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Zolmitriptan stimulates a Ca^{2+}-dependent K^+ current in C6 glioma cells stably expressing recombinant human 5-HT_{1B} receptors

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Abstract

Stimulation of a Ca^{2+}-dependent K^+ current by zolmitriptan, a 5-$HT_{1B/1D}$ receptor partial agonist, was investigated in C6 glioma cells stably expressing recombinant human 5-HT_{1B} receptors. Outward K^+ currents (I_K) were examined in non-transfected C6 glioma cells and in cells expressing cloned human 5-HT_{1B} receptors using the patch-clamp technique in the whole-cell configuration. In C6 glioma cells expressing recombinant human 5-HT_{1B} receptor, zolmitriptan increased I_K in a concentration-dependent manner (maximum increase $16.3 \pm 7.8\%$, $n = 5$, $p < 0.001$) with a pD_2 value (geometric mean with 95% confidence intervals) of 7.03 (7.90–6.10). Zolmitriptan failed to elicit increases in I_K in non-transfected C6 cells. In the presence of the mixed 5-$HT_{1B/1D}$ receptor antagonist, *N*-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2(-methyl-4(5-methyl-1,2,4)-oxadiazol-3-yl)[1,1-biphenyl]-4-carboxamide 2HCl (GR 127935, 0.1 μM), zolmitriptan (1 μM) failed to significantly increase I_K in C6 cells expressing human 5-HT_{1B} receptors confirming that zolmitriptan-evoked responses were indeed mediated by human 5-HT_{1B} receptors. In C6 cells expressing cloned human 5-HT_{1B} receptors, zolmitriptan-induced increases in I_K were prevented by the calcium chelator, EGTA (5 mM) when included in the patch pipette (maximum increase $-3.3 \pm 4.2\%$, $n = 4$, P = NS). The Ca^{2+}-dependent K^+ channel blockers, iberiotoxin (0.1 μM) and tetraethylammonium (TEA, 1 mM), abolished zolmitriptan-induced increases in I_K ($4.5 \pm 7.3\%$, $n = 4$ and $-0.8 \pm 1.7\%$, $n = 4$, respectively, P = NS in each case) in C6 cells expressing human 5-HT_{1B} receptors, confirming the involvement of Ca^{2+}-dependent K^+ channels. In conclusion, the 5-$HT_{1B/1D}$ receptor partial agonist, zolmitriptan, stimulates $I_{K/Ca}$ in C6 glioma cells stably transfected with human 5-HT_{1B} receptors suggesting an increase of hyperpolarizing current.

Keywords: Zolmitriptan; 5-HT_{1B} receptor, human; K^+ channel, Ca^{2+}-dependent; C6 glioma cell

1. Introduction

Zolmitriptan is a new 5-$HT_{1B/1D}$ receptor partial agonist (Martin et al., 1997) that has recently been approved for use in the acute treatment of migraine. Activation of the trigemino-vascular system is believed to be a key step in the initiation of migraine headache (Moskowitz, 1984; Goadsby et al., 1991). Therefore, like dihydroergotamine and other triptans, zolmitriptan inhibits trigeminal activation centrally as well as peripherally. Access to the central nervous system contributes to the effect of 5-$HT_{1B/1D}$ receptor agonists (Goadsby and Edvinsson, 1994; Goadsby and Hoskin, 1996; Goadsby and Knight, 1997) by reducing the excitability of central trigeminal neurons and thus nociception, of which the second-order neurons reside in the brain stem trigeminal nucleus caudalis (Hoskin et al., 1996). Moreover, trigeminal ganglion neurons could represent another key site of action for 5-$HT_{1B/1D}$ receptor agonists (John et al., 1999), in addition to the peripheral sensory nerve–vessel interface and central trigeminal nucleus caudalis. Collectively, activation of 5-$HT_{1B/1D}$ receptors reduce central and peripheral neuronal excitability (Goadsby and Hoskin, 1996; Martin, 1996; Martin et al., 1997) by aborting sensory neuronal firing in the trigemino-vascular system.

In a previous report, we proposed that the cellular mechanism that reduces neuronal excitability following 5-$HT_{1B/1D}$ receptor activation by sumatriptan was mediated by an increase of an hyperpolarizing K^+ current (Le Grand et al., 1998) indicating that recombinant human 5-HT_{1B} receptors are positively coupled to Ca^{2+}-dependent K^+ channels (Le Grand et al., 1998). The outward K^+

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current mediated by these channels is known to mediate hyperpolarization (Sah, 1996) and to play a key role in regulating neuronal excitability. Therefore, the aim of the present study was to investigate whether zolmitriptan, a 5-$HT_{1B/1D}$ receptor partial agonist (Martin et al., 1997), could similarly stimulate a Ca^{2+}-dependent K^+ current following human 5-HT_{1B} receptor activation.

