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Herkinorin dilates cerebral vessels via kappa opioid receptor and cyclic adenosine monophosphate (cAMP) in a piglet model

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Abstract

Since herkinorin is the first non-opioid mu agonist derived from salvinorin A that has the ability to induce cerebral vascular dilatation, we hypothesized that herkinorin could have similar vascular dilatation effect via the mu and kappa opioid receptors and the cAMP pathway. The binding affinities of herkinorin to kappa and mu opioid receptors were determined by in-vitro competition binding assays. The cerebral arteries were monitored in piglets equipped with a closed cranial window and the artery responses were recorded before and every 30 s after injection of artificial cerebrospinal fluid (CSF) in the presence or absence of the investigated drugs: herkinorin, norbinaltorphimine (NTP), a kappa opioid receptor antagonist, β -funaltrexamine (β -FNA), a mu opioid receptor antagonist, or Rp-8-Br-cAMPS (Rp-cAMPS), an inhibitor of protein kinase A (PKA). CSF samples were collected before and 10 min after herkinorin and NTP administration for the measurement of cAMP levels. Data were analyzed by repeated-measures analysis of variance. Our results show that herkinorin binds to both kappa and mu opioid receptors. Its vasodilation effect is totally abolished by NTP, but is not affected by β -FNA. The levels of cAMP in the CSF elevate after herkinorin administration, but are abolished with NTP administration. The cerebral vasodilative effect of herkinorin is also blunted by Rp-cAMPS. In conclusion, as a non-opioid kappa and mu opioid receptor agonist, herkinorin exhibits cerebral vascular dilatation effect. The dilatation is mediated through the kappa opioid receptor rather than the mu opioid receptor. cAMP signaling also plays an important role in this process.

Keywords

Herkinorin; Opioid receptors; Signal transduction; Cerebrovasodilation

1. Introduction

Herkinorin is the first non-opioid mu agonist derived from the structurally related compound salvinorin A (Butelman et al., 2008). Since kappa opioid receptor activation elicits pial artery dilation (Armstead, 1998) and salvinorin A is a potent cerebral vasculature dilator that activates nitric oxide synthases, kappa receptors, and adenosine triphosphate-sensitive potassium channels (Su et al., 2011), it is likely that herkinorin could also elicit cerebrovasodilation. Herkinorin has an approximately 8-fold selectivity for mu over kappa receptors and an approximately 98-fold selectivity for mu over delta receptors in competition binding assays (Harding et al., 2005). Thus, it is important to elucidate whether its mu agonism plays any role in the cerebral vasculature effects for compounds from this category due to their potential clinical implications as non-opioid receptor agonist.

cAMP is a key modulator downstream of opioid receptors (Liu and Anand, 2001) and activation of cAMP signaling elicits vascular smooth muscle relaxation, resulting in cerebrovasodilation in the pig brain (Parfenova et al., 1994). In addition, administration of opioid receptor antagonists attenuated cAMP analog-induced pial dilation (Wilderman and Armstead, 1996), suggesting a potential connection between cAMP-mediated and opioid-mediated vasodilations. It is possible that herkinorin could induce cerebral vascular dilation via cAMP pathway.

Here, we hypothesized that herkinorin, the first non-opioid mu agonist derived from salvinorin A, could dilate cerebral vasculature via mu and kappa opioid receptors and cAMP pathway. This hypothesis is distinctive from our previous study related to salvinorin A since herkinorin is categorized as a mu receptor agonist despite its structural similarity to the highly selective kappa opioid receptor agonist salvinorin A.

2. Results

2.1. Herkinorin binding with mu and kappa receptors

As shown in Fig. 1A, herkinorin has a relatively weaker binding affinity with the mu receptor ($K_i=45$ nM) compared with DAMGO ($K_i=2.5$ nM). The binding site of herkinorin overlaps with that of β -FNA, a selective mu opioid receptor ligand in the crystal structure shown in Fig. 1B. Similarly, herkinorin has a relatively weaker affinity with kappa receptor ($K_i=184$ nM) compared with U69593 ($K_i=0.8$ nM, Fig. 2A) and the binding site overlaps with JDTC, a selective kappa receptor ligand in the crystal structure shown in Fig. 2B. The binding affinity of herkinorin to mu receptor is approximately 4-fold stronger than that to kappa receptor.

