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5-Hydroxytryptamine Type 2A and 2C Receptors Linked to $Na^+/K^+/Cl^-$ Cotransport

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SUMMARY

The goal of the present paper was to investigate 5-hydroxytryptamine $(5HT)_{2A}$ and $5HT_{2C}$ receptor regulation of ion transport in fibroblast cell lines transfected with these receptors. Na^+/K^+-ATPase and $Na^+/K^+/2Cl^-$ cotransport were measured with $^{86}Rb^+$ as a surrogate for K^+ uptake. Serotonin agonists had no effect on Na^+/K^+-ATPase activity in either cell line. Bumetanide, an antagonist of $Na^+/K^+/2Cl^-$ cotransport, almost completely blocked ouabain-insensitive K^+ uptake in both cell lines, with an IC_{50} of about 1 μ M. $^{36}Cl^-$ uptake was 2-fold greater than $^{86}Rb^+$ uptake, consistent with the expected 2:1 stoichiometry. In addition, the Cl^-/HCO_3^- uptake blocker 4,4′-diisothiostilbene-2,2′-disulfonic acid had no effect on Cl^- uptake. The $5HT_{2A/2C}$ receptor agonist (−)-2,5-dimethoxy-4-bromoamphetamine increased ouabain-insensitive K^+ uptake, and this effect was blocked by bumetanide. The receptor antagonists mianserin and mesulergine, but not spiperone, blocked (−)-2,5-dimethoxy-4-bromoamphetamine responses in fibroblasts transfected with $5HT_{2C}$ receptors, and all three antagonists blocked the effects in cells expressing $5HT_{2A}$ receptors. Ouabain-insensitive $^{22}Na^+$ uptake was similarly affected. 5HT receptor-related actions were not observed in untransfected parent NIH/3T3 fibroblasts. Thus, we have demonstrated that $5HT_{2C}$ and $5HT_{2A}$ receptors are linked to activation of $Na^+/K^+/2Cl^-$ cotransport in transfected fibroblasts. This activity may be a factor in the pharmacological actions of 5HT agonists and antagonists.

Serotonin (5HT) receptors are subdivided into seven classes, $5HT_1$ through $5HT_7$, based on amino acid structure and signal transduction pathways. The $5HT_2$ receptor class was recently reclassified (1) and consists of three subtypes, i.e., $5HT_{2A}$ (formerly $5HT_2$), $5HT_{2B}$ (formerly $5HT_{2F}$), and $5HT_{2C}$ (formerly $5HT_{1C}$). These receptors activate the inositol lipid signaling cascade, with a subsequent rise in the intracellular calcium concentration. Receptor binding studies have shown markedly different patterns of distribution for the receptor subtypes. $5HT_{2A}$ receptors are found in highest density in the cerebral cortex, whereas the $5HT_{2B}$ receptor is localized primarily in the stomach fundus. $5HT_{2C}$ receptors are expressed in highest density in the choroid plexus (2), a transport-rich tissue in the brain that controls cerebrospinal fluid formation and composition (3).

We are interested in the function of $5HT_{2C}$ receptors in choroid plexus and have recently focused on the regulation of ion transport (4). $5HT_{2C}$ receptors are expressed in the major operative cell type of the choroid plexus, the epithelium, and recent evidence suggests an apical localization (5, 6). The driving force for cerebrospinal fluid formation is the movement of sodium ions across the choroid plexus epithelium. As a first step in probing the possible role of $5HT_{2C}$ receptors in cerebrospinal fluid secretion, the rat $5HT_{2C}$ receptor was expressed in fibroblasts, to investigate the regulation of sodium transport. Fibroblasts expressing the $5HT_{2A}$ receptor were also examined, to explore the generality of the effects on sodium transport. Two major sodium transport mechanisms were studied, Na^+/K^+-ATPase and $Na^+/K^+/2Cl^-$ cotransport. Evidence for the regulation of Na^+/K^+-ATPase by the phosphoinositide hydrolysis signal cascade is compelling. Lynch *et al.* (7) showed that agonists that increased phosphoinositide hydrolysis, as well as activation of protein kinase C, stimulate Na^+/K^+-ATPase in hepatocytes. In addition, dopamine regulates Na^+/K^+-ATPase in renal proximal tubule cells via products of the phosphoinositide hydrolysis cascade (8, 9). The present studies show that activation of serotonin receptors linked to phosphoinositide hydrolysis does not regulate Na^+/K^+-ATPase but markedly stimulates $Na^+/K^+/2Cl^-$ transport activity.

