenous human 2B receptors (Table 2) possibly due to differences in receptor density

or coupling efficiency.  However, our data here are in good agreement with those

obtained from cloned human $5\text{-HT}_{2B}$ receptors (r =0.84, p<0.01), and from rat stomach

fundus contraction studies (r = 0.91, p<0.001; Figure 5B), where endogenous $5HT_{2B}$

receptors are present.

Subtype-selective antagonists were utilized to more clearly define the $5\text{-HT}_2$

receptor subtype responsible for the observed 5-HT response in HUSMCs.  In contrast to

the lack of selective $5\text{-HT}_2$ agonists, potent subtype-specific antagonists are available and

routinely used to assign pharmacological actions of potential therapeutics to individual

receptor subtypes.  M-100907, a potent and selective antagonist for $5\text{-HT}_{2A}$ receptors

(Kehne et al., 1996) was ineffective as an inhibitor of 5-HT induced phosphoinositide

hydrolysis in HUSMCs ($pK_i$ =6.0).  This is 1000-fold weaker than binding affinities

reported for M-100907 using cloned human $5\text{-HT}_{2A}$ receptor, rat cortex tissues, or other

$5\text{-HT}_{2A}$ systems (Kehne et al., 1996).

Similarly, the $5\text{-HT}_{2C}$ selective antagonists, RS-102221 (Bonhaus et al., 1997) and

SB-242084 (Kennett et al., 1997) weakly inhibited the 5-HT response, exhibiting

potencies substantially less than would be expected from a $5\text{-HT}_{2C}$-mediated response.

Previous studies have identified RS-102221 as a $5\text{-HT}_{2C}$ selective compound with

nanomolar affinity for human $5\text{-HT}_{2C}$ receptor ($pK_i$ = 8.4) as well as nanomolar

antagonist potency in a cell-based microphysiometry assay ($pA_2$= 8.1) (Bonhaus et al.,

1997).  We found RS-102221 to be a very weak inhibitor of 5-HT action in HUSMCs

with a $pK_i$ = 5.6, approximately 300-fold weaker than observed in 5-HT$_{2C}$ systems.   A

second 5-HT$_{2C}$ specific antagonist, SB-242084, was a more effective inhibitor ($pK_i$ =

7.4), yet this potency was 50 – 100-fold weaker than the affinity and potency reported in

5-HT$_{2C}$ systems and correlated very well with binding affinities reported for this

compound at cloned human 5-HT$_{2B}$ receptors ($pK_i$ =7.0) (Kennett et al., 1997).

In contrast, the 5-HT$_{2B}$ receptor antagonist, RS-127445, was an extremely potent

inhibitor of 5-HT-induced PI turnover in HUSMCs ($pK_i$ = 9.9) and these results

compared favorably with previous studies where RS-127445 displayed subnanomolar

affinity at the 5-HT$_{2B}$ receptor ($pK_i$ = 9.5) and potently inhibited both 5-HT-evoked

inositol phosphate formation ($pK_B$ = 9.5) as well as increases in intracellular calcium

($pIC_{50}$ = 10.4) in cells expressing human recombinant 5-HT$_{2B}$ receptors (Bonhaus et al.,

1999).  RS-127445 has demonstrated 1000-fold selectivity for the 5HT$_{2B}$ subtype over

other 5-HT$_2$ subtypes (Bonhaus et al., 1999) and has recently been identified as the most

selective 2B receptor antagonist currently available (Knight et al., 2004).

Taken together, the pharmacological profile of the serotonergic agonists and

subtype-selective antagonists presented here as well as the strong correlation with

existing functional data for these compounds in a variety of assay systems provide

compelling evidence for the presence of a functional 5-HT$_{2B}$ receptor in these cultured

human uterine smooth muscle cells.  The human uterine smooth muscle cells provide a

convenient cell-based system for studying the biochemical and pharmacological

properties of a human, endogenous 5-HT$_{2B}$ receptor and allow for profiling the

bioactivity of compounds of interest.