2. Materials and methods

2.1. Cell culture

C6 glioma cells (ATCC, CL 107, rat) transfected (or not) with the human 5-HT_{1B} receptor gene were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated calf serum as previously described (Pauwels et al., 1996). They were plated in small volume chambers (100–200 μl) 48–96 h before use.

2.2. Whole-cell patch-clamp recordings

Outward K^+ currents (I_K) were measured at room temperature (20–24°C) using the patch-clamp technique in the whole-cell configuration. Pipettes with resistance of 3–5 MΩ were pulled from Pyrex capillaries not fire polished before use. A flow of solution (50–100 μl/min) from one of a series of five outlets continuously superfused the cell from which a recording was being made. A patch clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA, USA) was used. The resistance in series with the cell membrane was compensated using dynamic series resistance control compensation to provide the fastest possible capacity transient without ringing. Neither capacitive current nor leakage current was compensated. The cell capacitance was calculated as the ratio of the numerical integration of the averaged current transient (total charge) to the magnitude of the hyperpolarising pulse. Cell currents were further digitized at 1.6 kHz and analysed with a computer (Desk-Pro 486/33 M, Compaq, Houston, USA) with interactive software (ACQUIS1, G. Sadoc, Paris, France).

Cells were maintained at a holding potential of −60 mV. For these measurements, Na^+ current was inhibited by replacing NaCl by equimolar choline chloride. The external solution contained (in mM): choline chloride 135, $MgCl_2$ 1.1, $CaCl_2$ 0.8, HEPES 10, atropine sulphate 0.001, D-(+)glucose 10; pH adjusted to 7.4 with KOH. Atropine sulphate was employed to rule out any activation of muscarinic receptors by choline chloride. The internal solution contained (in mM): K-aspartate 115, KCl 10, $MgCl_2$ 3, KH_2PO_4 2, $CaCl_2$ 1, HEPES 10, D-(+)glucose 10; pH adjusted to 7.4 with KOH. Current amplitude was measured at the end of the depolarizing pulse with reference to the current before the beginning of the pulse.

2.3. Drugs

Zolmitriptan (base) and *N*-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2(-methyl-4(5-methyl-1,2,4)-oxadiazol-3-yl)[1,1-biphenyl]-4-carboxamide 2HCl (GR 127935) were synthesized by the Department of Analytical Chemistry and Division of Medicinal Chemistry IV at the Centre de Recherche Pierre Fabre. Zolmitriptan was dissolved in dimethyl sulphoxide as 10 mM stock solution and was then diluted in the final Krebs solution as required. The highest final concentration of dimethyl sulphoxide was 0.1% (i.e. zolmitriptan 10 μM). GR 127935 was dissolved in distilled water.

2.4. Data analysis

Data are expressed as mean values ± s.e. mean or 95% confidence intervals. Statistical significance was assessed by ANOVA with repeated measurements followed by Dunnett's test (intergroup analysis). $P < 0.05$ was consid-