Materials and Methods

Cell lines. The GF-6 cell line is a clone of NIH/3T3 fibroblasts transfected with $5HT_{2A}$ receptor cDNA, expressing about 10,000 fmol of receptors/mg of protein (10). The AW-1B cell line is a subclone of

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ABBREVIATIONS: 5HT, 5-hydroxytryptamine; DIDS, 4,4′-diisothiostilbene-2,2′-disulfonic acid; KRB, Krebs-Ringer-bicarbonate; KRH, Krebs-Ringer-HEPES; DOB, (−)-2,5-dimethoxy-4-bromoamphetamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

GF-6 cells, expressing a lower $5HT_{2A}$ receptor density (1000 fmol/mg of protein). The B3–1 cell line was prepared as described previously (11), by transfection of 3T3 fibroblasts with $5HT_{2C}$ receptor cDNA (12). The density of $5HT_{2C}$ receptors in these cells was approximately 600–800 fmol/mg of protein. After several months in culture, receptor density increased to 5000 fmol/mg of protein. The transfected cells were maintained in Dulbecco's modified Eagle medium with 10% bovine serum. Untransfected NIH/3T3 cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle medium with 10% serum. All fibroblast cultures were plated in 15-mm-diameter wells (coated with collagen to preserve cell adhesion) and allowed to grow to near confluence. Care was taken to avoid higher cell density, which resulted in sloughing, poor responses to drugs, and decreased intracellular volume.

Experimental protocol. The culture medium in each well was replaced with 1 ml of KRB saline solution (125 mM NaCl, 4.5 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.5 mM Na_2HPO_4, 18 mM $NaHCO_3$, 5 mM glucose). The medium was aerated with 95% O_2/5% CO_2 and the pH was adjusted to 7.35–7.38 at 37° with Tris base, using an Orion model 720A pH meter. In some experiments, 20 mM HEPES, adjusted to pH 7.37, was substituted for bicarbonate and 95% O_2/5% CO_2 (KRH medium). After 10–20 min of equilibration in a Dubnoff metabolic shaker gassed with 95% O_2/5% CO_2, at 37°, the KRB medium was replaced with 0.5 ml of fresh KRB or KRH medium containing 2 μCi of $^{86}Rb^+$ plus the drugs. $^{86}Rb^+$ uptake was assumed to be equivalent to K^+ influx (13). For measurements of $^{22}Na^+$ uptake the KRH medium was modified; the Na^+ concentration was reduced to 10 mM by isoosmotic replacement with *N*-methyl-D-glucamine (120 mM). The pH was adjusted to 8.0 with 20 mM glycylglycine. The reaction was initiated by addition of 4 μCi/ml $^{22}Na^+$ and 1 mM ouabain, unless otherwise noted. For experiments using $^{36}Cl^-$, the Cl^- concentration was 142 mM, with 1 μCi/ml $^{36}Cl^-$, in KRB saline. In all of the protocols triplicate wells were used for each treatment. After incubation for 0.5–10 min the medium was removed simultaneously from each triplicate set of wells with 18-gauge hypodermic tubing connected to a stainless steel manifold, under vacuum. The wells were placed in a crushed ice bath and rapidly washed four times with 3 ml of an ice-cold solution of 120 mM $MgCl_2$, 20 mM LiCl, and 25 mM HEPES, pH 7.4. The wash sequence was complete in 10 sec. Then, 1 ml of a solution of 20 mM NaCl, 5 mM EDTA, 10 mM HEPES, pH 7.4, was added to each well and sonicated at minimum power for 10 sec, with a microprobe (Microson ultrasonic cell disrupter; Heat Systems Inc., Farmingdale, NY). Aliquots of 500–800 μl of the sonicate were used for radioisotope counting and 100–500-μl aliquots were used for fluorometric assay of DNA with ethidium bromide (14). Isotope measurements were normalized to the DNA content of each well (1 μg of DNA corresponds to 30 μg of protein).

For the phosphoinositide hydrolysis assays, cells were labeled overnight with [^{3}H]inositol (1 μCi/ml) in CMRL medium that contains < 0.05 mg inositol/liter. Lithium chloride (10 mM) and pargyline (10 μM) were added 10 min before addition of agonist 5HT or (−)-DOB. Incubation with agonist proceeded for 45 min. The amount of [^{3}H] inositol monophosphate formed was determined by ion exchange chromatography, as described previously (15).

Intracellular volume was measured with 3-*O*-methyl[^{3}H]glucose (16), as previously described (4). Extracellular volume was determined with L-[^{3}H]glucose (17). Radioisotope measurements were made in a Beckman LS-3133 liquid scintillation counter for ^{3}H and a Beckman model 400 γ counter for $^{86}Rb^+$.

