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Short communication

Insulin induces internalization of the 5-HT_{2A} receptor expressed in HEK293 cells

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Abstract

To visualize the 5-hydroxytryptamine$_{2A}$ (5-HT_{2A}) receptor, we developed a 5-HT_{2A} receptor fused with yellow fluorescent protein (5-HT_{2A}–YFP) and expressed this receptor in HEK293 cells. In 5-HT_{2A}–YFP-expressing cells, but not in YFP-expressing or non-expressing cells, 5-HT induced a transient increase in the intracellular Ca^{2+} concentration (Ca^{2+} transient) in the Fluo 3 assay, suggesting that 5-HT_{2A}–YFP possesses a function similar to the wild-type 5-HT_{2A} receptor. Interestingly, not only 5-HT but also insulin induced the internalization of 5-HT_{2A}–YFP. Insulin also inhibited the 5-HT-induced Ca^{2+} transient. Genistein, an inhibitor of tyrosine kinase, blocked these insulin effects. Our results provide the first evidence that insulin receptor signaling via tyrosine kinase activation induces internalization of the plasma membrane 5-HT_{2A} receptor, and demonstrate crosstalk between the 5-HT_{2A} receptor and the insulin receptor.

Keywords: Insulin; 5-HT_{2A} receptor; Internalization; Ca^{2+} transient

1. Introduction

The 5-hydroxytryptamine$_{2A}$ (5-HT_{2A}) receptor, a Gq-coupled receptor, is involved in vascular smooth muscle contraction, platelet aggregation, and the modulation of mood and perception (Roth et al., 1998). Visualization of the localization of the 5-HT_{2A} receptor should increase the understanding of how 5-HT_{2A} receptor-associated cell functions are affected by various cell conditions. A green fluorescent protein (GFP)-fused 5-HT_{2A} receptor was recently developed and was shown to be expressed at the plasma membrane at rest and internalized upon stimulation with 5-HT (Bhattacharyya et al., 2002). It is known that plasma membrane receptors, such as 5-HT_{2A} receptor, are often internalized from the plasma membrane to the cytoplasm by agonist application. Interestingly, it is also known that agonist stimulation against a receptor induces the internalization or recycling of another receptor(s), revealing signaling crosstalk between multiple receptors. Insight into the regulation of plasma membrane 5-HT_{2A} receptor expression, such as by signaling crosstalk, is expected to provide new therapeutic strategies for diseases related to receptor abnormalities. Here we report that insulin induces 5-HT_{2A} receptor internalization, demonstrating signaling crosstalk between the 5-HT_{2A} receptor and the insulin receptor.

2. Materials and methods

2.1. Plasmid construction

To visualize the 5-HT_{2A} receptor in viable cells, pTriEx4neo–5HT_{2A}–mEYFP, an expression plasmid encoding yellow fluores-

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cent protein (YFP)-tagged 5-HT_{2A} receptor (5-HT_{2A}–YFP), was constructed as previously reported (Bhattacharyya et al., 2002), except for the use of YFP instead of GFP, as follows: first, pN1-mEYFP, a plasmid carrying the cDNA sequence for monomeric EYFP (Zacharias et al., 2002), was constructed by site-directed mutagenesis into pEYFP-N1 (BD Biosciences Clontech, Palo Alto, CA); GCCCTGAGCAAA encoding Ala-206/Leu-207/Ser-208/Lys-209 was changed to AAACTTTCGAAA, to replace Ala-206 with Lys (A206K; the amino-acid residue numbers described here correspond to those in EYFP). The 0.72-kb *Nco*I-*Not*I fragment of pN1-mEYFP was then ligated to the 6.36-kb *Nco*I-*Not*I fragment of pTriEx4neo (Novagen, Madison, WI) to yield pTriEx4neo–mEYFP. The cDNA sequence for the 5-HT_{2A} receptor was then amplified by polymerase chain reaction (PCR) using HTR2A cDNA (GenBank accession number NM_000621, Toyobo, Osaka, Japan) as a template; the PCR primers used were 5′-GCGGATCCCGCCACCATGGATATTCTTTGTGAA-GAAAATACTTCTTTG-3′ (forward) and 5′-GGCCATGGTGGC-GACCGGTGGATCCCGCACACAGCTCACCTTTTCATT-CACTCC-3′ (reverse); the 1.42-kb PCR product was digested with *Nco*I and inserted into the *Nco*I site of the pTriEx4neo–mEYFP. The orientation of the insert in pTriEx4neo–5HT_{2A}–mEYFP was checked by digestion with restriction enzymes.