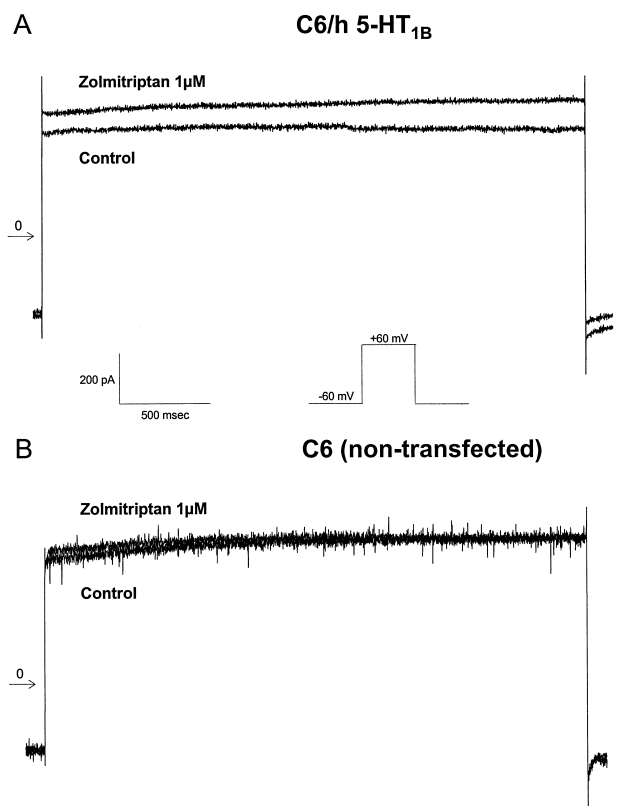

Fig. 1. Effects of the 5-$HT_{1B/1D}$ receptor agonist zolmitriptan on typical I_K recordings obtained with depolarizing steps from −60 to +60 mV in C6 glioma cells. Superimposed recordings of $I_{K/Ca}$ either in the presence or absence of zolmitriptan (applied for 3 min) in a C6 glioma cell expressing human 5-HT_{1B} receptors (upper panel) and in a non-transfected cell (lower panel).

ered the minimum level of significance (StatView 4.1, Abacus Concepts, Berkeley, CA). Curve fitting was performed using the non-linear least square gradient-expansion Marquardt algorithm (Origin 6.0, Microcal Software, Northampton, MA), which gave geometric EC_{50} values with 95% confidence intervals.

3. Results

3.1. Zolmitriptan-induced increases in I_K in C6 cells stably expressing human 5-HT_{1B} receptors

Baseline current in C6 cells stably expressing human 5-HT_{1B} receptors (152.7 ± 21.5 pA/pF, $n = 25$) was not statistically significantly different from that in non-transfected C6 cells (217.1 ± 79.1 pA/pF, $n = 8$, NS). Fig. 1 shows typical recordings of I_K obtained with a depolarizing step from -60 to $+60$ mV in the presence or absence of zolmitriptan (1 μM). In transfected C6 glioma cells, human 5-HT_{1B} receptor activation with zolmitriptan (1 μM) increases I_K (Fig. 1, upper panel). The current responses evoked by zolmitriptan began within 30 s of agonist application, were maximal at approximately 1 min perfusion and were fully reversible during washout. The absence of zolmitriptan-induced increases in I_K in non-transfected C6 glioma cells (Fig. 1, lower panel) confirmed a specific involvement of the membrane human 5-HT_{1B} receptor in transfected C6 glioma cells. Thus, activation of human 5-HT_{1B} receptors by zolmitriptan enhances I_K (Fig. 1, upper panel and Fig. 2A; maximum increases were $16.3 \pm 7.8\%$, $n = 5$, $P < 0.001$). The pD_2 value for zolmitriptan in increasing I_K was 7.03 (7.9–6.1) in C6 glioma cells expressing human 5-HT_{1B} receptors.