Materials. Culture media and bovine serum were obtained from Hyclone Laboratories, Inc. (Logan, UT) or from the Core Culture Facility at Vanderbilt University. Reagent-grade salts for the buffer solutions were obtained from Fisher or Baker. $^{86}Rb^+$, $^{22}Na^+$, and $^{36}Cl^-$ were procured from DuPont-New England Nuclear (Boston, MA). Bumetanide, ouabain, DIDS, 5HT, Tris base, and HEPES were obtained from Sigma Chemical Co. (St. Louis, MO). (−)-DOB was kindly furnished by the National Institute on Drug Abuse. Mianserin, mesulergine, ketanserin, and spiperone were purchased from Research Biochemicals (Natick, MA).

Statistical analysis. The Student *t* test was used when only two sets of data were compared. For multiple columns of normally distributed data a one-way analysis of variance, followed by Bonferroni or Student-Newman-Kuels *post hoc* tests, was used. Nonparametric tests, such as the Mann-Witney *U* test, were applied when data were not normally distributed. All statistical procedures were done with Instat software (GraphPAD, La Jolla, CA). Data are reported as means ± standard errors. Differences between groups with p values of <0.05 were considered significant.

Results

General aspects. The criteria for determining viability of the fibroblast cultures were similar to those previously described for choroid plexus epithelial cells (4). Cultures were used only if the wells contained microscopically homogeneous cell populations grown to partial or near confluence. Little change in morphology or DNA was noted in the successive steps of the experiments. The intracellular volume of fibroblast cells was 0.13 μl/μg of DNA, and the extracellular volume was ≤0.01 μl/μg of DNA. Uptake of the radioisotopes ^{86}Rb, ^{22}Na, and ^{36}Cl was linear for at least 10 min. The concentration of ouabain needed to produce almost complete inhibition of Na^+/K^+-ATPase, 1 mM, appeared to have no toxic effect on morphology or intra- or extracellular volume, provided that the cells were exposed to ouabain for no more than 20 min. (−)-DOB was used as a prototypic 5HT agonist that did not require a monoamine oxidase inhibitor and that had potent and selective actions at $5HT_{2C}$ and $5HT_{2A}$ receptors. An indication that the cells used for the present experiments expressed the appropriate 5HT receptors was their ability to respond to 5HT or DOB with elevated inositol phosphate hydrolysis. The data in Fig. 1 exemplify such a response by B3–1 cells. The EC_{50} for 5HT was 20 nM and that for DOB was 40 nM.

K^+ uptake in fibroblast cell lines transfected with 5HT receptor genes and effects of 5HT agonists. DOB markedly stimulated ^{86}Rb uptake in transfected fibroblast cell lines. The B3–1 line expressing the $5HT_{2C}$ receptor (≈600 fmol/mg of protein) responded to 1 μM DOB with a 50% increase in K^+ uptake (Fig. 2). After 1 mM ouabain the response to DOB

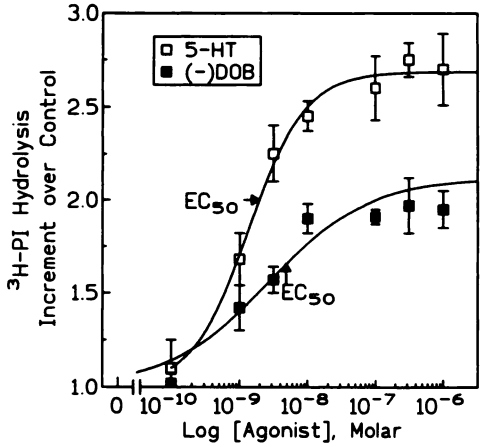

Fig. 1. Inositol monophosphate (*PI*) formation in fibroblasts transfected with the $5HT_{2C}$ receptor gene (cell line B3–1), expressing 600 fmol/mg of protein. The EC_{50} of 5HT was 20 ± 9 nM and that of (−)-DOB was 40 ± 11 nM. Each *point* represents triplicate determinations in each of three experiments. The data were fitted to sigmoid curves with a nonlinear regression program (GraphPAD).

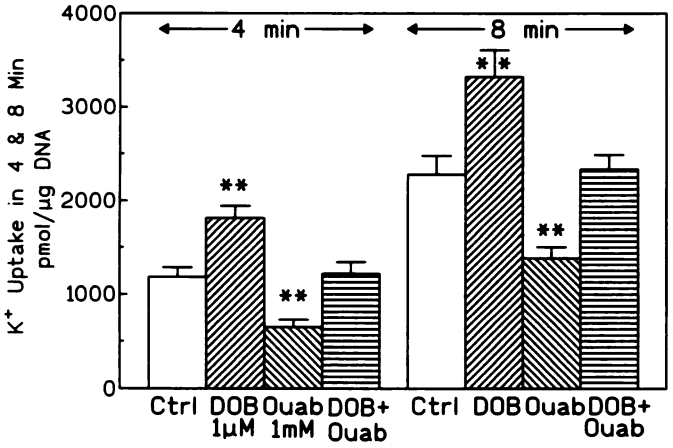

Fig. 2. Stimulation of K^+ (i.e., $^{86}Rb^+$) influx into B3–1 fibroblasts, which express $5HT_{2C}$ receptors (600 fmol/mg of protein). Experiments were terminated after 4 or 8 min, as described in Materials and Methods. The initial velocity of K^+ influx was 292 ± 13 pmol/µg of DNA/min. *Ctrl*, no drugs added. All treatments except control versus DOB plus ouabain (*Ouab*) differed from one another by analysis of variance followed by Bonferroni *post hoc* tests ($p < 0.0001$, nine to 12 experiments for each *bar*). *Error bars*, standard error. **, $p < 0.01$.