As a plasma membrane marker, we constructed pN1-mECFPNT, a plasmid that expresses cyan fluorescent protein targeted to the plasma membrane (CFP_{PM}) as previously reported (Zacharias et al., 2002): first, the cDNA sequence encoding the sorting signal peptide for the plasma membrane (LCCMRRTKQVEKNDEDQKI; deduced amino-acid residues in single-letter codes) was amplified by PCR. The PCR primers were designed to anneal themselves, as follows: 5′-GGAGATCTGGATCCGATATCCGCCAC-CATGCTGTGCTGTATGAGAAGAACCAAACAGG-3′ and 5′-GGGTCGACAATCTTTTGGTCCTCATCATTCTTTT-CAACCTGTTTGGTTCTTCTCATACAGC-3′. The 0.09-kb PCR product was digested with *Bgl*II and *Sal*I, and then the cDNA sequence encoding monomeric ECFP (Zacharias et al., 2002) was amplified by PCR using pN1–mECFP, which was constructed similarly to pN1–mEYFP except for the use of pECFP–N1 (BD Biosciences Clontech) instead of pEYFP–N1. The PCR primers used were 5′-GGGTCGACGAGCTCACCATGGTGAG-CAAGGGCGAG-3′ (forward) and 5′-GCGAATTCGGATCCGC-GGCCGCTTACTTGTACAGCTCGTCCAT-3′ (reverse); the 0.72-kb PCR product was digested with *Sal*I and *Not*I. The above 0.09-kb *Bgl*II-*Sal*I fragment and the 0.72-kb *Sal*I-*Not*I fragment were then ligated with the 6.36-kb *Bgl*II-*Not*I fragment of pEGFP-N1 (BD Biosciences Clontech), yielding pN1–mECFPNT. All the constructs described above were verified by DNA sequencing.

2.2. Fluorescence measurements in HEK293 cells

HEK293 cells were cultured in growth medium composed of Dulbecco's modified Eagle's medium and 10% fetal bovine serum (Nakai et al., 2001). To express 5HT_{2A}–YFP, YFP, or CFP_{PM}, cells were transfected with pTriEx4neo–5HT_{2A}–mEYFP, pTriEx4neo–mEYFP, or pN1–mECFPNT, respectively (1 μg each/3.5-cm dish), using FuGENE 6 (Roche, Basel, Switzerland) according to the manufacturer's manual. Transfected cells were incubated at 37 °C for 1–4 days before testing. After exchanging the culture media for HEPES-buffered saline (HBS) containing 107 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 11.5 mM glucose, and 20 mM HEPES (pH 7.4), pre-warmed to room temperature, cells were moved to the microscope.

Cells co-expressing 5-HT_{2A}–YFP and CFP_{PM} were monitored by exciting at 514 and 458 nm, respectively, and detected at longer than 530 and 500±25 nm, respectively, with a confocal laser scanning microscope (LSM510Meta, Zeiss, Oberkochen, Germany). To observe changes in the localization of 5-HT_{2A}–YFP, 5-HT (0.01 nM–10 μM) or insulin (0.1–100 nM) was bath-applied to the transfected cells. Images were taken at 30 s intervals.

To test 5-HT-induced transient changes in intracellular Ca^{2+} concentration (Ca^{2+} transient), HEK293 cells were transfected with pTriEx4neo–5HT_{2A}–mEYFP or pTriEx4neo–mEYFP and loaded with Fluo3-AM (Dojindo, Kumamoto, Japan), a Ca^{2+}-sensitive fluorescent dye, as previously described (Nakai et al., 1997). Cells loaded with Fluo3 were set to the microscope stage as described above, and Fluo3 fluorescence excited at 458 nm was detected at 500±25 nm. To induce the Ca^{2+} transient in cells expressing 5-HT_{2A}–YFP, 5-HT (0.01 nM–10 μM) was bath-applied to the cells. In some experiments, cells were pretreated with the vehicle or insulin (10 nM) for 30 min, and with the vehicle, genistein (1 μM), an inhibitor of tyrosine kinase, or genistin (1 μM), an inactive analogue of genistein, for 1 h, prior to the application of 5-HT. Images were taken at 5 s intervals.