2.2. Herkinorin-induced kappa receptor-dependent vasodilation upon administration

The pial artery diameters increased after herkinorin administration without significant systemic blood pressure variation. Applying 0.1 nM herkinorin induced a 10.6% diameter dilation while 10 nM herkinorin induced a 17.8% diameter dilation on average. The dilation effects were totally abolished by norbinaltorphimine (NTP), a kappa receptor antagonist (Fig. 3A, $P<0.05$ compared with herkinorin administration groups), but not affected by β -FNA (Fig. 3B). Isoproterenol-induced pial artery dilation was unchanged by either NTP or β -FNA. β -FNA itself elicited minimal pial artery dilation ($P<0.05$, Dunnett's multiple comparison tests). These results indicate that the herkinorin-induced vasodilation is mediated via kappa, but not mu opioid receptor.

2.3. Pial artery dilation by herkinorin was mediated via cAMP signaling

Administration of herkinorin significantly increased cAMP levels in the CSF. The elevated cAMP levels were blocked by NTP, but not affected after β -FNA administration (Fig. 4A). Furthermore, the PKA antagonist Rp-cAMPS blunted herkinorin-mediated pial artery dilation. Co-administration of 10 μ M Rp-cAMPS with 0.1 nM or 10 nM herkinorin attenuated the changes in pial artery diameters (Fig. 4B). The artery dilation induced by cAMP analog Sp-cAMPS is similar to that of isoproterenol ($P>0.05$). These data suggest that cAMP/PKA pathway modulates the herkinorin-mediated cerebrovasodilation.

3. Discussion

There are three new major findings in this study. First, herkinorin is a potent pial artery dilator despite its classification as a non-opioid mu receptor agonist. Second, the herkinorin-induced pial artery dilation effects are modulated via kappa opioid receptors. No significant involvement of the mu opioid receptor is observed. Third, cAMP is demonstrated to be involved in the kappa agonist induced cerebral vascular dilatation. This study also confirms previous findings that herkinorin interacts with both mu and kappa opioid receptors and the binding site for this interaction overlaps with that of other traditional opioid receptor ligands.

3.1. Herkinorin as a non-nitrogenous opioid receptor agonist

Although herkinorin is an opioid receptor agonist, it does not contain nitrogen, an essential element for the traditional nitrogenous opioid ligand. Thus, herkinorin is the first non-opioid mu opioid receptor ligand (Butelman et al., 2008). Herkinorin was discovered in 2005 when various analogues of the natural product Salvinorin A were synthesized to study the structure and the function of neoclerodane diterpenes (Harding et al., 2005; Tidgewell et al., 2006). While salvinorin A is a selective kappa opioid agonist with no significant mu opioid receptor affinity (Roth et al., 2002), herkinorin acts on both mu and kappa receptors. Its affinity for the mu receptor is much stronger than that for the kappa receptor as demonstrated here and in other studies (Tidgewell et al., 2006). Thus, unlike salvinorin A, herkinorin is categorized as a mu opioid receptor ligand. Its binding site overlaps well with the sites for other receptor ligands as demonstrated in the docking experiments.

Interestingly, the fact that herkinorin does not induce β -arrestin recruitment or promote receptor internalization (Groer et al., 2007; Tidgewell et al., 2008) suggests that herkinorin may not induce significant tolerance or dependence as traditional opioids do. A recent study indicates that herkinorin could produce a dose-dependent antinociceptive effect in a rat pain model, suggesting that herkinorin may be a promising starting point for developing novel analgesics without significant risk of dependence or tolerance (Lamb et al., 2012).

3.2. The effects on brain vessels and the role of receptors

In the present study, herkinorin exhibits similar pharmacological features for cerebral vasculature to salvinorin A as we demonstrated previously (Su et al., 2011). Herkinorin seems to be a more potent artery dilator than salvinorin A because the concentration required to effectively dilate pial arteries (10–16% changes compared to the baseline) is much lower for herkinorin (0.1 nM) compared to that of salvinorin A (10 nM). The cerebrovasodilation effect of herkinorin is blocked by NTP, but not β -FNA. Similar to our previous study, neither NTP nor β -FNA shows any effect on pial diameter by itself (Armstead, 1998). Thus, the cerebral vascular dilatation effects of herkinorin are mediated through the kappa opioid receptor rather than the mu receptor.

3.3. The role of cAMP

Cerebrovasodilation is mediated through several mechanisms, including cGMP, cAMP, and K^+ channels (Faraci and Heistad, 1998). Isoproterenol and cAMP increase the activities of calcium-dependent potassium channels in the cerebral vascular muscle, which is believed to induce vasodilatation. In this study, we observed that cerebrovasodilation is associated with the elevation of cAMP levels in the CSF after administration of herkinorin. Moreover, the cerebrovasodilation induced by herkinorin administration is abolished by the cAMP antagonist Rp-cAMPS. This observation is consistent with reports that administration of cAMP analog Sp-cAMPS elicits vasodilation, which can be blunted by Rp-cAMPS (Wilderman and Armstead, 1996). Together, these findings indicate that cAMP plays an important role in the herkinorin induced cerebral vascular dilatation. We previously demonstrated the involvement of cGMP in salvinorin A-mediated vascular dilatation (Su et al., 2011). It is highly likely that cGMP might also be involved in the dilatation effects of herkinorin though further study is warranted.