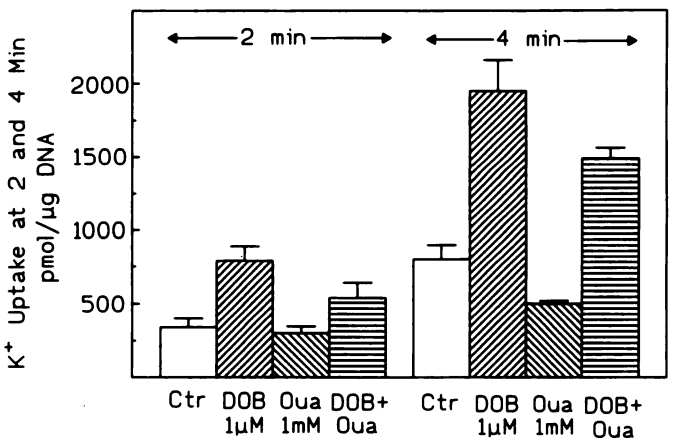

Fig. 4. Stimulation of K^+ influx in GF-6 fibroblasts expressing the $5HT_{2A}$ receptor at a density of about 20,000 fmol/mg of protein. The initial velocity of K^+ influx was 212 ± 16 pmol/µg of DNA/min. Cells were incubated for 2 or 4 min with ^{86}Rb. All treatments differed from one another with $p < 0.01$, except ouabain (*Oua*) versus control (*Ctr*) at 2 min and DOB plus ouabain at 2 min versus ouabain alone at 4 min ($p > 0.05$) (nine to 12 experiments for each *bar*).

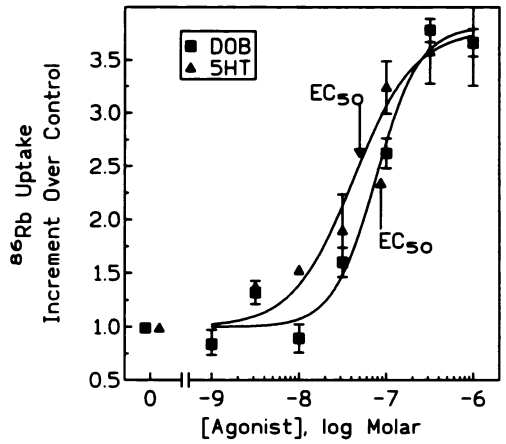

Fig. 3. Concentration-response curves for (−)-DOB and 5HT in experiments with B3–1 cells. Data were obtained after a 4-min incubation in the presence of 1 mM ouabain. Each *bar* represents the mean ± standard error of data obtained in triplicate in each of three experiments. The EC_{50} values were 70 ± 11 nM for DOB and 30 ± 9 nM for 5HT, which were not significantly different.

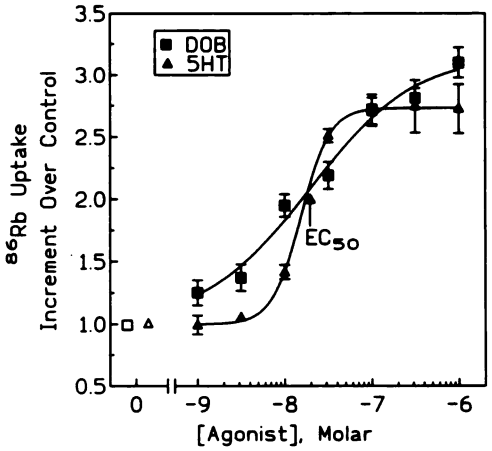

Fig. 5. Concentration-response curves for $^{86}Rb^+$ uptake in GF-6 cells (expressing $5HT_{2A}$ receptors) in response to varying concentrations of 5HT and (−)-DOB. All data were obtained with 4-min incubations in the presence of 1 mM ouabain. Each *point* represents four experiments, with triplicates in each experiment. The EC_{50} values for both agonists were 20 ± 5 nM. Open square and triangle represent controls.

appeared to be attenuated, relative to control, but the fractional increase relative to ouabain alone remained high, because ouabain lowered the base-line value. DOB at 1 µM produced a maximum increase in K^+ influx. The EC_{50} was 20 nM, the same as that observed with 5HT (Fig. 3). The apparent discrepancy in maximum responses to DOB in Figs. 2 and 3 could be attributed to increased $5HT_{2C}$ receptor density that developed in this cell line during the time that elapsed between the two sets of experiments (several months).

