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ACCELERATED COMMUNICATION

RNA Editing of the Human Serotonin 5-HT$_{2C}$ Receptor Delays Agonist-Stimulated Calcium Release

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ABSTRACT

RNA encoding the human 5-HT$_{2C}$ receptor undergoes adenosine-to-inosine RNA editing events at five positions in the putative second intracellular loop, with a corresponding reduction in receptor/G-protein coupling. Agonist-stimulated calcium release was examined in NIH-3T3 fibroblasts stably expressing the nonedited human INI (hINI) or the edited hVSV or hVGV variants. We hypothesized that different receptor isoforms would show altered dynamics of agonist-induced calcium release. The three isoforms showed a rightward shift in agonist concentration-response curves for eliciting calcium release (EC_{50} values: hINI, 2.2 nM; hVSV, 15 nM; hVGV, 49 nM). Additionally, the hVGV receptor showed a blunted and delayed $[Ca^{2+}]_i$ peak compared with the hINI or hVSV receptor isoforms. These distinctions in agonist-induced $[Ca^{2+}]_i$ release imply that edited 5-HT$_{2C}$ receptors may produce distinct physiological responses within the central nervous system.

The serotonin 5-HT$_{2C}$ receptor (5-HT$_{2C}$R) signals through the heterotrimeric G-protein, G_q, to activate phospholipase C (Chang et al., 2000), leading to the intracellular accumulation of inositol trisphosphate and subsequent calcium release. RNA editing within the putative second intracellular loop of the 5-HT$_{2C}$R produces at least 14 different receptor isoforms (Burns et al., 1997; Niswender et al., 1998). In the human 5-HT$_{2C}$R, editing occurs at five potential sites named A, B, C, D, and E (Fig. 1; Fitzgerald et al., 1999; Niswender et al., 1999). Editing at A, B, C, and D sites changes amino acids 156, 158, and 160 from INI (hINI) to VSV (hVSV); editing at all five sites produces VGV (hVGV). Since hVSV is the predominant isoform in human brain and hVGV has the most prominent phenotypic differences (Niswender et al., 1999), the present study focuses on calcium responses produced by these two edited receptors.

Previous work has shown that 5-HT and (±)-1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane (DOI), a 5-HT$_{2A/2C}$ receptor agonist, exhibit decreased potency when interacting with the hVSV and hVGV isoforms of the 5-HT$_{2C}$R, reflected in a rightward shift in the dose-response curve for [^{3}H]inositol monophosphate generation (Fitzgerald et al., 1999; Niswender et al., 1999) and decreased efficiency of receptor/G-protein coupling (Herrick-Davis et al., 1999; Niswender et al., 1999). In the current study, we examine the timing of agonist-induced increases in levels of $[Ca^{2+}]_i$, hypothesizing that edited isoforms will exhibit altered calcium release dynamics. Such alterations in the dynamics of calcium signaling have the potential to dramatically alter neuronal function (for review, see Berridge, 1998).

Experimental Procedures

Materials. Ionomycin was purchased from Sigma Chemical Co. (St. Louis, MO). Fura-2/acetoxymethyl ester (fura-2/AM) and pluronic acid were purchased from Molecular Probes (Eugene, OR). DOI was purchased from Research Biochemicals, Inc. (Natick, MA).

Cell Culture. NIH-3T3 cells stably expressing hINI, hVSV, and hVGV 5-HT$_{2C}$R s were generated as described previously (Niswender et al., 1999); receptor densities were 2047, 1292, and 5375 fmol/mg of protein, respectively. Cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units of penicillin/ml and 100 μg of streptomycin/ml under 5% CO_2 at 37°C.

Calcium Imaging. Cells were plated on 35-mm tissue culture plates and incubated in serum-free Dulbecco's modified Eagle's medium overnight. They were then loaded with fura-2 by incubation in

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ABBREVIATIONS: 5-HT$_{2C}$R, serotonin 5-HT$_{2C}$ receptor; HBSS, Hanks' balanced salt solution; DOI, (±)-1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane; fura-2/AM, fura-2 acetoxymethyl ester.