2.3. Internalization quantification

5-HT_{2A}–YFP internalization was quantified using an image analysis system (LSM5 Image Browser, Zeiss) as follows: whole cell fluorescence and intracellular fluorescence were measured in a cell of interest by manually setting the measurement regions of interest (ROIs) to include the whole cell region and the intracellular region deeper than 0.5 μm from the plasma membrane, respectively. Thirty minutes after the application of the vehicle or insulin, whole cell fluorescence and intracellular fluorescence in the same cell were again measured by setting new ROIs based on the criteria described above. After each measurement, the ratio of intracellular fluorescence against whole cell fluorescence was determined, and the increased percentage of the ratio after 30 min was defined as the extent of 5-HT_{2A}–YFP internalization.

2.4. Statistical analysis

Values are expressed as the mean±S.E.M. for 30–50 cells. Significance ($P<0.05$) was determined by one-way ANOVA with the Newman–Keuls multiple range test.

3. Results

5-HT_{2A}–YFP expressed in HEK293 cells was mainly localized at the plasma membrane (Figs. 1Aa, 2A and B). 5-HT (10 nM) triggered the Ca^{2+} transient in 5-HT_{2A}–YFP-expressing cells (Fig. 1Aa), but not in non-expressing (Fig. 1Aa) or YFP-expressing (Fig. 1Ab) cells.

5-HT did not induce the internalization of 5-HT_{2A}–YFP during the initial 60 s (Fig. 1Aa), but caused internalization from 3 to 10 min (Fig. 2A). The 5-HT-induced 5-HT_{2A}–YFP internalization was blocked by preincubation with sarpogrelate (1 μM), a 5-HT_{2A}-specific antagonist (data not shown).

To test for crosstalk between the 5-HT_{2A} receptor and the insulin receptor, we investigated the effect of insulin on 5-HT_{2A}–YFP trafficking. Interestingly, as shown in Fig. 2B, insulin (10 nM) induced the internalization of 5-HT_{2A}–YFP but not CFP_{PM} used as a plasma membrane marker. As expected, pretreatment with insulin (10 nM) reduced the 5-HT (0.01 nM)-induced Ca^{2+} transient in 5-HT_{2A}–YFP-expressing cells (Fig. 1B). The ratio of 5-HT_{2A}–YFP internalized increased by approximately 25% on cell stimulation with insulin (10 nM) for 30 min (Fig. 2C). These effects of insulin were blocked by pretreatment with genistein (1 μM) but not with genistin (1 μM) (Figs. 1C and 2C).