JPET #100172

# References

Baxter GS, Murphy OE and Blackburn TP (1994) Further characterization of 5-hydroxytryptamine receptors (putative 5-HT$_{2B}$) in rat stomach fundus longitudinal muscle.  *Br J Pharmacol* 112:323-331.

Baxter GS (1996) Novel discriminatory ligands for 5-HT$_{2B}$ receptors. *Behavioural Brain Res* 73:149-152.

Bonhaus DW, Weinhardt KK, Taylor M, Desouza A, McNeeley PM, Szczepanski K, Fontana DJ, Trinh J, Rocha CL, Dawson MW, Flippin LA and Eglen RM (1997) RS-10221: A novel high affinity and selective, 5-HT2C receptor antagonist. *Neuropharmacology* 36:621-629.

Bonhaus DW, Flippin LA, Greenhouse RJ, Jaime S, Rocha C, Dawson M, Van Natta K, Chang LK, Pulido-Rios T, Webber A, Leung E, Eglen RM and Martin GR (1999) RS-127445: a selective, high affinity, orally bioavailable 5-HT$_{2B}$ receptor antagonist. *Br J Pharmacol* 127:1075-1082.

Cheng Y, Prusoff WH (1973) Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* 22:3099-3108.

JPET #100172

Connolly HM, Crary JL, McGoon MD, Hensrud DD, Edwards BS, Edwards WD, Schaff HV. (1997) Valvular heart disease associated with fenfluramine-phentermine. *New Engl J Med* 337:581-588.

Cussac D, Newman-Tancredi A, Quentric Y, Carpentier N, Poissonnet G, Parmentier J-G, Goldstein S and Millan MJ (2002) Characterization of phospholipase C activity at h5-HT$_{2C}$ compared with h5-HT$_{2B}$ receptors: influence of novel ligands upon membrane-bound levels of [$^3$H]-phosphatidylinositols. *Naunyn-Schmiedeberg's Arch Pharmacol* 365:242-252.

Doyle VM, Creba JA, Ruegg UT, and Hoyer D (1986) Serotonin increases the production of inositol phosphates and mobilizes calcium via the 5-HT2 receptor in A7r5 smooth muscle cells. *Naunyn-Schmiedeberg's Arch Pharmacol* 333:98-103.

Fitzgerald LW, Burn TC, Brown BS, Patterson JP, Corjay MH, Valentine PA, Sun J-H, Link JR, Abbaszade I, Hollis JM, Largen BzLZ, Hartig PR, Hollis GF, Meunier PC, Robichaud AJ and Robertson DW (2000) Possible role of valvular serotonin 5-HT$_{2B}$ receptors in the cardiopathy associated with fenfluramine. *Mol Pharmacol* 57:75-81.

Griffin BW, Magnino PE, Pang I-H and Sharif NA (1998) Pharmacological characterization of an FP prostaglandin receptor on rat vascular smooth muscle cells (A7r5) coupled to phosphoinositide turnover and intracellular calcium mobilization. *J Pharmacol Exp Ther* 286:411-418.

JPET #100172

Hoyer, D., Clarke, D.E., Fozard, J.R., Hartig, P.R., Martin, G.H., Mylecharane, E.J., Saxena, P.R. and Humphrey, P.P.A. (1994) VII. International union of pharmacology classification of receptors for 5-hydroxytryptamine (serotonin). *Pharm. Rev.* 46:157-203.

Hoyer, D., Hannon, J.P. and Martin, G.R. (2002) Molecular, pharmacological and functional diversity of 5HT receptors. *Pharmacol. Biochem. Behav*. 71: 533-554.

Inoue-Matsuhisa E, Moroi SE, Takenaka H, Sogo S, and Mano T. (2003) 5-HT$_2$ receptor-mediated phosphoinositide hydrolysis in bovine ciliary epithelium. *J Ocul Pharmacol Ther* 19:55-62.