0.5 mM fura-2/AM in Hanks' balanced salt solution (HBSS) for 60 min at room temperature, followed by two washes with HBSS. Cells were superfused with either HBSS (with or without calcium), DOI, or ionomycin. $[Ca^{2+}]_i$ was measured in individual cells by dual-wavelength spectrofluorometry using a Nikon inverted microscope attached to a Compix Calcium Imaging System consisting of a charge coupled device camera (Dage-MTI CCD-72, Michigan City, IN) attached to an IBM compatible computer executing SIMCA C-Imaging software (Compix, Cranberry Township, PA). Cells were exposed to excitation wavelengths of 340 and 380 nm every 2 s and the emitted fluorescence was measured in real time at 510 nm. The ratio of emission at 340 and 380 nm excitation was used as an index of $[Ca^{2+}]_i$.

Data Analysis. Data were analyzed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA) and Microsoft Excel 97. Statistical analyses were performed by two-way ANOVA with Tamhane post-tests. Statistics were only calculated for these doses of DOI: −8, −7.5, −7, −6.5, −6, −5.5 (log M). For $t_{1/2}$ decay data, doses were grouped by low (log M −8, −7.5), medium (−7, −6.5), and high (−6, −5.5) doses. Level of significance was set at $P < .05$.

Results

Agonist-stimulated increases in $[Ca^{2+}]_i$ were measured using ratiometric imaging with fura-2 in cells expressing either hINI, hVSV, or hVGV. Since shifts in agonist potency to induce accumulation of [^{3}H]inositol phosphate have been described for the different receptor isoforms (Fitzgerald et al., 1999; Niswender et al., 1999), we first determined if there was a similar shift in agonist potency for increasing $[Ca^{2+}]_i$, a downstream consequence of inositol trisphosphate release. DOI-induced increases in $[Ca^{2+}]_i$ were measured and the ratios of DOI/ionomycin were normalized to percentage of maximum response for the highest concentration of DOI used in the same experiment. The dose-response curves of DOI-induced increases in $[Ca^{2+}]_i$ were shifted rightward for the edited isoforms (Fig. 1).

To determine whether there were differences in the time course of the DOI-stimulated increase in $[Ca^{2+}]_i$, three parameters were measured: peak latency (time from baseline to maximal response), half-time of decay (time from maximal response to half-maximal decay), and basal/postdrug ratio (ratio of baseline calcium level to postagonist calcium level) (Table 1). DOI-stimulated increases in $[Ca^{2+}]_i$ at the hVGV receptor showed a dramatic increase in peak latency compared with the hINI and hVSV receptor isoforms (Fig. 2). All isoforms showed a dose-dependent decrease in latency that plateaued at maximal concentrations of DOI, but the prolonged latency was still evident for the hVGV isoform (Table 1).

In some systems, the agonist-stimulated increase in $[Ca^{2+}]_i$ by the 5-HT_{2C}R has been shown to have two phases: a rapid increase to peak followed by a plateau level of higher $[Ca^{2+}]_i$ than before agonist was applied that is dependent on extracellular calcium (Watson et al., 1995). Although not all 5-HT_{2C}R systems demonstrate this biphasic response (Albert et al., 1999), it has been postulated that these two phases are mediated by different G-proteins (Macrez-Lepretre et al., 1997). To test whether 5-HT_{2C}R isoforms could modulate the

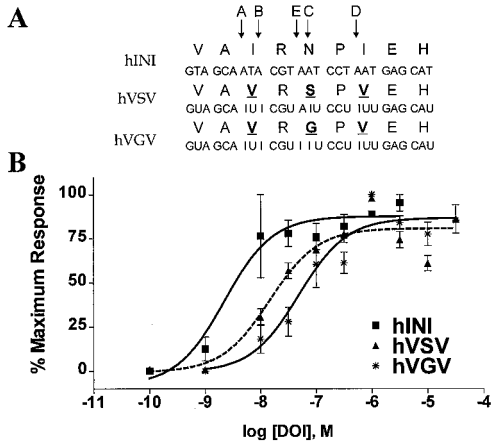

Fig. 1. Dose-dependent calcium signals of 5-HT_{2C}R isoforms. A, the positions of the editing sites within human 5-HT_{2C}R RNA and amino acid sequence are shown for the hINI RNA at the top, hVSV in the middle, and the hVGV on the bottom. B, NIH-3T3 cells expressing hINI, hVSV, or hVGV 5-HT_{2C}R s were loaded with fura-2 to measure increases in intracellular calcium. Cells were challenged with DOI, and the height of the calcium response was measured. To allow comparison between experiments, the peak ratio value of DOI challenge was normalized to an ionomycin challenge performed at the end of each experiment. Responses were normalized to percentage of maximum peak height in response to the highest concentration of DOI for each isoform. Data are mean ± S.E.M. values of at least seven independent experiments. Mean EC_{50} values: 2.2 nM, 15 nM, and 49 nM for hINI, hVSV, and hVGV, respectively.