3.4. Potential implications and future studies

Because salvinorin A could dilate cerebral vasculature (Su et al., 2011) and preserve cerebral autoregulation from cerebral hypoxia/ischemia injury in a piglet model, it is highly likely that herkinorin could have similar properties given that its cerebrovasodilative effects are modulated in a manner similar to salvinorin A. Thus, it might be an alternative non-opioid medication to be used during the perioperative period for patients who are at risk for cerebral vascular spasm or ischemia. Such potential clinical implications need to be investigated. We suspect that the effects and mechanisms of herkinorin-induced cerebrovasodilation are similar in adults. However, this will need to be fully addressed in future experiments using juvenile/adult animal models.

In conclusion, as a non-opioid kappa and mu opioid receptor agonist, herkinorin exhibits a cerebral vascular dilation effect. The dilation is mediated through kappa opioid receptor rather than mu opioid receptor. cAMP signaling also plays an important role in this process.

4. Experimental procedures

Herkinorin (purity 99%) was obtained from Ascent Scientific LLC (Cambridge, MA, USA). Isoproterenol, NTP, β -FNA, Rp-cAMPS and Sp-8-Br-2'-O-Me-cAMPS (Sp-cAMPS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of reagent grade and were obtained from Sigma as well.

4.1. In-vitro affinity determination

Cell lines transfected with cloned rat kappa receptor and cloned human mu receptor were used for affinity determination as described previously (Roth et al., 1981). [3H]U69593, a potent kappa receptor agonist, and [3H]DAMGO, a μ receptor agonist, were used as competitors of the testing compounds. Herkinorin was prepared as 1 mg/ml stock in DMSO. A similar stock of salvinorin A (positive control) was also prepared. K_i determinations were performed at the National Institute of Mental Health's Psychoactive Drug Screening Program (Contract no. HHSN-271-2008-00025-C, NIMH PDSP; <http://pdsp.med.unc.edu/>).

4.2. Binding site location with computational docking

Docking calculations were carried out using Docking server as described previously (<http://www.dockingserver.com>) (Liu et al., 2012) to locate ligand binding site. The herkinorin coordinates were downloaded from the PubChem server (<http://pubchem.ncbi.nlm.nih.gov/>). The coordinates of the high-resolution structures of the murine μ opioid receptor (PDB code: 4DKL (Manglik et al., 2012)) and kappa opioid receptor (PDB code: 4DJH (Wu et al.,

2012)) were downloaded from the protein data bank (PDB) server. We added Gasteiger partial charges to the herkinorin atoms through the server. Affinity (grid) maps of $30 \times 30 \times 30$ Å grid points and 0.375 Å spacing were used in this study. The top ranked pose of the docking results were used to compare the overlapping of the ligand for each receptor. We used PyMOL (<http://www.pymol.org/>, Version 1.3, Schrödinger, LLC) to generate the graphical renderings.

4.3. Animals and surgery

Newborn pigs (aged up to 6 days, 1.1–2.0 kg) of both genders were used for this study. Protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania (Philadelphia). The newborn piglet model was used because its brain is gyrencephalic and contains more white matter than grey matter, which is similar to that of humans. Furthermore, the head is large enough for the insertion of a cranial window and vascular visualization. Animals were induced with isoflurane (1–2 minimum alveolar concentration) and maintained with α -chloralose (80–100 mg/kg supplemented with 5 mg/kg h, IV). Both femoral arteries were catheterized to monitor blood pressure and blood gas to maintain a constant carbon dioxide and pH. A catheter was inserted into the right femoral vein for medication administration. Animals were ventilated with room air after tracheal intubation. A heating pad was used to maintain the rectal temperature of animals at 37–39 °C. A closed cranial window on the top of the head of the piglet was placed for direct pial artery visualization and diameter measurement (Devine and Armstead, 1995). The closed cranial window consisted of three parts: a stainless steel ring, a circular glass cover slip, and three ports consisting of 17-ga hypodermic needles attached to three precut holes in the stainless steel ring. CSF was collected through a cranial window port for cAMP measurement in some animals. The space under the window was filled with artificial CSF with the following composition (in mM): 3.0KCl; 1.5MgCl₂; 1.5calcium chloride; 132NaCl; 6.6urea; 3.7dextrose; 24.6NaHCO₃; pH 7.33; PaCO₂, 46 mm Hg; and PO₂ 43 mm Hg. Artificial CSF was warmed to 37–38 °C before applying to the cerebral cortical surface. Pial arteries were observed with a video camera mounted on a dissecting microscope. Vascular diameter was measured from a video monitor that was connected to the camera with a video microscaler (VPA 550; For-A-Corp., Los Angeles, CA) by the investigator who administered the medication.