Jerman JC, Brough SJ, Gager T, Wood M, Coldwell MC, Smart D, and Middlemiss DN (2001) Pharmacological characterisation of human 5-HT$_2$ receptor subtypes. *Eur J Pharmacol* 414:23-30.

Kehne JH, Baron BM, Carr AA, Chaney SF, Elands J, Feldman DJ, Frank RA, Van Giersbergen PLM, McCloskey TC, Johnson MP, McCarty DR, Poirot M, Senyah Y, Siegel BW and Widmaier C (1996) Preclinical characterization of the potential of the putative atypical antipsychotic MDL 100,907 as a potent 5-HT$_{2A}$ antagonist with a favorable CNS safety profile. *J Pharmacol Exp Ther* 277:968-981.

JPET #100172

Kelly CR, Williams GW and Sharif NA (2003) Real-time intracellular $Ca^{2+}$ mobilization by travoprost acid, bimatoprost, unoprostone, and other analogs via endogenous mouse, rat, and cloned human FP prostaglandin receptors. *J Pharmacol Exp Ther* 304:238-245.

Kennett GA, Wood MD, Bright F, Trail B, Riley G, Holland V, Avenell KY, Stean T, Upton N, Bromidge S, Forbes IT, Brown AM, Middlemiss DN and Blackburn TP (1997) SB 242084, a selective and brain penetrant 5-HT$_{2C}$ receptor antagonist. *Neuropharmacology* 36:609-620.

Knight AR, Misra A, Quirk K, Benwell K, Revell D, Kennett G, and Bickerdike M (2004) Pharmacological characterisation of the agonist radioligand binding site of 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors. *Naunyn-Schmiedeberg's Arch Pharmacol* 370:114-123.

Kursar JD, Nelson DL, Wainscott DB and Baez M (1994) Molecular cloning, functional expression, and mRNA tissue distribution of the human 5-hydroxytryptamine$_{2B}$ receptor *Mol Pharmacol* 46:227-234.

May JA, McLaughlin MA, Sharif NA, Hellberg MR and Dean TR (2003)  Evaluation of the ocular hypotensive response of serotonin 5-HT$_{1A}$ and 5-HT$_2$ receptor ligands in conscious ocular hypertensive cynomolgus monkeys.  *J Pharmacol Exp Ther* 306:301-309.

Osborne NN, Fitzgibbon F, Nash M, Liu NP, Leslie R, Cholewinski A. (1993)
Serotonergic, 5-HT$_2$, receptor-mediated phosphoinositide turnover and mobilization of
calcium in cultured rat retinal pigment epithelium cells. *Vision Res* 33:2171-2179.

Porter RHP, Benwell KR, Lamb H, Malcolm CS, Allen NH, Revell DF, Adams DR, and
Sheardown MJ (1999) Functional characterization of agonists at recombinant human 5-
HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors in CHO-K1 cells. *Brit J Pharmacol* 128:13-20.

Rothman RB, Baumann MH, Savage JE, Rauser L, McBride A, Hufeisen SJ, and Roth
BL (2000) Evidence for possible involvement of 5-HT$_{2B}$ receptors in the cardiac
valvulopathy associated with fenfluramine and other serotonergic medications.
*Circulation* 102:2836-2841.

Rydelek-Fitzgerald L, Wilcox BD, Teitler M, and Jeffrey JJ (1993) Serotonin-dependent
collagenase induction in rat myometrial smooth muscle cells: mediation by the 5-HT$_2$
receptor. *Mol Cell Endocrinol* 91:67-74.

Schmuck K, Ullmer C, Engels P, and Lubbert H (1994) Cloning and functional
characterization of the human 5-HT$_{2B}$ serotonin receptor. *FEBS Lett* 342:85-90.

Schroeder KS and Neagle BD (1996) FLIPR: A new instrument for accurate, high
throughput optical screening. *J Biomol Screen* 1:75-80.