TABLE 1

Analysis of receptor-stimulated calcium release

Cells stably expressing hINI, hVSV, or hVGV 5-HT_{2C}Rs were incubated in serum-free medium overnight and then loaded with fura-2 for 1 h. Cells were then treated with various concentrations of DOI, and the latency (time from baseline to maximal response), half-time of decay (time from maximal response to half-maximal decay), and basal/postdrug ratio (ratio of baseline calcium level to postdrug calcium level) parameters were measured. Data are mean ± S.E.M. values of at least three independent experiments.

	Isoform	Dose (log [DOI], M)								
		−10	−9	−8	−7.5	−7	−6.5	−6	−5.5	−5
Latency (s)[a]	INI	38.8 ± 16.9	33.0 ± 5.7	15.1 ± 1.6	10.4 ± 1.1	11.3 ± 1.5	8.8 ± 1.1	13.0 ± 1.7	7.9 ± 0.8	N.D.
	VSV	N.D.	45.0 ± 3.2	22.3 ± 2.8	18.4 ± 2.1	19.5 ± 3.3	13.5 ± 2.2	26.4 ± 2.7	11.9 ± 1.2	17.2 ± 2.2
	VGV	N.D.	N.D.	40.0 ± 6.9	43.6 ± 3.3	25.3 ± 4.2	33.8 ± 3.7	34.0 ± 1.8	35.7 ± 2.6	34.1 ± 3.1
Decay ($t_{1/2}$, s)	INI	N.D.	56.8 ± 22.8	57.0 ± 10.6	41.4 ± 7.3	37.5 ± 5.6	56.9 ± 29	39.2 ± 3.6	N.D.	N.D.
	VSV	N.D.	34.7 ± 6.7	51.7 ± 7.2	54.7 ± 12.7	58.6 ± 4.5	51.1 ± 12.5	35.5 ± 2.6	58.6 ± 14.4	56.6 ± 8.5
	VGV	N.D.	N.D.	62.1 ± 31.7	78.4 ± 10.4	43.9 ± 5.9	59.0 ± 20.5	55.6 ± 18.3	63.2 ± 11.2	70.4 ± 8.9
Basal/postdrug ratio	INI	1.3 ± 0.1	1.0 ± 0.1	1.3 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.01	0.8 ± 0.02	N.D.
	VSV[b]	N.D.	1.1 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	1.1 ± 0.2	0.9 ± 0.1	1.3 ± 0.1	1.1 ± 0.2	1.1 ± 0.1
	VGV	N.D.	0.9 ± 0.001	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.04	0.9 ± 0.1	1.0 ± 0.2	0.8 ± 0.1	0.9 ± 0.04

N.D., not determined.
[a] All isoforms were significantly different with respect to latency ($P < .001$).
[b] VSV was significantly different than VGV ($P = .003$).

second phase of agonist-induced calcium release, we generated a ratio by measuring levels of $[Ca^{2+}]_i$ at the end of agonist treatment and dividing that value by baseline (preagonist) $[Ca^{2+}]_i$. Thus, basal/postdrug ratios near one indicate that the contribution of the second phase of $[Ca^{2+}]_i$ elevation is negligible. As can be seen in Table 1, these ratios had a value near one for each isoform, suggesting that the second phase of calcium release was negligible in these cells. The time needed from peak maximum to reach ½ peak height is $t_{1/2}$ decay and is used as a measure of calcium release dynamics (Okamoto et al., 1995; Ozaki et al., 1997). It is proposed to reflect a mixture of calcium release from intracellular stores as well as calcium influx (Berridge, 1997). There were no significant differences among the $t_{1/2}$ of the edited isoforms. In addition, the shapes of the curves were not affected by removing calcium from the extracellular perfusate (Fig. 2, inset) suggesting that the DOI-induced increase in $[Ca^{2+}]_i$ was predominately a result of release from intracellular stores and not calcium influx.

