In Silico Screening Identified Novel Small-molecule Antagonists of PAC1 Receptor[S]

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ABSTRACT

Pituitary adenylate cyclase-activating polypeptide (PACAP) and its receptors are present in the spinal dorsal horn and dorsal root ganglia, suggesting an important role of PACAP signaling systems in the modulation of spinal nociceptive transmission. Previously, we found that intrathecal injection of PACAP or maxadilan, a selective PACAP type I (PAC1) receptor agonist, induced transient aversive responses followed by a long-lasting mechanical allodynia in mice, suggesting that PACAP-PAC1 receptor systems are involved in chronic pain and that selective PAC1 antagonists may become a new class of analgesics. Although several PAC1 antagonists, such as PACAP 6–38, have been reported, all of them are peptide compounds. In the present study, we identified new small-molecule antagonists of the PAC1 receptor using in silico screening and in vitro/vivo pharmacological assays. The identified small-molecule compounds, named PA-8 and PA-9, dose dependently inhibited the phosphorylation of CREB induced by PACAP in PAC1-, but not VPAC$_1$- or VPAC$_2$-receptor-expressing CHO cells. PA-8 and PA-9 also dose dependently inhibited PACAP-induced cAMP elevation with an IC$_{50}$ of 2.0 and 5.6 nM, respectively. In vivo pharmacological assays showed that intrathecal injection of these compounds blocked the induction of PACAP-induced aversive responses and mechanical allodynia in mice. In contrast, the compounds when administered alone exerted neither agonistic nor algesic actions in the in vitro/vivo assays. The compounds identified in the present study are new and the first small-molecule antagonists of the PAC1 receptor; they may become seed compounds for developing novel analgesics.

Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) was originally isolated from ovine hypothalamic extracts based on its ability to stimulate adenylate cyclase in rat anterior pituitary cell cultures (Miyata et al., 1989). PACAP exists as two variants, a 38-amino acid form (PACAP38) and a C-terminal truncated form (PACAP27) (Miyata et al., 1989, 1990), both of which share 68% homology with vasoactive intestinal polypeptide (VIP), suggesting that PACAP belongs to the VIP/secretin/glucagon superfamily (Miyata et al., 1989, 1990;

Vaudry et al., 2009). Three distinct G-protein-coupled receptors mediate the actions of PACAP and VIP. The PACAP type I (PAC1) receptor, which is coupled mainly to adenylate cyclase/protein kinase A, binds the two forms of PACAP with high affinity and selectivity. VPAC$_1$ and VPAC$_2$ receptors, which are also primarily coupled to adenylate cyclase, can bind both PACAP and VIP with similar affinities (Dickson and Finlayson, 2009; Vaudry et al., 2009).

PAC1 receptor is particularly abundant in the central nervous system, including the spinal dorsal horn (Dickson and Finlayson, 2009; Vaudry et al., 2009), where PACAP-immunoreactive fibers are also considerably localized (Moller et al., 1993; Dun et al., 1996). These fibers are thought to be predominantly primary afferents in origin, because capsaicin treatment induces the release of PACAP from rat spinal cord (Zhang et al., 1997; Sakashita et al., 2001) and a significant decrease in the number of PACAP-immunoreactive nerve

ABBREVIATIONS: AR, aromatic; BNST, bed nucleus of the stria terminalis; CHO, Chinese hamster ovary; CREB, cAMP response element binding protein; 3D, three-dimensional; EC, extracellular; HBD, hydrogen bond donor; HP, hydrophobic; PACAP, pituitary adenylate cyclase-activating polypeptide; PAC1, pituitary adenylate cyclase-activating polypeptide type I; TBST, Tris-buffered saline/Tween 20; VIP, vasoactive intestinal polypeptide.

fibers within the spinal cord (Moller et al., 1993). Furthermore, PACAP mRNA/immunoreactivity is localized primarily in calcitonin gene-related peptide or substance P-immunoreactive neurons in rat dorsal root ganglia (Moller et al., 1993; Mulder et al., 1994) and is markedly upregulated after peripheral nerve injury or inflammation (Zhang et al., 1995, 1996, 1998; Jongsma Wallin et al., 2003; Mabuchi et al., 2004). Several lines of evidence indicate that PACAP and its receptor systems could play an important role in the modulation of spinal nociceptive transmission. It has been reported that intrathecal injection of PACAP induces thermal hyperalgesia in mice (Narita et al., 1996; Ohsawa et al., 2002) and that formalin-induced nociceptive responses are ameliorated by pretreatment with intrathecal PACAP 6–38, an antagonist of the PACAP receptor (Ohsawa et al., 2002; Davis-Taber et al., 2008). Formalin- or carrageenan-induced inflammatory pain and nerve injury-induced neuropathic pain have been found to decrease in mice deficient in PACAP and/or the PAC1 receptor (Jongsma et al., 2001; Mabuchi et al., 2004; Sándor et al., 2010).