4.4. Protocol

Two types of pial vessels, small arteries (resting diameter 120–160 μ m) and arterioles (resting diameter 50–70 μ m), were monitored and recorded every 30 s after injection of artificial CSF in the presence or absence of the investigated drugs. Typically, the window was flushed with 1–2 ml CSF through the port in 30 s. Responses to herkinorin (0.1 nM and 10 nM, dissolved with DMSO) and isoproterenol (10 nM and 1 μ M), were obtained with and without β -FNA, NTP, Sp-cAMPS and Rp-cAMPS. All tested drug solutions were made fresh on the day of use.

4.5. cAMP assay

The cerebral cortical periarachnoid CSF samples were collected before and 10 min afterward administration of herkinorin and NTP to measure the cAMP level. Artificial CSF was slowly infused into one port of the window, allowing the CSF to drip freely into a collection tube on the opposite port. Commercially available ELISA kits (Assay Designs, Ann Arbor, MI) were used to quantify cAMP concentrations.

4.6. Statistical analysis

All data were analyzed with one way-ANOVA (two-tailed) followed by Bonferroni post hoc test or by Dunnett's multiple comparison tests (SPSS 11.0 for Windows). An alpha level of $P < 0.05$ was considered significant in all statistical tests. All values are represented as means $\pm$ standard error. All P -values reported in the paper have been corrected for the effect of multiple comparisons. Although the sample size in this study was rather small, there was no apparent violation of the assumptions of lack of interaction, homogeneity of variance, and normal distribution.

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Fig. 1. Affinity determination for herkinorin in HEK cells over-expressed with mu and kappa opioid receptor. Part (A) demonstrates the binding affinity of herkinorin with the mu receptor as compared to DAMGO, a potent mu agonist. The K_i is 2.5 nM for DAMGO and 45 nM for herkinorin. The model illustrated in (B) suggests that herkinorin (labeled as H over the red sphere ligand in the binding pocket) binds to the same binding site as that for β -funaltrexamine (labeled as β over the light blue sphere ligand in the binding pocket), a selective mu opioid receptor ligand found in the crystal structure.

Fig. 2. Affinity determination for herkinorin in HEK cells over-expressed with kappa opioid receptor and the location of the binding site. Part (A) demonstrates the binding affinity of herkinorin with kappa receptor as compare to U69593, a potent kappa agonist. The K_i is 0.8 nM for U69593 and 184 nM for herkinorin. The model illustrated in (B) suggests that herkinorin (labeled as H over the red sphere ligand in the binding pocket) binds to the same binding site as that for JDtic (labeled as J over the green sphere ligand in the binding pocket), a selective kappa opioid receptor ligand found in the crystal structure.

Fig. 3.

The cerebrovasodilation effects of herkinorin is mediated through kappa opioid receptor. Part (A) demonstrates the dilatation effect of herkinoin (Herk) on pial artery is blocked by the kappa receptor antagonist norbinaltorphimine (NTP) (A); but not blocked by the mu receptor antagonist β -funaltrexamine (β -FNA, B). The dilatation effect of herkinoin is equivalent to that of isoproterenol (ISO), a potent beta adrenergic agonist (A) ($P>0.05$). Administration of NTP or β -FNA alone does not have any dilatation effects (one way Anova followed by Dunnett's multiple comparison tests). $n=5$ for each group. *For $p<0.05$, **for $p<0.01$, ***for $p<0.005$ in t -tests, and # for $p<0.05$ in ANOVA tests.

Fig. 4. Cerebrovasodilation effects of herkinorin is mediated via cAMP signaling. Part (A) demonstrates that the levels of cAMP in CSF elevated with herkinorin administration, which were abolished with administration of the kappa antagonist NTP. Part (B) demonstrates that the cerebrovasodilation effects of herkinorin were abolished by administration of 10 μ M Rp-cAMPS, a cAMP antagonist ($n=5$) Sp: Sp-cAMPS, Rp: Rp-8-Br-cAMPS. *For $p<0.05$, **for $p<0.01$, ***for $p<0.005$.