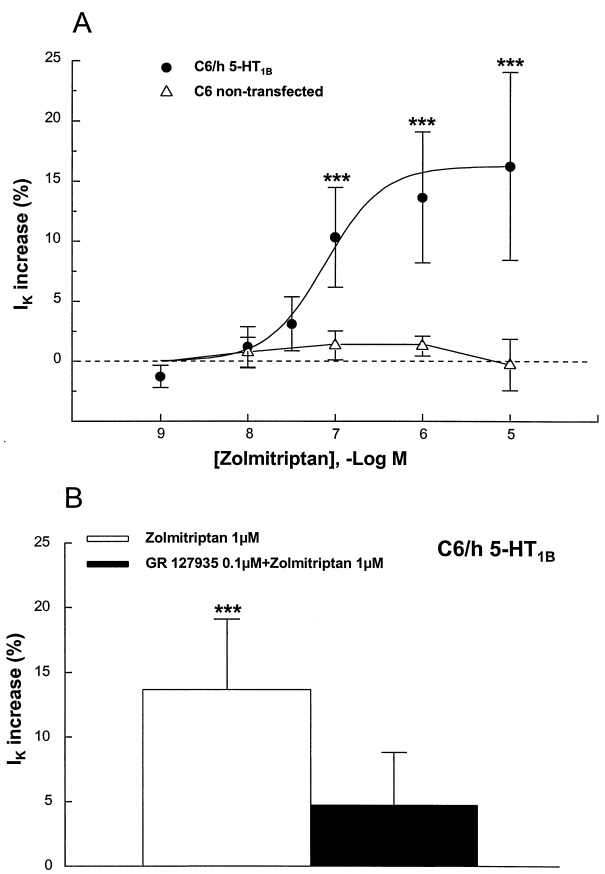

Fig. 2. (A) Effect of zolmitriptan on $I_{K/Ca}$ in non-transfected C6 glioma cells, and in C6 cells stably transfected with human 5-HT_{1B} receptor genes ($n = 5$). I_K amplitude obtained at the end of the pulse was normalized with respect to control values. (B) Inhibition of zolmitriptan-stimulated I_K by the 5-$HT_{1B/1D}$ receptor antagonist GR 127935 (0.1 μM). Cells were superfused with zolmitriptan (1 μM) before and after GR 127935 ($n = 5$, in each case). I_K was evoked by depolarizing pulses from a holding potential of -60 to $+60$ mV. Statistical significance was assessed between vehicle and zolmitriptan groups by one-way ANOVA followed by Dunnett's test. $^{***}p < 0.001$.

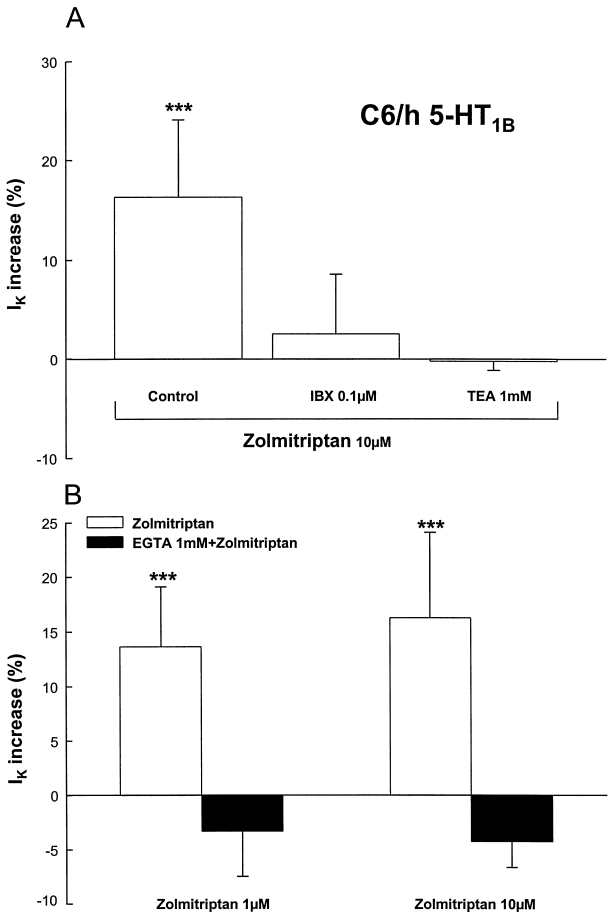

Fig. 3. Characterization of I_K stimulated by zolmitriptan in C6 glioma cells stably expressing cloned human 5-HT_{1B} receptors. (A) Changes in $I_{K/Ca}$ induced by zolmitriptan after 3-min application of iberiotoxin (IBX; 0.1 μM, $n = 4$) and TEA (1 mM, $n = 4$) in cells expressing human 5-HT_{1B} receptors. (B) Inhibition of zolmitriptan-stimulated $I_{K/Ca}$ after loading with EGTA in cells. Cells were perfused with zolmitriptan (1 μM, $n = 4$ and 10 μM, $n = 4$) in the absence (open bars) and in the presence of EGTA 5 mM (filled bars). $I_{K/Ca}$ was evoked by depolarizing pulses from a holding potential of -60 to $+60$ mV. Statistical significance was assessed between vehicle and zolmitriptan groups by one-way ANOVA followed by Dunnett's test. $^{***}p < 0.001$.

3.2. Inhibition of zolmitriptan-induced increases in I_K by the 5-$HT_{1B/1D}$ receptor antagonist GR 127935

The mixed 5-$HT_{1B/1D}$ receptor antagonist, GR 127935 (Clitherow et al., 1994), per se had no effect on I_K in either non-transfected C6 glioma cells ($-1.2 \pm 1.3\%$, $n = 5$, NS), or those transfected with the human 5-HT_{1B} receptor gene ($3.2 \pm 3.5\%$, $n = 5$, NS). In C6 glioma cells expressing human 5-HT_{1B} receptors, GR 127935 (0.1 μM) significantly reduced zolmitriptan (1 μM)-evoked increases in I_K (Fig. 2B, $n = 5$). These results provide further evidence that zolmitriptan-induced increases in I_K in transfected C6 glioma cells were mediated by human 5-HT_{1B} receptors.

