The mRNA Expression of Serotonin 2C Subtype Receptors Uncoupled With Inositol Hydrolysis in NG108-15 Cells

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ABSTRACT—Cell culture systems seem to be useful for clarifying the cellular physiological mechanisms of serotonin 2C subtype receptors (5-HT₂CR) and related drug action mechanisms. However, there are still few reports about cells that contain intrinsic 5-HT₂CR. This report demonstrates by using RT/PCR that 5-HT₂CR mRNA exists in splicing variant forms in NG108-15 cells. The PCR results using a pair of primers that recognized sequences near the third intracellular loop site showed two neighboring bands at about 500 bp upon electrophoresis in acrylamide gels. The sequence analysis demonstrated that one band was the rat 5-HT₂CR sequence and the other one was that of the mouse. Serotonin, however, did not enhance the inositol phosphates formation in NG108-15 cells. It has been reported that post-translational modifications of RNA, splicing and editing, occur at the site of the second intracellular loop domain in 5-HT₂CR mRNA. Accordingly, a pair of primers that recognized this site were designed. The molecular size of the PCR product was shorter than that expected based on the sequence of the native 5-HT₂CR. The fragment lacked the 95 nucleotides of native 5-HT₂CR mRNA. This seems to be the reason why serotonin did not enhance inositol phosphates formation in NG108-15 cells.

Keywords: Serotonin 2C subtype receptor, Inositolphosphate formation, Modification of RNA, RNA editing, NG108-15

The serotonin 2C subtype receptor (5-HT₂CR) is the first cloned one among over 14 subtypes of serotonin receptors (1). 5-HT₂CR is functionally linked to the phosphoinositide (PI) hydrolysis pathway (1) as shown in the *Xenopus* oocytes functional expression system, in which 5-HT₂CR expression caused an outward current of Cl[−] in response to PI. This oocyte system was used to perform 5-HT₂CR cloning (2). 5-HT₂CR is widely distributed throughout the brain (3). The physiological functions of 5-HT₂CR are involved in feeding behavior and epilepsy, as shown by results obtained with knockout mice (4). Pharmacological studies have shown that 5-HT₂CR appears to be the binding site for antipsychotic drugs (5), hallucinogens (6) and antidepressants (7, 8). We also reported that repeated treatment with antidepressants stimulates the expression of 5-HT₂CR mRNA in the rat brain (9, 10). To clarify the detailed mechanisms of these pharmacological actions, cultured cell systems that intrinsically express 5-HT₂CR

would be useful. There is only one report (11) mentioning the endogenous expression of 5-HT₂CR in cells (Balb/c-3T3 cells), and the amount of 5-HT₂CR in those cells was smaller than that of serotonin 2A subtype receptor (5-HT₂AR).

In this study, we found that 5-HT₂CR mRNAs are expressed in NG108-15 cells, hybrid cells of mouse neuroblastoma and rat glioma, although PI turnover was not stimulated by serotonin.

MATERIALS AND METHODS

Cell culture

NG108-15 cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing HAT (100 μ M hypoxanthine, 1 μ M aminopterin and 16 μ M thymidine) and 5% fetal bovine serum at 37°C in a humidified atmosphere of 90% air, 10% CO₂. The culture dishes (35 mm) were coated with 0.1 mg/ml polyornithine (polymerization about 50) in 150 mM borate buffer (pH 8.4) to prevent detachment of the cells from the dishes.

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Isolation of RNA

Total RNA from the cells was isolated by the methods of Chomczynski and Sacchi (12). The medium was removed from the 35-mm dish. The cells were washed once with phosphate buffered saline (PBS) and then 1 ml of Isogen[®] (Nippon Gene, Toyama) was added per dish. The cell lysate was transferred to a microtube, added 0.2 ml CHCl₃, and then centrifuged. The resultant aqueous phase was precipitated with 0.5 ml isopropanol. The pellet was used as total RNA after washing with 70% ethanol.