With the GF-6 clone, which expresses the $5HT_{2A}$ receptor at high density (20,000 fmol/mg of protein), DOB had effects analogous to those described above (Fig. 4). The magnitude of the response to a saturating concentration of DOB (1 µM) was >2-fold greater in GF-6 cells. The EC_{50} of DOB was 70 nM and that of 5HT was 40 nM (Fig. 5). In a subclone of the GF-6 cell line, AW-1B, DOB elicited a similar response but with a lesser magnitude (data not shown). This was consistent with the lower receptor density in these cells (≈1000 fmol/mg of protein). With both of the $5HT_{2A}$ receptor-transfected cell lines, the response to DOB in the presence of ouabain was more prominent at the longer of the two incubation times used in each set of experiments, because K^+ uptake did not increase with time in the presence of ouabain alone.

Na^+/K^+-ATPase activity can be estimated by the difference between $^{86}Rb^+$ uptake in the absence and presence of ouabain. However, an even more sensitive index is $^{86}Rb^+$ influx in the presence of bumetanide, which eliminates uptake mediated by $Na^+/K^+/2Cl^-$ cotransport. As can be seen in Table 1, DOB had no effect on bumetanide-insensitive $^{86}Rb^+$ uptake in either cell line. Because this remaining uptake is essentially eliminated by ouabain, this suggests that neither the $5HT_{2A}$ nor $5HT_{2C}$ receptor regulates Na^+/K^+-ATPase activity.

Role of 5HT receptors. 5HT antagonists were used to further define the specificity of receptors linked to K^+ influx. With the cell line expressing $5HT_{2C}$ receptors (B3–1), 10 µM mianserin and mesulergine completely blocked the response to

TABLE 1

Bumetanide inhibition of K^+ (^{86}Rb) uptake by cells transfected with the $5HT_{2A}$ or $5HT_{2C}$ receptor gene

Cultures were incubated for 4 min in the absence of ouabain (total activity) or with 1 mM ouabain (ouabain-insensitive activity). The values are means ± standard errors of six to 12 determinations. All treatments differed from control with $p < 0.001$.

Treatment	^{86}Rb uptake	
	Total activity	Ouabain-insensitive activity
	pmol/μg of DNA/min	
B3-1 ($5HT_{2C}$)		
Control	308 ± 9	198 ± 9
DOB, 1 μM	420 ± 24	295 ± 9
Bumetanide, 10 μM	118 ± 4	38 ± 2
DOB + bumetanide	101 ± 6	28 ± 1
GF-6 ($5HT_{2A}$)		
Control	234 ± 16	124 ± 12
DOB, 1 μM	602 ± 38	418 ± 42
Bumetanide, 10 μM	106 ± 15	22 ± 2
DOB + bumetanide	114 ± 14	30 ± 4

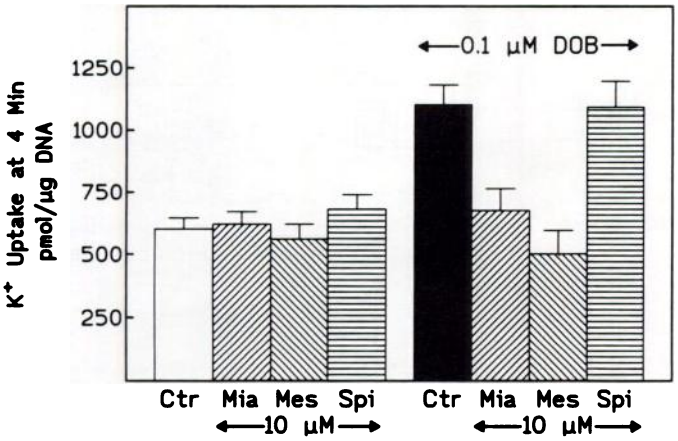

Fig. 6. Actions of 5HT antagonists on K^+ influx into B3–1 cells transfected with the $5HT_{2C}$ receptor gene. The $5HT_{2C}$ antagonists mianserin (*Mia*) and mesulergine (*Mes*) and the $5HT_{2A}$ blocker spiperone (*Spi*), at 10 μM, had no significant intrinsic action on base-line $^{86}Rb^+$ uptake during 4-min incubations in the presence of 1 mM ouabain. The reduction with mesulergine was borderline insignificant ($p = 0.06$). Mianserin and mesulergine (10 μM) completely blocked the stimulatory effect of (−)-DOB on $^{86}Rb^+$ uptake, but spiperone was without effect. Results are from four experiments, with triplicate determinations in each. *Ctr*, control.