3.3. Characterisation of outward K^+ current stimulated by zolmitriptan in C6 glioma cells

In a previous study, we demonstrated that activation of human 5-HT_{1B} receptors produces an increase in Ca^{2+}-dependent K^+ current. Therefore, the nature of the zolmitriptan-induced increases in outward current in transfected C6 cells was then explored. Zolmitriptan-induced increases in I_K were prevented by iberiotoxin (0.1 μM), a specific blocker of calcium-activated K^+ channels and by tetraethylammonium (TEA, 1mM), a non-specific blocker of calcium-activated K^+ channels (Fig. 3A; $4.5 \pm 7.3\%$, $n = 5$ and $-0.8 \pm 1.7\%$, $n = 4$, respectively, $P =$ NS in each case). In addition, zolmitriptan-induced increases in I_K were abolished by the calcium chelator EGTA (5 mM) included in the patch pipette (Fig. 3B; $-3.3 \pm 4.2\%$ with zolmitriptan 1 μM, $n = 4$, $P =$ NS and $-4.2 \pm 2.4\%$ with zolmitriptan 10 μM, $n = 4$, $P =$ NS). Collectively, these results indicate that zolmitriptan-mediated an increase in I_K occur in a Ca^{2+}-dependent manner ($I_{K/Ca}$).

4. Discussion

In C6 glioma cells stably expressing cloned human 5-HT_{1B} receptors, zolmitriptan increased an outward K^+ current that required Ca^{2+} ions for activation ($I_{K/Ca}$). The relatively selective 5-$HT_{1B/1D}$ receptor antagonist GR 127935 (Clitherow et al., 1994) prevented zolmitriptan-induced increases in $I_{K/Ca}$. When EGTA was included in the patch pipette, zolmitriptan failed to enhance $I_{K/Ca}$. In addition, the Ca^{2+}-dependent K^+ channel blockers iberiotoxin and TEA abolished zolmitriptan-induced increases in $I_{K/Ca}$. Collectively, our findings indicate that zolmitriptan, a 5-$HT_{1B/1D}$ receptor partial agonist stimulated a Ca^{2+}-dependent K^+ current in C6 glioma cells stably expressing recombinant human 5-HT_{1B} receptors. The data confirm previous findings in which human 5-HT_{1B} receptors were shown to be positively coupled to Ca^{2+}-dependent K^+ channels (Le Grand et al., 1998).

4.1. Involvement of the transfected human 5-HT_{1B} receptor

In C6 glioma cells stably expressing cloned human 5-HT_{1B} receptors, zolmitriptan concentration-dependently increased $I_{K/Ca}$. The absence of zolmitriptan-induced increases in $I_{K/Ca}$ in non-transfected C6 cells provides firm evidence for a specific involvement of human 5-HT_{1B} receptors in mediating zolmitriptan current responses in transfected cells. In addition, this observation excludes any involvement of native 5-$HT_{2A/C}$ receptors known to stimulate $I_{K/Ca}$ in C6 glioma cells (Manor et al., 1992; Elliot et al., 1995). The potency value obtained for zolmitriptan in augmenting $I_{K/Ca}$ following activation of human 5-HT_{1B} receptors (pD_2 7.03) is in agreement with potency values reported for inhibition of forskolin-stimulated cAMP accumulation (pD_2 7.51; Pauwels et al., 1997) and enhancement of specific GTPγ^{35}S binding (pD_2 7.22; Pauwels et al., 1997, 1998). The potency value reported for activation of $I_{K/Ca}$ in transfected C6 glioma cells is also consistent with potency values reported for zolmitriptan-induced contraction of primate basilar artery (pD_2 6.92; Martin et al., 1997), human epicardial coronary artery (pD_2 7.3; Martin et al., 1997) and rabbit saphenous vein rings (pD_2 6.79; Martin et al., 1997) consecutive to activation of 5-HT_{1B} receptors. In addition, the potency value and the maximum I_K increases obtained for zolmitriptan (pD_2 7.03, maximum increase $16.3 \pm 7.8\%$) are in the same rank of order with that obtained for sumatriptan (pD_2 7.21, maximum I_K increase $19.4 \pm 7.2\%$; Le Grand et al., 1998). In the presence of GR 127935, a mixed 5-$HT_{1B/1D}$ receptor antagonist, zolmitriptan failed to significantly increase $I_{K/Ca}$ in C6 glioma cells stably transfected with the human 5-HT_{1B} receptor. Collectively, these results demonstrate that zolmitriptan stimulates $I_{K/Ca}$ following activation of recombinant human 5-HT_{1B} receptors.