RT/PCR

The total RNA (2 μ g) was incubated with oligo(dT) primer and superscript II (Gibco, Rockville, MD, USA) in a 20 μ l reaction mixture to make single-stranded complementary DNA (ss-cDNA). The PCR was carried out in a 10- μ l reaction mixture, containing 1 μ l of template ss-cDNA, 1 μ M each of forward and reverse 20-mer primer with 50% GC content (T_m = 55.4°C), dNTPS (2.5 mM) and 2 units of Taq DNA polymerase (Promega, Madison, WI, USA) in the reaction buffer with 2.5 mM MgCl₂. The primer sets for detection around the third intracellular loop site of 5-HT₂CR are 5'-TTA CTT CgA ggT CAC ACC gA-3' (forward) and 5'-TAA CAT TgA gCT CCC TCC CA-3' (backward) to produce a product of about 500 base pairs. Primers for around the second intracellular loop site of 5-HT₂CR are 5'-Tgg CAg TAA gCA Tgg AgA Ag-3' (forward) and 5'-gAC CCg ATg AgA ACg AAg TT-3' (backward) to produce an approximately 430 base pair product of the 5-HT₂CR long variant sequence. Thermocycling was performed as follows: 1) 94°C for 10 min; 2) the indicated number of cycles of 94°C for 1 min, 56°C for 2 min and 72°C for 2 min; and then 3) 72°C for 10 min, followed by cooling to 4°C. The products were resolved by 6% polyacrylamide gel electrophoresis and stained with SYBR gold[®] (Molecular Probes, Inc., Eugene, OR, USA).

Sequence of the isolated cDNA

The cDNA bands amplified by PCR were cut out of the gels under UV transillumination. Each gel slice was heated to 90°C in 100 μ l of water for 12 min. Using the supernatant as template, reamplifying PCR was carried out with the same pair of primers used in the initial reaction. The amplified DNA fragments were purified by 0.6% agarose gel electrophoresis and subcloned into the pBluescript II KS(-) vector/EcoRV site after T4 DNA polymerase treatment. The resultant vector was transfected into competent DH5a cells (Toyobo, Tsuruga) to isolate and to amplify the clone. Finally, the isolated clone was sequenced using a Hitachi-5500 sequencer (Hitachi, Tokyo).

Quantification of inositol phosphates

When the cells reached subconfluency, the culture medi-

um was replaced by fresh Dulbecco's modified Eagle's medium containing HAT as well as [³H]inositol (74 kBq/35-mm-diameter dish) instead of unlabeled inositol, and the cultivation was continued for 17 h. After one wash with Hepes buffered saline (HBS), cells labeled with [³H]inositol were preincubated with 950 μ M HBS containing 10 μ M LiCl for 5 min at 37°C, and then 50 μ l of 20-fold concentrated 5-HT or bradykinin was added, and the mixtures were then further incubated for 30 s at 37°C. The reaction was terminated by addition of 1 ml of 20% trichloroacetic acid, and the supernatant was washed twice with 2 ml of water-saturated ether to remove trichloroacetic acid from the solution. The resultant solution was applied to a column (0.6 $\times$ 4.0 cm) of Dowex I $\times$ 8 (formate form, 200 – 400 mesh). After inositol and glycerophosphoinositol were eluted with 16 ml of 5 mM sodium tetraborate/60 mM ammonium formate, inositol phosphates were eluted sequentially by an ammonium formate step-gradient in 0.1 M formic acid as follows: three washes with 4 ml each of 0.2 M for inositol monophosphate (IP₁) three washes with each 4 ml of 0.4 M for inositol bisphosphate (IP₂) and 4 ml of 1.0 M for inositol trisphosphate (IP₃). The radioactivity of each fraction (totally 7 fractions for each sample) was measured after addition of the scintillation cocktail, Clear-sol[®] (Nacalai Tesque, Kyoto). The blank values which were obtained from the same volume of elution solution were subtracted from each value, and three values in the case of IP₁ and IP₂ were added up to result. Statistical significance was assessed by Student's *t*-test.

RESULTS

The total RNA isolated from confluent NG108-15 cells was used to perform RT/PCR. The PCR products amplified using the primer set for the third intracellular loop site of 5-HT₂CR showed a double band at about 500 bp in polyacrylamide gel electrophoresis (Fig. 1A). The PCR products were not amplified from contaminating genomic DNA, since they were not detected in samples without RT reaction (Fig. 1A, RNA 1) even from 20-fold concentrated samples without RT (Fig. 1A, RNA 20). Each band was subjected to reamplification by PCR and the resultant products also showed a small difference in size (Fig. 1B). These two PCR products were subcloned and subjected to nucleotide sequencing. The sequencing results showed that the longer product had the same sequence as the rat 5-HT₂CR sequence (accession No. M21410), while the sequence of the shorter product was identical with that of mouse 5-HT₂CR (M63685) (Fig. 2A). As shown in Fig. 2A, the length of the reported mouse sequence is three bases shorter than the rat sequence in the third intracellular loop site. The sequencing gel electrophoresis image also showed a three-base difference at this site (Fig. 2B). These results