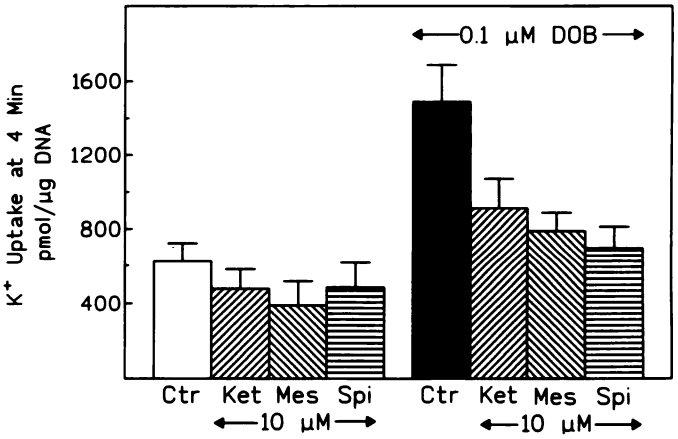

Fig. 7. Actions of 5HT antagonists on K^+ influx into GF-6 cells, which express the $5HT_{2A}$ receptor. The $5HT_2$ antagonists ketanserin (*Ket*), mesulergine (*Mes*), and spiperone (*Spi*) had no inherent activity, but after 0.1 μM (−)-DOB treatment >50% inhibition was observed in 4-min incubations with $^{86}Rb^+$. Ouabain at 1 mM was present in all experiments. *Ctr*, control.

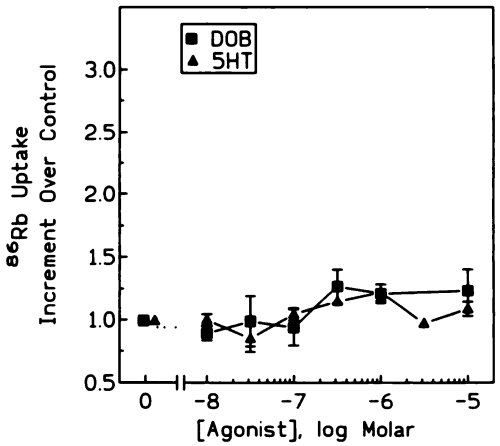

Fig. 8. Concentration-response relationship for (−)-DOB and 5HT effects on $^{86}Rb^+$ uptake by the parent NIH/3T3 fibroblast cell line. No significant effects were observed with the agonists in the range of 10^{-8} to 10^{-5} M after 4-min incubations in the presence of ouabain. Data represent four experiments with 5HT and three with (−)-DOB, with each concentration being assayed in triplicate in each experiment.

0.1 μM DOB without altering basal K^+ uptake (Fig. 6). Spiperone, a $5HT_{2A}$ receptor antagonist, had no effect. In the GF-6 cell line, expressing $5HT_{2A}$ receptors, ketanserin, mesulergine, and spiperone partly inhibited the K^+ uptake response to 0.1 μM DOB, with spiperone producing the largest degree of inhibition (Fig. 7). These data, taken as a whole, indicate that the ouabain-insensitive K^+ uptake by these two distinct cell lines is regulated by specific 5HT receptor subtypes.

Additional evidence that the response to DOB in transfected cell lines was mediated by $5HT_{2C}$ and $5HT_{2A}$ receptors is shown in Fig. 8. The untransformed parent cell line, NIH/3T3 fibroblasts, did not respond to either DOB or 5HT in the range of 10^{-8} to 10^{-5} M, in the presence of 1 mM ouabain.

Characterization of $Na^+/K^+/2Cl^-$ cotransport. The effects of $5HT_{2C}$ and $5HT_{2A}$ agonists and antagonists on K^+ uptake by transformed cell lines in the presence of ouabain are presumptive evidence of an action on $Na^+/K^+/2Cl^-$ cotransport. Bumetanide, an inhibitor of $Na^+/K^+/2Cl^-$ cotransport, reduced both basal K^+ uptake and the response to 1 μM DOB in B3–1 and GF-6 cell lines (Table 1). These effects were associated with the ouabain-insensitive fraction of K^+ influx. The concentration-response relationship for bumetanide is shown in Fig. 9. The IC_{50} values were 400 nM in B3–1 cells and 1 μM in GF-6 cells.