Setola V, Hufeisen SJ, Grande-Allen KJ, Vesely I, Glennon RA, Blough B, Rothman RB and Roth BL (2003) 3,4-Methylenedioxymethamphetamine (MDMA, "Ecstasy") induces fenfluramine-like proliferative actions on human cardiac valvular interstitial cells in vitro. *Mol Pharmacol* 63:1223-1229.

Sharif NA & Xu SX (1996) Pharmacological characterization of bradykinin receptors coupled to phosphoinositide turnover in SV40-immortalized human trabecular meshwork cells. *Exp Eye Res* 63, 631-637.

Thomas DR, Gager TL, Holland V, Brown AM and Wood MD (1996) m-Chlorophenylpiperazine (mCPP) is an antagonist at the cloned human 5-HT$_{2B}$ receptor. *NeuroReport* 7:1457-1460.

Veglio F, DeSanctis U, Schiavone D, Cavallone S, Mulatero P, Grignolo FM and Chiandussi L. (1998) Evaluation of serotonin levels in human aqueous humor. *Ophthalmologica* 212:160-163.

Vickers SP, Easton N, Malcolm CS, Allen NH, Porter RH, Bickerdike MJ, and Kennett GA (2001) Modulation of 5-HT$_{2A}$ receptor-mediated head-twitch behaviour in the rat by 5-HT2C receptor agonists. *Pharmacology, Biochemistry and Behavior* 69:643-652.

Wood MD, Thomas DR, Gager TL, Browne MJ, Carey JE, Chambers JK, Gale DG, Hastwell C, Muir AI, Pritchard TR, Robinson JH, Wilson S, and Brown AM (1997)

JPET #100172

Pharmacological characterisation of the human 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors in functional studies. *Pharmacology Reviews and Communications* 9:259-268.


Wainscott DB, Lucaites VL, Kursar JD, Baez M and Nelson DL (1996) Pharmacologic characterization of the human 5-hydroxytryptamine$_{2B}$ receptor:  evidence for species differences. *J Pharmacol and Exp Ther* 276:720-726.

26

## Legends for Figures

Figure 1.  Concentration-dependent phosphoinositide hydrolysis and subsequent $[^3H]$-inositol phosphates accumulation induced by 5-HT and other serotonergic agonist compounds in human uterine smooth muscle cells. (A)  (■) serotonin (5-HT); (●) α-methyl-5-HT; (□) BW-723C86; (○) R-DOI. (B)  (▼) (+)-norfenfluramine; (▽) (–)-norfenfluramine; (▲) MK-212; (△) mCPP.  Responses induced by the test compounds were represented as a percentage of the maximal response generated by 10μM 5-HT. Data are mean ± S.E.M. from n ≥ 3 independent experiments.

Figure 2.  Concentration-dependent mobilization of $[Ca^{2+}]_i$ in response to 5-HT in human uterine smooth muscle cells.  Representative fluorescence traces of real-time $[Ca^{2+}]_i$ mobilization in HUSMCs loaded with a fluorescent $Ca^{2+}$-sensitive dye and exposed to test compounds for 180 seconds.  Trace data is from a single representative experiment that was repeated at least three times.  Each data point of the trace is the average fluorescence change from multiple wells.

Figure 3.  Concentration-dependent intracellular calcium mobilization induced by 5-HT and other serotonergic agonist compounds in HUSMCs.  (A)  (■) serotonin (5-HT); (●) α-methyl-5-HT; (□) BW-723C86; (○) R-DOI. (B)  (▼) (+)-norfenfluramine; (▽) (–)-norfenfluramine; (▲) MK-212; (△) mCPP.  Responses induced by the test compounds

27

were represented as a percentage of the maximal response generated by 10μM 5-HT.

Data are mean ± S.E.M. from n ≥ 6 independent experiments.