Fig. 1. The PCR detection of 5-HT₂CR mRNA in NG108-15 cells. RNAs were isolated from NG108-15 cells by the acidic phenol method. RT/PCR was performed using a primer set that detected a region around the third intracellular loop of 5-HT₂CR (sscDNA). A) Two bands were seen near the 527-bp molecular marker (M). To certify that there was no contamination of genomic DNA in RNA samples, PCR was performed without RT. The same amount (1) and a 20-fold greater amount (20) of RNA was subjected to the PCR. B) The two products obtained by PCR shown in A were recovered from the gel individually and reamplification by PCR was carried out.

indicate that the both mouse and rat 5-HT₂CR are expressed in NG108-15 cells.

It is well known that 5-HT₂CR mediates PI turnover. Therefore the synthesis of inositol phosphates induced by 5-HT stimulation in NG108-15 cells was examined. Contrary to our expectation, inositol phosphate formation was not enhanced by 5-HT treatment (Fig. 3).

The short variant and the substitution of some bases in the structure around the second intracellular loop site of 5-HT₂CR have been reported (13). To examine this region, we designed an appropriate primer set to detect the second intracellular loop site and performed RT-PCR. The RT/PCR product from the rat brain total RNA consisted of one major band on polyacrylamide gel electrophoresis; this band was about 430-bp long, which was the expected size according to the reported 5-HT₂CR sequence (Fig. 4, lane Rat). On the other hand, the RT/PCR product from the RNA of NG108-15 cells showed a shorter main band at around 340 bp (Fig. 4, lane NG). This shorter band was isolated, reamplified and sequenced as in the case of the third intracellular loop domain described above. The sequence analysis revealed a 95-nucleotides (nt) deletion in this PCR product compared to that from rat brain (Fig. 5).

DISCUSSION

NG108-15 cells intrinsically express some receptor

subtypes that stimulate PI turnover, including receptors of bradykinin B₂ (14), histamine H₁ (15), purinergic P₂ (16), opiate (17), endothelin (18) and angiotensin (19). Since the intracellular signaling systems of PI turnover in NG108-15 are highly developed, the cell is often used to study the cellular physiological functions of inositolphosphate and related systems (20, 21), and it is used as a host cell for transfection studies of PI turnover-coupled receptors (22, 23). The 5-HT₂CR are of the PI turnover-coupled type, and in this report the mRNA for these receptors was detected by RT/PCR in NG108-15 cells. Contaminating genomic DNA did not affect the detection, because PCR performed without RT did not amplify the 5-HT₂CR band (Fig. 1). Although 5-HT₂CR mRNA exists in NG108-15 cells when a primer set detecting around the third intracellular domains was used, PI turnover was not enhanced by the addition of 1 to 1,000 μ M 5-HT. There are some possible reasons for this. One possibility is that other categories of 5-HT receptors possessing in NG108-15 cells, such as 5-HT₃ (24) and 5-HT₇ (25, 26), inhibit PI turnover by raising intracellular Ca²⁺ or cGMP concentrations. We also detected 5-HT_{1A} mRNA, but not 5-HT_{2A} mRNA, by the same methods as used in this report (data not shown). The possibility that other types of 5-HT receptors may contribute to inhibition of PI turnover may be denied from our preliminary results that 1-(*m*-chlorophenyl)piperazine (*m*-CPP), a selective agonist of 5-HT₂CR, could not enhance the intra-

A)