4.2. Stimulation of $I_{K/Ca}$ in transfected C6 glioma cells

The present data indicate that zolmitriptan-induced increases in $I_{K/Ca}$ in C6 glioma cells are sensitive to blockade by iberiotoxin, a specific blocker calcium-activated K^+ channels, and by TEA, a non-specific blocker of calcium-activated K^+ channels. These observations are in close agreement with our results which show that zolmitriptan-induced increases in $I_{K/Ca}$ were significantly reduced by dialysing cells with the calcium chelator, EGTA. This indicates that in C6 glioma cells, zolmitriptan-evoked increases in $I_{K/Ca}$ can be activated by small changes in intracellular free Ca^{2+} concentrations, in agreement with activation of a specific $I_{K/Ca}$ (Xia et al., 1998). Such positive coupling of 5-HT_{1B} receptors to Ca^{2+}-dependent K^+ channels therefore requires Ca^{2+} release from intracellular stores. Thus, increases in intracellular free Ca^{2+} concentrations consecutive to human 5-HT_{1B} activa-

tion by sumatriptan, another 5-$HT_{1B/1D}$ receptor partial agonist, in C6 glioma cells resulted from the internal liberation of Ca^{2+} ions from thapsigargin-sensitive Ca^{2+} stores (Le Grand et al., 1998). In addition, blockade of sumatriptan-induced increases in $I_{K/Ca}$ by heparin (Le Grand et al., 1998), an $InsP_3$ receptor blocker (Kobayashi et al., 1988; Hirose and Iino, 1994), clearly suggests that inositol phospholipid hydrolysis is the major signalling pathway by which increases in intracellular free Ca^{2+} concentrations occur consecutive to human 5-HT_{1B} receptor activation by sumatriptan in C6 glioma cells. Therefore, an identical positive signalling pathway between phospholipid hydrolysis and Ca^{2+}-dependent K^+ channel opening also occurs following activation of the human 5-HT_{1B} receptor by zolmitriptan.

A consequence of zolmitriptan-induced increases in Ca^{2+}-dependent K^+ current following 5-HT_{1B} receptor activation could be membrane hyperpolarization. This could constitute a potential mechanism by which zolmitriptan reduces neuronal excitability (Goadsby and Hoskin, 1996; Martin et al., 1997; Werhahn et al., 1998). Thus, 5-$HT_{1B/1D}$ receptor mRNA (Bruinvels et al., 1992; Bouchelet et al., 1996) and immunoreactivity (Longmore et al., 1997) have been detected in trigeminal neurons, both peripherally and centrally, suggesting that these receptors may be involved in the modulation of trigeminal nociceptive neurotransmission. In isolated guinea-pig trigeminal ganglion neurons, Ca^{2+}-dependent K^+ currents involved in regulating excitability were increased by the 5-$HT_{1B/1D}$ receptor agonists, sumatriptan and F 11356 (John et al., 1999). Therefore, a native system that constitutively expresses 5-$HT_{1B/1D}$ receptors has therefore been reported to exhibit identical coupling with Ca^{2+}-dependent K^+ channels. In addition, current-clamp experiments conducted in trigeminal ganglion neurons showed that activation of 5-$HT_{1B/1D}$ receptors by sumatriptan induced an hyperpolarization of the resting membrane potential (-2.4 ± 0.8 mV, $n = 5$, unpublished data). However, further studies are required to clearly identify which receptor subtype, 5-HT_{1B}, 5-HT_{1D} or both, modulates electrical activity in the trigemino-vascular system. Nevertheless, activation of 5-$HT_{1B/1D}$ receptors by zolmitriptan significantly reduced firing probability of trigeminal neurons (Goadsby and Hoskin, 1996). It is well-established that reduction of cellular firing frequently results from neuronal hyperpolarization. Stimulation of $I_{K/Ca}$ is therefore one possible, major mechanism by which hyperpolarization can be induced (Sah, 1996; Xia et al., 1998). Increases in $I_{K/Ca}$ following activation of 5-HT_{1B} receptors is thus a potential key mechanism that could explain reduction of cellular firing and neuronal excitability.

In conclusion, the present findings provide evidence that the 5-$HT_{1B/1D}$ receptor partial agonist, zolmitriptan, like sumatriptan, stimulates $I_{K/Ca}$ in C6 glioma cells stably transfected with human 5-HT_{1B} receptors suggesting increases in hyperpolarizing current.

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