Recently, we demonstrated that a single intrathecal injection of PACAP or maxadilan, a PAC1 receptor specific agonist (Uchida et al., 1998; Moro et al., 1999), induced transient nociceptive behaviors followed by a long-lasting mechanical allodynia in mice (Ohnou et al., 2016; Yokai et al., 2016), suggesting that spinal PACAP-PAC1 receptor systems play an important role in the modulation of spinal nociceptive transmission and induction of chronic pain. Our results, together with the previous studies, indicate that the PAC1 receptor has great potential as a drug target for treating both inflammatory and chronic neuropathic pain. Although several PAC1 receptor antagonists, such as PACAP 6–38, M65, and max.d.4 (Robberecht et al., 1992; Uchida et al., 1998; Moro et al., 1999), are used as pharmacological tools, all of them are peptide compounds. They are not practical as therapeutic agents because of their half-lives, lack of oral bioavailability, and difficulty in penetrating the blood-brain barrier. Therefore, the development of specific nonpeptide antagonists of the PAC1 receptor has been desired. However, to date, there have been no reports on the development of such PAC1 receptor antagonists.

In the present study, we attempted to identify potential antagonists of the PAC1 receptor using in silico screening followed by in vitro/vivo pharmacological assays. Here we report the development of novel small-molecule antagonists of the PAC1 receptor.

Materials and Methods

In Silico Screening. We performed a multifilter virtual screening protocol to explore candidate compounds for novel PAC1 receptor antagonists, as shown in Fig. 1. For this purpose, we used an NMR structure of the *N*-terminal extracellular (EC) domain of PAC1 receptor in complex with its peptide antagonist PACAP 6–38 (PDB ID: 2JOD) (Fig. 2A). When we examined the NMR structure, the residues Y22, V26, and R30 of PACAP 6–38 were found to be located on one side of its helical structure interacting with the PAC1 receptor, as shown in Fig. 2B. It has also been reported that a single point mutation of the residues Y22A, V26G, or R30A of PACAP 6–38 results in a drastic loss in affinity to the receptor (Sun et al., 2007), indicating that these residues play important roles in the antagonist activity of PACAP 6–38. Therefore, we explored compounds that were expected to interact with the PAC1 receptor in a manner similar to Y22, V26, and R30 of PACAP 6–38 (Fig. 2B).

Materials. PACAP (38-amino acid form), VIP, and PACAP 6–38 were purchased from Peptide Institute Inc. (Osaka, Japan). These drugs were made up as concentrated stock solutions in phosphate-buffered saline or physiologic saline, and stored at −30°C. An aliquot was diluted to the desired concentration in phosphate-buffered saline or artificial cerebrospinal fluid (NaCl 138 mM, KCl 3 mM, CaCl$_2$ 1.25 mM, MgCl$_2$ 1 mM, D-glucose 1 mM) immediately before use.

Cell Culture. PAC1, VPAC$_1$, and VPAC$_2$ receptor-expressing CHO cells (PAC1/CHO, VPAC$_1$/CHO, and VPAC$_2$/CHO cells, respectively) were cultured in Dulbecco's modified Eagle's medium/F-12 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 200 μg/ml G418 or 5 μg/ml blasticidin S. The cells were propagated in a humidified 37°C incubator in 5% CO$_2$. VPAC$_1$/CHO and VPAC$_2$/CHO cells were kindly provided by Dr. I. Tatsuno (Toho University, Tokyo, Japan).

Western Blot Analysis. Cells were seeded onto 12-well plates at 1×10^5 cells/well and incubated for 48 hours. After treatment with candidate compounds or 0.1% dimethylsulfoxide (vehicle control) for 30 minutes, the cells were stimulated with PACAP for 30 minutes.

Cellular materials were lysed in lysis buffer [150 mM NaCl, 1% NP-40, 10% glycerol, protease inhibitor mix (Nacalai Tesque, Kyoto, Japan), and 20 mM Tris-HCl (pH 7.4)]. The lysate (20 μg total protein per lane) was subjected to SDS-polyacrylamide gel electrophoresis (10% gel) and then blotted onto PVDF membranes (GE healthcare, Buckinghamshire, UK). The membranes were blocked with Blocking One (Nacalai Tesque) at room temperature for 1 hour. The membranes were then incubated with primary antibodies in Tris-buffered saline 0.1% Tween 20 (TBST) and 5% Blocking One at 4°C for 16–18 hour, washed three times with TBST, and exposed to peroxidase-conjugated secondary antibody in TBST containing 5% Blocking One at room temperature for 1 to 2 hours. Immunoreactive proteins were visualized by a luminescent image analyzer (LAS-1000 plus; Fujifilm, Tokyo, Japan) using an enhanced chemiluminescence detection system (Chemi-Lumi One L; Nacalai Tesque). The following primary antibodies were used; anti-phospho-CREB (Ser133) and anti-CREB (Cell Signaling Technology, Danvers, MA). The phosphorylation level of CREB was calculated as the ratio of the intensity of the band relative to that of total CREB protein in each sample.