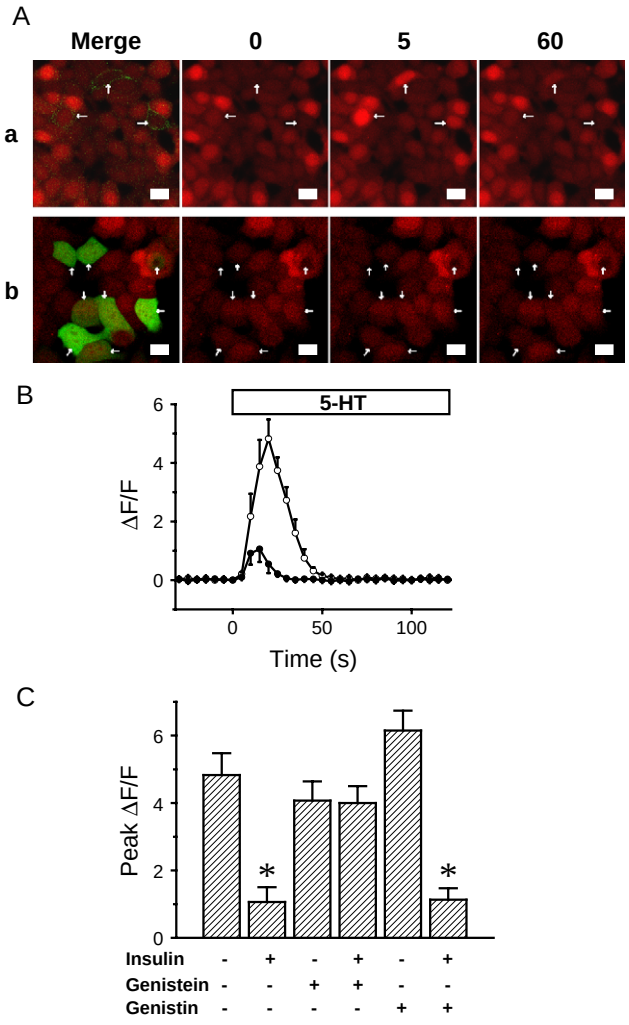

Fig. 1. (A) Typical 5-HT-induced Ca^{2+} transient in HEK293 cells expressing 5-HT_{2A}–YFP. Fluorescence images of cells expressing 5-HT_{2A}–YFP (a) or YFP (b) were taken before and after the application of 5-HT (10 nM). Green, fluorescence of 5-HT_{2A}–YFP (a) or YFP (b); red, fluorescence of Fluo3. Images in the left lane, fluorescence merges between green and red before 5-HT application. Arrows, cells expressing 5-HT_{2A}–YFP (a) or YFP (b). Numbers above images, length of 5-HT exposure in seconds. Bars, 5 μm. (B) Inhibitory effect of insulin on the 5-HT-induced Ca^{2+} transient. Cells expressing 5-HT_{2A}–YFP were pretreated with the vehicle (open circle) or insulin (10 nM, 30 min; closed circle) and changes in Fluo3 fluorescence were measured before and after the application of 5-HT (0.01 nM). $\Delta F/F$, change in Fluo3 fluorescence against its initial fluorescence. Bar, duration of 5-HT application. (C) Involvement of tyrosine kinase in the insulin effect on the 5-HT-induced Ca^{2+} transient. Peak $\Delta F/F$ reflecting the peak change in the 5-HT (0.01 nM)-induced Ca^{2+} transient with pretreatment of the vehicle (control) or drugs is shown. $^*P<0.05$ versus the control.