Figure 4.  Concentration-dependent inhibition of phosphoinositide hydrolysis by 5-HT$_2$ receptor subtype-selective antagonists.  After a 15-min preincubation with varying concentrations of antagonist, cells were exposed to a submaximal concentration of 5-HT agonist (30 nM).  (■) RS-127445 (5-HT$_{2B}$); (▲) SB-242084 (5-HT$_{2C}$); (●) M-100907 (5-HT$_{2A}$); (○) RS-102221 (5-HT$_{2C}$).  Values were expressed as a percentage of the response obtained with 30 nM 5-HT alone.  Data are mean ± S.E.M. from n ≥ 3 independent experiments.

Figure 5.  Correlation of the functional agonist and antagonist potencies of selected serotonergic compounds at the putative 5-HT$_{2B}$ receptors in HUSMC from the present study and functional data from contraction assays in rat stomach fundus.  (A) Correlation of agonist potencies determined from the PI turnover assay with agonist potencies determined using the $[Ca^{2+}]_i$ mobilization assay.  The pEC$_{50}$ was calculated as -log of the EC$_{50}$ for each agonist determined in the two assays.  (B) Correlation of functional agonist and antagonist potencies using PI turnover assay in the present study (expressed as pEC$_{50}$; pK$_i$) with rat stomach fundus contraction studies (pEC$_{50}$; pA$_2$) from Baxter et al., (1994) and MDS PanLabs.

Table 1. Functional agonist activity of serotonergic compounds at the 5-HT$_2$ receptor in human uterine smooth muscle cells (Phosphoinositide hydrolysis). Data are mean ± SEM from n ≥ 3 independent PI turnover experiments. Efficacy values (% E$_{max}$) are determined relative to 10µM 5-HT as control.

| Compound | EC$_{50}$ ± SEM (nM) | E$_{max}$ ± SEM (%) | pEC$_{50}$ | Rat Fundus Contraction (pEC$_{50}$)[a] |
|---|---|---|---|---|
| 5-hydroxytryptamine | 20 ± 2.8 | 104.8 ± 5.7 | 7.7 | 8.64 |
| α-methyl-5-HT | 4.1 ± 2.1 | 93.2 ± 11.6 | 8.4 | 8.42 |
| BW-723C86 | 18 ± 2.5 | 104.1 ± 4 | 7.74 | 7.9 |
| R-DOI | 63 ± 41 | 87.8 ± 11.4 | 7.2 | 7.92[b] |
| mCPP | 110 ± 32 | 36.7 ± 4.5 | 6.96 | 7.68 |
| MK-212 | 880 ± 320 | 74 ± 8.4 | 6.06 | 6.43 |
| (+)-norfenfluramine | 33 ± 2.6 | 106.7 ± 5.8 | 7.48 | 7.16[b] |
| (–)-norfenfluramine | 500 ± 100 | 83.8 ± 10.8 | 6.3 | 6.42[b] |
| RS-127445 | K$_i$ ± SEM (nM)  0.13 ± 0.04 | | pK$_i$  9.9 | pA$_2$  9.5[c] |

[a]Rat fundus contraction pEC$_{50}$ values were taken from Baxter et al., 1994 and Baxter, 1996.

[b]Rat fundus contraction values were determined at MDS Pharma Services, Taipei, Taiwan.

[c]Schild regression analysis value for inhibition of contraction obtained from Bonhaus et al., 1999.

Table 2.  Functional agonist activity of serotonergic compounds at the 5-HT$_2$ receptor in human uterine smooth muscle cells (Intracellular Ca$^{2+}$ Mobilization data).  Data are mean ± SEM from n ≥ 6 independent [Ca$^{2+}$]$_i$ mobilization experiments.  Efficacy values (% E$_{max}$) are determined relative to 10μM 5-HT as control.