To characterize the ouabain-insensitive transport more thoroughly, the uptake of $^{22}Na^+$ and $^{36}Cl^-$ was investigated. The pH was adjusted to 8.0 and the Na^+ concentration to 10 mM, to eliminate Na^+ entry via the pH-dependent Na^+/H^+ antiport and to obtain sensitivity and linearity. These conditions did not allow calculation of the stoichiometry of the cotransport of Na^+. The rate of $^{22}Na^+$ uptake was linear for 5 min after addition of Na^+. DOB (1 μM) doubled $^{22}Na^+$ influx, and this was totally blocked by bumetanide. Ketanserin similarly blocked the effect of DOB. Neither blocking agent had endogenous activity (Table 2). These effects were ouabain insensitive. Exclusion of K^+ from the medium virtually abolished $^{22}Na^+$

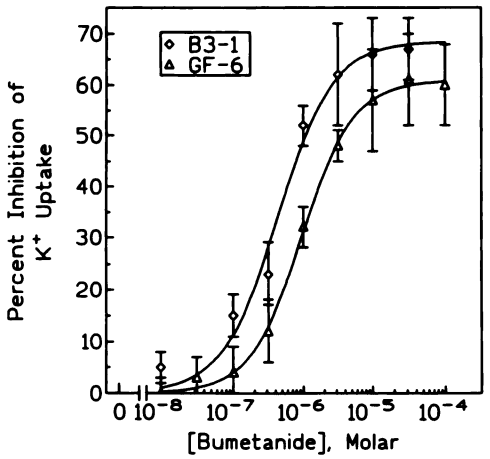

Fig. 9. Inhibition by bumetanide of K^+ uptake by B3–1 (expressing $5HT_{2C}$ receptors) and GF-6 (expressing $5HT_{2A}$ receptors) fibroblast cell lines during 4-min incubations in the presence of 1 mM ouabain. The IC_{50} in B3–1 cells was 400 ± 120 nM and that in GF-6 cells was 1.1 ± 0.2 μM ($p < 0.05$, 12 experiments for each data point).

TABLE 2

$^{22}Na^+$ uptake by fibroblasts transfected with $5HT_{2C}$ receptors (B3-1 cells)

Incubation was for 5 min at pH 8.0, with 10 mM Na^+ and 1 mM ouabain. Values are means ± standard errors of 10–12 measurements in four experiments, with triplicate determinations in each experiment.

Treatment	$^{22}Na^+$ uptake
	pmol/μg of DNA/min
Control	23 ± 2
DOB, 1 μM	49 ± 3[a]
DOB + ouabain, 1 mM	50 ± 9[a]
Bumetanide, 10 μM	18 ± 2
DOB + bumetanide	25 ± 2
Ketanserin, 10 μM	22 ± 2
DOB + ketanserin	26 ± 3
DOB with $[K^+] = 0$	5 ± 2[a]

[a] Different from control, $p < 0.001$.

TABLE 3

$^{36}Cl^-$ uptake by fibroblasts transfected with the $5HT_{2C}$ receptor gene (B3-1 cells)

Data were obtained from experiments in which cells were incubated for 4 min with KRB buffer containing 142 mM Cl^- and 2 μCi/ml ^{36}Cl. Data are means ± standard errors of the number of replicate determinations given in parentheses. B3-1 cells showed a stimulatory effect of DOB ($p < 0.001$) that was blocked by bumetanide ($p < 0.001$).

Treatment	$^{36}Cl^-$ uptake
	pmol/μg of DNA/min
None	460 ± 32 (9)
(−)-DOB, 1 μM	830 ± 26 (6)
Ouabain, 1 mM	489 ± 26 (6)
Bumetanide, 10 μM	263 ± 30 (6)
DOB + bumetanide	337 ± 18 (6)
DOB + DIDS, 30 μM	879 ± 31 (3)

uptake, evidence that supports the premise of a cotransport system.

Measurement of $^{36}Cl^-$ uptake (Table 3) demonstrated a rate approximately twice that of K^+ entry into the B3–1 cells that expressed $5HT_{1C}$ receptors. This indicates a stoichiometry of uptake of 1 K^+:2 Cl^-. $^{36}Cl^-$ uptake was not sensitive to ouabain but was reduced about one half by bumetanide. The observation that DIDS had no effect on Cl^- uptake indicates that there was no inward flux of Cl^- mediated by Cl^-/HCO_3^- exchange.

Discussion

The regulation of Na^+/K^+-ATPase and $Na^+/K^+/2Cl^-$ cotransport by transfected $5HT_{2A}$ and $5HT_{2C}$ receptors was studied as a first step in defining the role of 5HT in choroid plexus. These two transport mechanisms are highly active in the choroid plexus[1] and are involved in cerebrospinal fluid formation (3). The choroid plexus contains a single 5HT receptor subtype, $5HT_{2C}$, that is linked to phosphoinositide hydrolysis (18). This signaling cascade has been shown to control Na^+ transport in other systems (7–9) and could have comparable effects in choroid plexus.