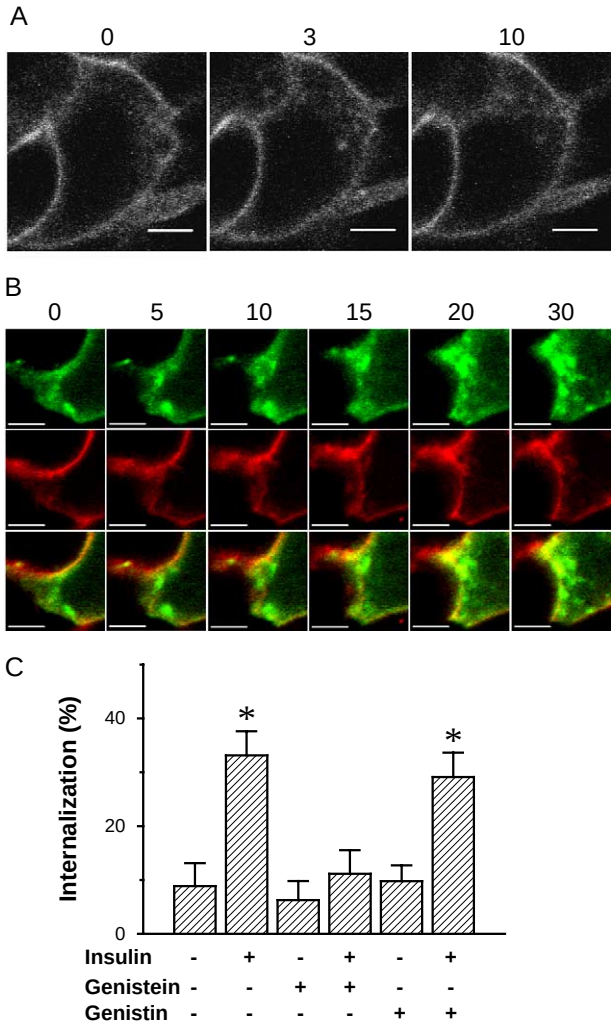

Fig. 2. (A) Typical 5-HT-induced 5-HT_{2A}–YFP internalization. Fluorescence images were taken before and 3 or 10 min after the application of 5-HT (10 nM). Numbers above images, time of 5-HT exposure in minutes. Bars, 5 μm. (B) Typical insulin-induced 5-HT_{2A}–YFP internalization. Fluorescence images were taken before and 5–30 min after the application of insulin (10 nM). Top row, 5-HT_{2A}–YFP in green; middle row, CFP_{PM} in red; bottom row, merge. Numbers above images, time of insulin exposure in minutes. Bars, 5 μm. (C) Involvement of tyrosine kinase in the insulin-induced 5-HT_{2A}–YFP internalization. HEK293 cells expressing 5-HT_{2A}–YFP were stimulated with the vehicle (control) or insulin (10 nM) for 30 min after treatment with the vehicle, genistein (1 μM), or genistin (1 μM) for 1 h. $^*P<0.05$ versus each control.

4. Discussion

To visualize the 5-HT_{2A} receptor, we constructed a 5-HT_{2A}–YFP, a YFP-fusion 5-HT_{2A} receptor, similar to the GFP-fusion 5-HT_{2A} receptor reported by Bhattacharyya et al. (2002), except for the use of YFP instead of GFP. As with the GFP-fusion 5-HT_{2A} receptor, our 5-HT_{2A}–YFP also showed internalization and Ca^{2+} transient on stimula-

tion with 5-HT, suggesting that the function of 5-HT_{2A}–YFP is similar to that of the GFP-fusion 5-HT_{2A} receptor, as well as the wild-type 5-HT_{2A} receptor.

Despite their practical benefits as fluorescent markers, GFP and variants such as CFP and YFP are known to make dimers when their intracellular concentrations reach micromolar ranges, and such a property is apt to produce artifact protein–protein interactions when proteins of interest are tagged with GFP and analyzed for their interactions. Recently, an A206K mutation in GFP protein was discovered to reduce dimerization and promote monomerization (Zacharias et al., 2002). As we used monomeric A206K YFP or CFP to design 5-HT_{2A}–YFP or CFP_{PM}, respectively, our results should not reflect such artifacts.

Recently, Doronin et al. (2002) reported that activation of the insulin receptor, a tyrosine kinase-coupled receptor, induced internalization of the β_2-adrenergic receptor, a Gs-coupled receptor, demonstrating crosstalk between these receptors expressed in A431 or CHO cells. They also reported that such treatment with insulin did not induce internalization of the 5-HT_{2A} receptor, unlike the β_2-adrenergic receptor. However, we surprisingly found that incubating HEK293 cells expressing 5-HT_{2A}–YFP with insulin (10 nM) induced the internalization of 5-HT_{2A}–YFP, thereby inhibiting the 5-HT-induced Ca^{2+} transient (Figs. 1B and 2B). These results demonstrate signaling crosstalk between the 5-HT_{2A} receptor and the insulin receptor. As the activation of tyrosine kinase following insulin receptor stimulation was shown to be necessary for this 5-HT_{2A} receptor internalization using an inhibitor of tyrosine kinase, down-stream signaling of the insulin receptor is probably associated. Similar signaling crosstalk has been shown between the 5-HT_{2C} receptor, which is another 5-HT receptor subtype coupled with Gq protein, and the insulin receptor, from evidence that insulin inhibited the 5-HT_{2C} receptor function observed with a reduction in the 5-HT-induced Ca^{2+} transient in rat choroid plexus cells (Hurley et al., 2003). It has not been clarified whether this insulin-induced inhibition is a result of 5-HT_{2C} receptor internalization, but considering our findings, 5-HT_{2C} receptor internalization is a plausible mechanism.

In conclusion, our results provide the first evidence that insulin receptor signaling via tyrosine kinase activation induces internalization of the plasma membrane 5-HT_{2A} receptor, and demonstrate crosstalk between the 5-HT_{2A} receptor and the insulin receptor.

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