| Compound | EC$_{50}$ ± SEM (nM) | E$_{max}$ ± SEM (%) | pEC$_{50}$ | Cloned human 5-HT$_{2B}$ [Ca$^{2+}$]$_i$ Mobilization pEC$_{50}$ [a] |
|---|---|---|---|---|
| 5-hydroxytryptamine | 3.2 ± 1.2 | 92.3 ± 4.9 | 8.49 | 8.68 |
| α-methyl-5-HT | 1.6 ± 0.5 | 79.9 ± 3.3 | 8.8 | 8.7 |
| BW-723C86 | 69.4 ± 27 | 84.9 ± 3.7 | 7.16 | 8.97 |
| R-DOI | 72.6 ± 17.4 | 86.1 ± 6.2 | 7.14 | 8.37[b] |
| mCPP | 1110 ± 471 | 54.0 ± 6.6 | 5.96 | 7.2 |
| MK-212 | 1300 ± 236 | 73.4 ± 5.8 | 5.88 | 6.53 |
| (+)-norfenfluramine | 71.8 ± 24.6 | 79.3 ± 6.2 | 7.14 | 8.06 |
| (−)-norfenfluramine | 1660 ± 486 | 84.8 ± 6.7 | 5.78 | 6.9[b] |

[a]Cloned human 5-HT$_{2B}$ FLIPR pEC$_{50}$ data is from Porter et al., 1999 except where noted.

[b]Cloned human 5-HT$_{2B}$ pEC$_{50}$ data determined using the Aequorin screening assay at Euroscreen S.A., Brussels, Belgium.

Table 3.  Inhibition of 5-HT-induced phosphoinositide hydrolysis by 5-HT$_2$ receptor subtype selective antagonists in human uterine smooth muscle cells.   Data are mean $\pm$ SEM from n $\geq$ 3 independent PI turnover antagonist experiments.   A submaximal 5-HT agonist concentration of 30nM was used for the inhibition studies.

| Antagonist Compound | Reported Selectivity | K$_i$ $\pm$ SEM $(nM)$ | $n$ |
|---|---|---|---|
| RS-127445 | 5-HT$_{2B}$ | 0.13 $\pm$ 0.04 | 5 |
| SB-242084 | 5-HT$_{2C}$ | 42.4 $\pm$ 21.3 | 5 |
| M-100907 | 5-HT$_{2A}$ | 914 $\pm$ 155 | 6 |
| RS-102221 | 5-HT$_{2C}$ | 2560 $\pm$ 964 | 4 |