1) tttct gaactgctgc tgcaagaaga atggtggtga
 2) tttct gaactgctgc tgcaagaaga atggtggtga
 3) tttct aaagtgctgc tgcaagaag ggtgatga
 4) tttct aaagtgctgc tgcaagaag ggtgatga

ggaagagaac gctccgaacc ctaatccaga tcagaaacca cgtcgaaaga
 ggaagagaac gctccgaacc ctaatccaga tcagaaacca cgtcgaaaga
 ggaagagaac gctcccaacc ccaatccaga tcagaagcca cgtcgaaaga
 ggaagagaac gctcccaacc ccaatccaga tcagaagcca cgtcgaaaga

agaaagaaaa gcgtcccaga ggcaacatgc aagctatcaa caacgaaaag
 agaaagaaaa gcgtcccaga ggcaacatgc aagctatcaa caacgaaaag
 agaaagaaaa gcggcctaga ggcaacatgc aagctatcaa caatgagaag
 agaaagaaaa gcggcctaga ggcaacatgc aagctatcaa caatgagaag

aaagcttcca aagtccttgg cattgtattc tttgtgtttc tgatcatgtg
 aaagcttcca aagtccttgg cattgtattc tttgtgtttc tgatcatgtg
 aaagcttcca aagtccttgg cattgtattc tttgtgtttc tgatcatgtg
 aaagcttcca aagtccttgg cattgtattc tttgtgtttc tgatcatgtg

gtgcccgttt ttcataccca atatcctgtc ggttccttgt gggaaggcct
 gtgcccgttt ttcataccca atatcctgtc ggttccttgt gggaaggcct
 gtgcccgttt ttcataccca atatcctgtc ggttccttgt gggaaggcct
 gtgcccgttt ttcataccca atatcctgtc ggttccttgt gggaaggcct

gtaacaaaaa gctaattggag aagcttctca atgtgtttgt gtggattggc
 gtaacaaaaa gctaattggag aagcttctca atgtgtttgt gtggattggc
 gtaacaaaaa gctaattggag aaacttctca atgtgtttgt ttggattggc
 gtaacaaaaa gctaattggag aaacttctca atgtgtttgt ttggattggc

tatgtgtgtt caggcatcaa tcctctggtg tacactctct ttaataaaat
 tatgtgtgtt caggcatcaa tcctctggtg tacactctct ttaataaaat
 tatgtgtgtt caggcatcaa tcctctggtg tacactctct tcaacaaaat
 tatgtgtgtt caggcatcaa tcctctggtg tacactctct tcaacaaaat

B)

Fig. 2. Sequence analysis of the PCR products. A) The sequences are 1) rat 5-HT₂CR cDNA reported by Julius et al. (M21410) (ref. 33), 2) high molecular weight band amplified from NG108-15 cell RNA (shown in Fig. 1B, lane H), 3) low molecular weight band amplified from NG108-15 cell RNA (shown in Fig. 1B, lane L) and 4) mouse 5-HT₂CR cDNA sequence reported by Yu et al. (M63685) (unpublished). The transmembrane domains are underlined. These sequences correspond to the area between the third and fourth intracellular domains of 5-HT₂CR. Notice that the mouse sequence is three bases shorter than the rat sequence. B) Sequencing gel images showing the three-base deletion in mouse 5-HT₂CR cDNA. The upper image is from the high molecular weight band from NG108-15 cells and the lower is from the low molecular weight band. The bases "att" (indicated by a bar) can not be seen in the lower image.

cellular Ca²⁺ concentration in fura-2AM preloaded NG108-15 cells (data not shown). The inhibitory effect of 5-HT on PI turnover at 1,000 μ M seems to be a non-specific one due to this very high concentration of 5-HT. The possibility that

some kind of factor inhibited the translation of 5-HT₂CR mRNA to protein is also not ruled out. However, Canton et al. (13) detected a short variant of 5-HT₂CR mRNA produced by alternative splicing. This short variant con-

Fig. 3. Effects of 5-HT and bradykinin on inositol phosphate formation in NG108-15 cells. Cellular phosphoinositides were prelabeled by incubation of cells for 17 h in culture medium containing [³H]inositol without unlabeled inositol. The inositol phosphates (IP₁, IP₂ and IP₃) were quantified at 30 s after addition of 5-HT or bradykinin in buffer containing 10 mM LiCl to inhibit IP₁ phosphatase. Data are the mean $\pm$ S.E.M. values of 4 experiments. **P* < 0.01 vs control.

Fig. 4. RT-PCR detection of 5-HT₂CR mRNA in rat brain and NG108-15 cells. RNA was isolated from rat brain (Rat) and NG108-15 (NG) cells by the acidic phenol method. RT/PCR was performed using a primer set that detected the region around the second intracellular loop of 5-HT₂CR. M, molecular marker: Msp I-digested pBR322 plasmid with bands of 622, 527, 404, 307 bp and other sizes.

tains a deletion of a 95 nt sequence in the region between the second intracellular and fourth transmembrane domains of 5-HT₂CR. Therefore, we designed primer sets that recognized this area. Surprisingly, only the short variant of 5-HT₂CR mRNA could be detected in NG108-15 cells. The deletion leads to a frameshift. Since 5-HT₂CR protein can not be produced from the deleted mRNA (13), this deletion may be the reason why PI turnover was not stimulated by 5-HT in NG108-15 cells. Studies using antibodies would be able to examine this possibility.