Discussion

The human 5-HT$_{2C}$R undergoes adenosine-to-inosine RNA editing to generate distinct amino acids within the second intracellular loop of the protein (Burns et al., 1997), a region known to be important for G-protein coupling (Arora et al., 1995, 1997; Blin et al., 1995; Gomeza et al., 1996; Liu and Wess, 1996; Iida-Klein et al., 1997; Verrall et al., 1997; Ballesteros et al., 1998; Burstein et al., 1998). These editing events are conserved among rodent and human species (Burns et al., 1997; Niswender et al., 1998) and exhibit region-specific regulation (Burns et al., 1997), suggesting that they may serve an important role in receptor function. Previous work has shown that the agonist DOI exhibits a decreased potency for eliciting inositol phosphate production when interacting with the edited receptor isoforms (hINI > hVSV > hVGV) stably expressed in an NIH-3T3 fibroblast cell line (Niswender et al., 1999). The results presented here are in agreement, demonstrating rightward shifts in concentration-response curves for calcium mobilization of 7- and 22-fold for hVSV and hVGV receptors, respectively.

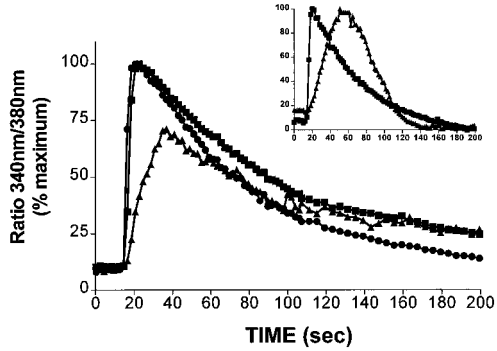

Fig. 2. Typical time courses of DOI-induced intracellular calcium mobilization. NIH-3T3 cells expressing hINI (■), hVSV (●), or hVGV (▲) 5-HT$_{2C}$Rs were loaded with fura-2 to measure increases in intracellular calcium. Cells were challenged with 1 μM DOI (hINI, hVSV) or 10 μM DOI (hVGV). The ratio of excitations at 340 and 380 nm gives an indication of intracellular calcium concentration. Data are representative traces of the responses of at least 50 cells/experiment in at least three independent experiments and are normalized to the maximum hINI response. Inset, similar experiments in hINI and hVGV cells using calcium-free medium. Responses are normalized to the maximum response for each isoform.

The current study compared the dynamics of calcium release in cells expressing different 5-HT$_{2C}$R isoforms. The hVGV isoform shows a different pattern of calcium release compared with the hINI and hVSV isoforms, with a smaller peak height and longer latency. One explanation for these alterations in latency is that the intensity of receptor response may alter the ratio of external to internal calcium release. If the response of hVGV is biased toward calcium entry, as opposed to release from intracellular stores, then this could explain why the response develops more slowly and has a lower amplitude. However, our results suggest that the increased latency does not reflect a difference in the relative contributions of internal stores versus calcium influx since the latency difference was retained in calcium-free medium.

A similar alteration in peak latency has been found for genetic polymorphisms of the 5-HT$_{2A}$ receptor in platelets. Ozaki et al. (1997) showed that His^{452}Tyr, an abundant, naturally occurring structural polymorphism in the C-terminal tail of the 5-HT$_{2A}$ receptor, resulted in functional differences in 5-HT-induced Ca^{2+} mobilization in platelets. His^{452}/Tyr^{452} heterozygotes showed a smaller peak amplitude of Ca^{2+} mobilization, a longer peak latency, and longer half-time relative to His^{452}/His^{452} homozygotes (Ozaki et al., 1997). In addition, a change in the dynamics of 5-HT$_{2A}$-induced $[Ca^{2+}]_i$ mobilization has been demonstrated in psychiatric diseases (Yamawaki et al., 1998). The present work demonstrates that RNA editing of the 5-HT$_{2C}$R produces a similar change in the kinetics of calcium release. The mechanism of the differential calcium signaling properties of the edited isoforms is not known. It is tempting to speculate that the structural difference in the second intracellular loop or more global alterations in conformation of the receptor presents distinct interfacial domains with differing protein:protein interaction properties. For example, differential interaction of the edited receptors with scaffolding proteins, such as InaD (Ullmer et al., 1998), may dictate kinetics of calcium mobilization.

The 5-HT$_{2C}$R is the first, and so far the only known, G-protein-coupled receptor regulated by RNA editing, a post-transcriptional event that changes the genetic code at the level of RNA. Although it has been documented that RNA editing of the 5-HT$_{2C}$R reduces agonist potency and the efficiency of G-protein coupling, the current study is the first to show that RNA editing modifies the *dynamics* of signal generation. Thus, the release of intracellular calcium by the fully edited 5-HT$_{2C}$R isoform is temporally delayed in conjunction with a reduced magnitude. In a dynamic situation, such as synaptic transmission, this altered temporal pattern of calcium release has the potential to differentially regulate physiological functions.

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