31

Fig. 1A
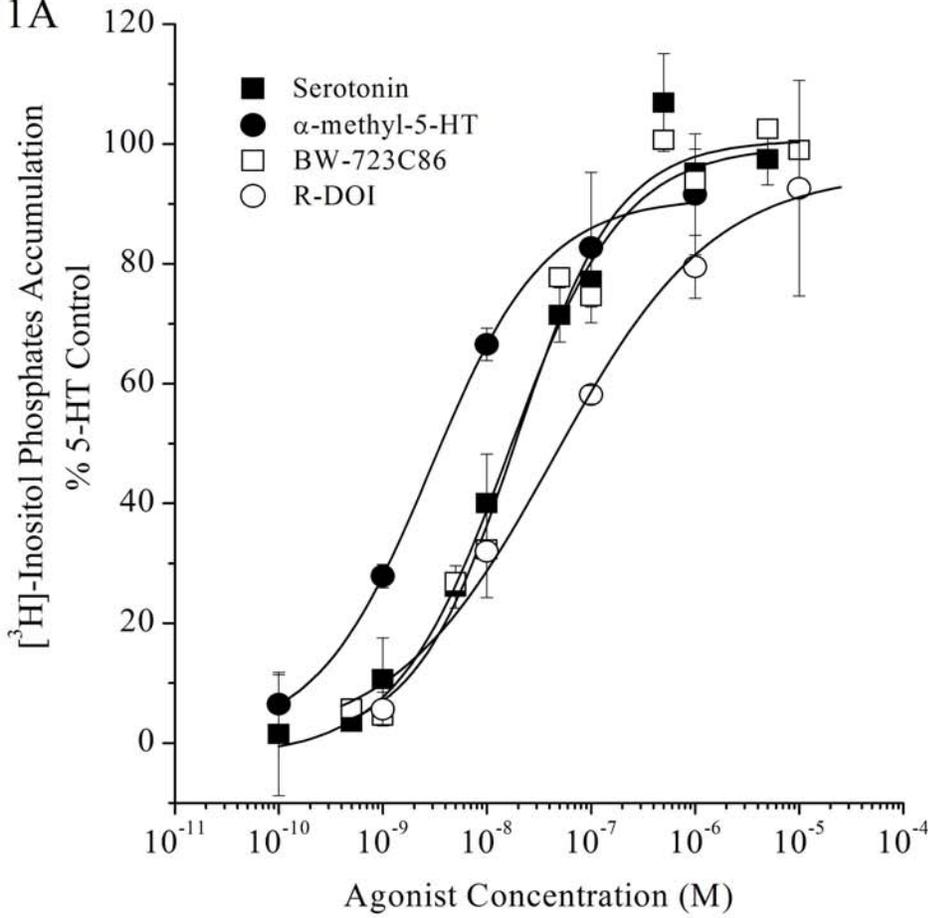


Fig. 1B
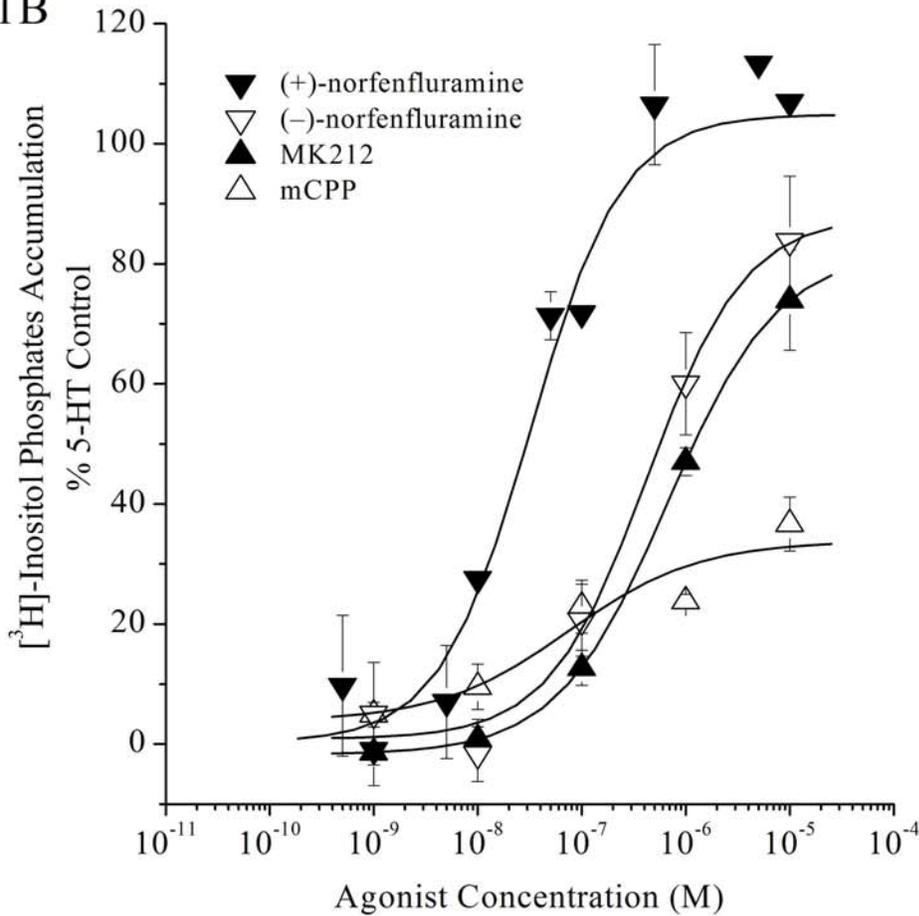