The deleted 95 nt includes the RNA editing site. In RNA editing, genomically encoded adenosine residues are converted to inosines by a specific adenosine deaminase (27). The edited RNA thus generates proteins that contain amino acids not encoded by the original transcript. Among receptors, the AMPA type of glutamate receptor (28, 29) and 5-HT₂CR (30) are affected by this event. In the case of 5-HT₂CR, five residues in the second transmembrane coding region can be edited, resulting in a change of three amino acids (31). It has been suggested that this editing in 5-HT₂CR affects the G-protein coupling with the receptor (32). Since both events are mediated by the second transmembrane domain, it will be of interest to determine whether RNA editing is involved in the 95 nt deletion of 5-HT₂CR.

A)

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781  tggcagtaag catggagaag aaactgcaca atgctaccaa ttattttctta atgtccctag
    tggcagtaag catggagaag aaactgcaca atgctaccaa ttattttctta atgtccctag

841  ccattgctga tatgctggtg ggactacttg tcatgcccct gtctctgctt gcaattcttt
    ccattgctga tatgctggtg ggactacttg tcatgcccct gtctctgctt gcaattcttt

901  atgattatgt ctggccttta cctagatatt tgtgccccgt ctggatttca ctagatgtgc
    atgattatgt ctggccttta cctagatatt tgtgccccgt ctggatttca ctagatgtgc

961  tattttcaac tgcgtccatc atgcacctct gcgccatata gctggaccgg tatgtagcag
    tattttcaac tgcgtccatc atgcacctct gcgccatata gctggaccgg

1021 tgcgtagtcc tgttgagcat agccggttca attcgcggac taaggccatc atgaagattg

1081 ccategtttg ggcaatatca ataggagttt cagttcctat ccctgtgatt ggactgaggg
    agttt cagttcctat ccctgtgatt ggactgaggg

1141 acgaaagcaa agtgttcgtg aataatacta cctgcgtgct caatgaccgg aacttcgttc
    acgaaagcaa agtgttcgtg aataatacta cctgcgtgct caatgaccgg aacttcgttc

1201 tcatecgggtc cttcgtggca ttcttcatcc cgttgacaat tatgggtgatc acctacttct
    tcatecgggtc

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B)

A
C
G
T

Fig. 5. The sequence of PCR products highly expressed in NG108-15 cells detected using a primer set for the region around the second intracellular domain of 5-HT2CR. The upper sequence is that of mouse 5-HT2CR cDNA reported by Yu et al. (M63685, unpublished). The lower one is that of the isolated PCR product of NG108-15 cells. Notice that 95 nt were deleted from the sequence of NG108-15 compared with the mouse sequence. The deleted sequence contained the second intracellular domain of 5-HT2CR. B) The sequencing gel image in the region of the deletion in NG108-15 cells. The deleted sequence region is indicated by a triangle.

Julius et al., who cloned 5-HT2CR (the previous name was 5-HT1C) (2), showed that the transfection of 5-HT2CR cDNA into NIH3T3 fibroblast cells resulted in the generation of transformed foci (33). They probably transfected the edited sequence, because the sequence that they cloned was reported to contain guanosine instead of adenosine residues in 4 of the 5 editable positions. We propose the following hypothesis. The edited mRNA and the protein translated from this mRNA may stimulate cellular growth rather than transmembrane signaling. If the editing is involved in the short variant formation, the protein product of the short variant, which is clearly a different protein from 5-HT2CR, may have a role in regulating growth. NG108-15 and other cloned cell lines have high growth potential, and NG108-15 cells were found here to contain the short variant of 5-HT2CR mRNA. This might be the reason why there are few reports about endogenous expression of 5-HT2CR in cloned cell lines, although the distribution of 5-HT2CR

within brain is considerable and widespread (3). Further studies are necessary to clarify the cellular function of the short variant of 5-HT2CR including the relationship with cell growth and RNA editing. NG108-15 cells may be a useful tool to know that.

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