The uptake of K^+ into transfected NIH/3T3 fibroblasts appeared to be mediated entirely by two Na^+-dependent transport mechanisms, Na^+/K^+-ATPase and $Na^+/K^+/2Cl^-$ cotransport. Ouabain inhibited K^+ uptake by 30–40% and the remaining activity was blocked by bumetanide, a furosemide-like diuretic. The discovery of ouabain-insensitive K^+ uptake in a variety of epithelia (19), which was inhibited by furosemide and related drugs, lead to the initial description of $Na^+/K^+/2Cl^-$ cotransport. O'Grady *et al.* (20) proposed criteria for the presence of such a cotransport system, i.e., 1) interdependencies of ion fluxes, 2) ion selectivity, and 3) specificity of pharmacological inhibition by loop diuretics, such as bumetanide, but not by DIDS, an inhibitor of Cl^-/HCO_3^- exchange, or amilorides, inhibitors of Na^+/H^+ antiporters. These criteria were addressed in the present study. The stoichiometry of uptake of $^{86}Rb^+/^{36}Cl^-$ was 1:2. $^{22}Na^+$ uptake was almost completely blocked when K^+ was omitted from the incubation medium. The ouabain-insensitive component of K^+ and Cl^- uptake was essentially eliminated by bumetanide, the most widely used inhibitor of $Na^+/K^+/2Cl^-$ cotransport, whereas DIDS had no effect on Cl^- uptake, suggesting that Cl^- uptake in these cells was not influenced by a Cl^-/HCO_3^- antiport. Thus, ouabain-insensitive and bumetanide-insensitive ^{86}Rb uptake was used to estimate $Na^+/K^+/2Cl^-$ cotransport and Na^+/K^+-ATPase activity, respectively.

5HT and the selective $5HT_{2A/C}$ agonist DOB did not alter the bumetanide-insensitive component of $^{86}Rb^+$ uptake in fibroblasts expressing a high density of either $5HT_{2A}$ or $5HT_{2C}$ receptors, even though both agonists markedly increased phosphoinositide hydrolysis (Ref. 15 and present results). It appears, therefore, that 5HT-mediated phosphoinositide hydrolysis is incapable of activating Na^+/K^+-ATPase in these fibroblast cell lines. In other systems, phosphoinositide hydrolysis-linked receptors and/or activation of protein kinase C regulate Na^+/K^+-ATPase (7, 9, 21). Interestingly, the cloned $5HT_{1A}$ receptor expressed in HeLa cells stimulates Na^+/K^+-ATPase activity (22). Although inhibition of adenylate cyclase is the primary signaling mechanism for the $5HT_{1A}$ receptor, a second pathway, involving calcium-dependent phosphoinositide hydrolysis, is thought to mediate the activation of Na^+/K^+-ATPase. The present results show that stimulation of phosphoinositide hydrolysis is not sufficient to activate Na^+/K^+-ATPase, at least in fibroblasts, suggesting a possible synergistic interaction between signaling mechanisms in cells expressing the $5HT_{1A}$ receptor.

In contrast to the absence of effects on Na^+/K^+-ATPase, 5HT and DOB markedly enhanced $Na^+/K^+/2Cl^-$ cotransport in transfected fibroblasts expressing either $5HT_{2A}$ or $5HT_{2C}$

[1] S. E. Mayer and E. Sanders-Bush, unpublished observations.

receptors. These effects were blocked by appropriate antagonists. Mesulergine, a nonselective $5HT_{2A/2C}$ receptor antagonist, blocked DOB activation of $Na^+/K^+/2Cl^-$ cotransport in both cell lines, whereas spiperone, a $5HT_{2A}$-selective antagonist, blocked activation of $Na^+/K^+/2Cl^-$ cotransport by DOB in cells expressing the $5HT_{2A}$ but not the $5HT_{2C}$ receptor. This activation of $Na^+/K^+/2Cl^-$ cotransport by 5HT may be comparable to that described in other cell lines in which $Na^+/K^+/2Cl^-$ cotransport is regulated by humoral agents (23, 24) in a calmodulin-dependent process (25). 5HT was nearly equipotent in GF-6 and B3–1 cells, which express $5HT_{2A}$ and $5HT_{2C}$ receptors, respectively, even though 5HT has a much lower binding affinity for the $5HT_{2A}$ versus $5HT_{2C}$ receptor (26). The equal potency in our functional analyses suggests a large $5HT_{2A}$ receptor reserve in GF-6 cells. This has been confirmed for the phosphoinositide hydrolysis response, using phenoxybenzamine to inactivate reserve receptors.[1]

In conclusion, 5HT potently regulates $Na^+/K^+/2Cl^-$ cotransport in cells expressing cloned $5HT_{2A}$ or $5HT_{2C}$ receptors, but Na^+/K^+-ATPase is resistant to activation of these receptors. To our knowledge this is the first demonstration of 5HT regulation of $Na^+/K^+/2Cl^-$ cotransport. If this occurs in the choroid plexus, then it may have important implications for the control of cerebrospinal fluid formation.

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