Fig. 2
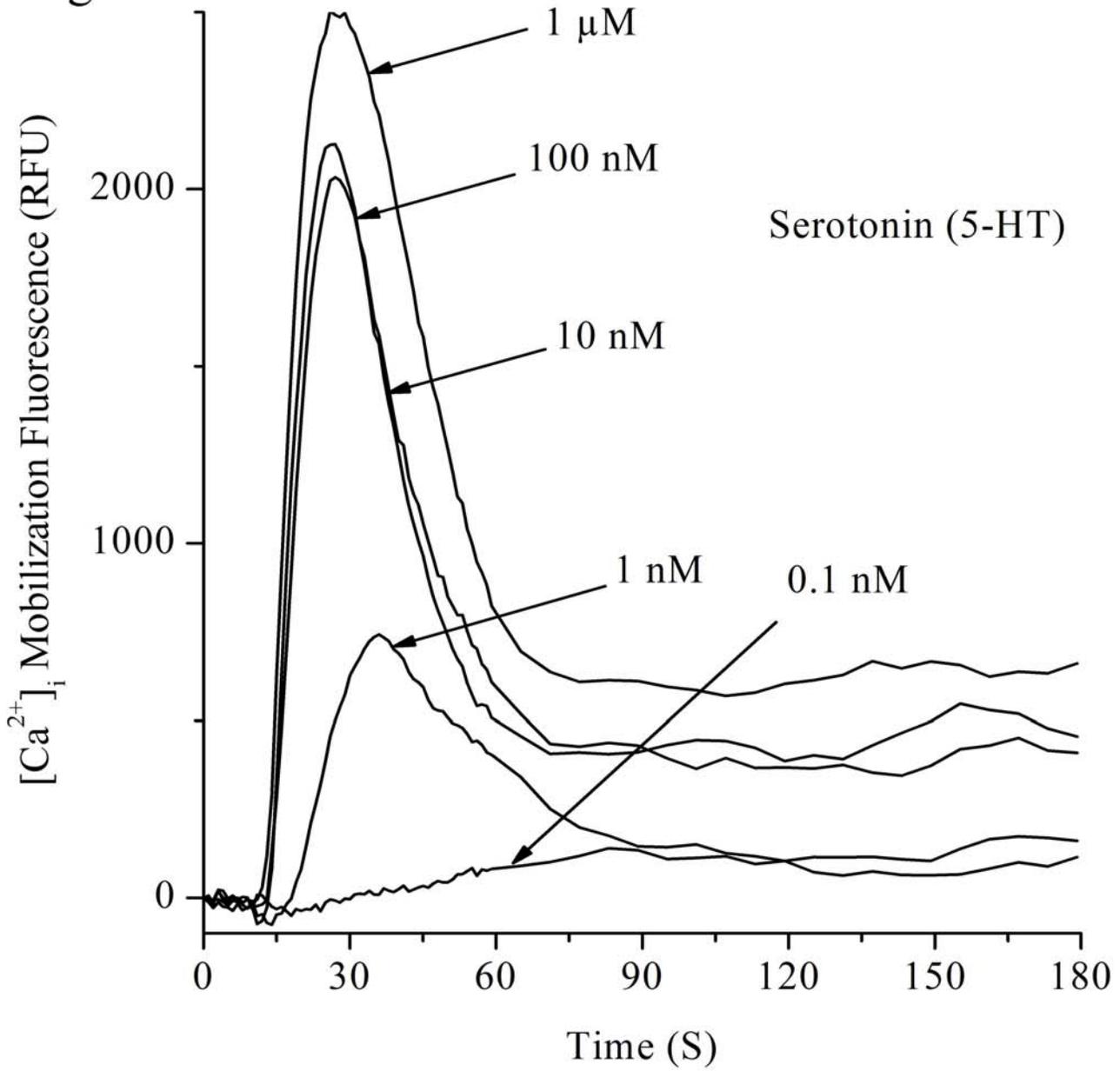
Fig. 3A



Fig. 3B

Fig. 4

Fig. 5A

Fig. 5B