Measurement of cAMP. cAMP-Glo Assay kit (Promega, Madison, WI) was used to measure intracellular cAMP levels according to the manufacturer's instructions. Briefly, the PAC1/CHO cells were
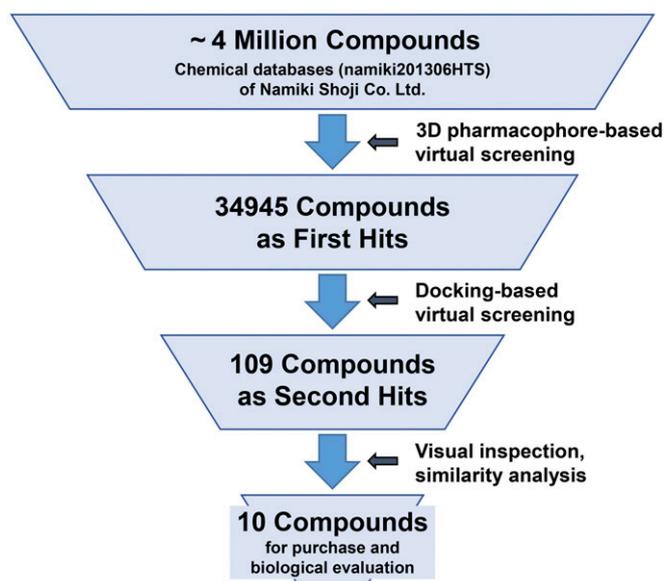

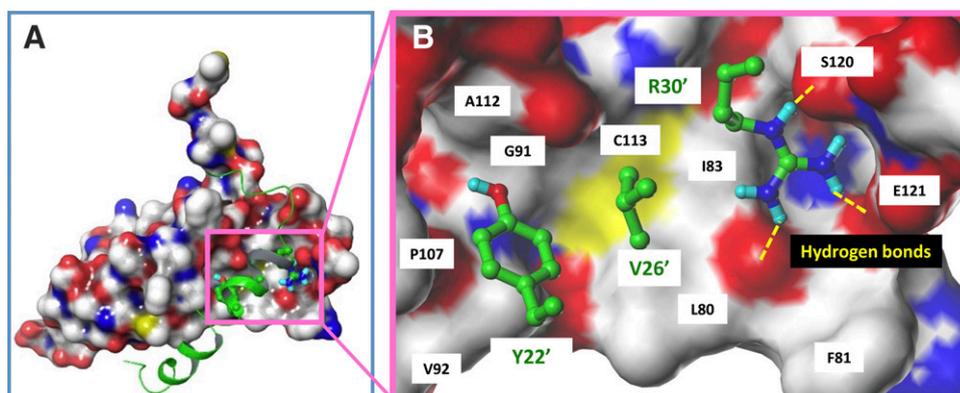**Fig. 1.** Multifilter virtual screening.

Fig. 2. (A) NMR structure of the *N*-terminal extracellular (EC) domain of PAC1 receptor in complex with PACAP 6–38 (green). (B) Interaction of Y22, V26, and R30 of PACAP 6–38 with PAC1 receptor. Hydrogen-bonding interactions are indicated by dashed yellow lines.

plated onto 96-well plates at 1×10^4 cells/well and incubated for 48 hours. After preincubation with the incubation medium [Dulbecco's modified Eagle's medium/F-12, 500 μM isobutyl-1-methylxanthine, and 100 μM 4-(3-butoxy-4-methoxy-benzyl) imidazolidone (Ro 20-1724)] for 1 hour, the cells were stimulated with PACAP (1 nM) for 1 hour. All candidate compounds were pretreated for 30 minutes before PACAP stimulation.

Animals. Male ddY mice (6 weeks old at the start of experiments) were purchased from Japan SLC Inc. (Shizuoka, Japan) and housed in standard polycarbonate cages (four mice per cage) under controlled temperature (24 ± 1°C) and humidity (55% ± 10%) with a 12-hour light–dark cycle (lights on at 0700) with food and water freely available. Mice were habituated to the animal facility for at least 1 week before experimentation. The animal experiments were approved by the Animal Care Committee of University of Toyama (approval no. 2014ENG-2) or Kagoshima University (approval no. MD 15011) and were conducted in accordance with the ethical guidelines for the study of experimental pain in conscious animals of the International Association for the Study of Pain (Zimmermann, 1983).

Intrathecal Injection and Behavioral Observation. Intrathecal injection was given in a volume of 5 μl by percutaneous puncture through an intervertebral space at the level of the fifth or sixth lumbar vertebra, according to a previously reported procedure (Hylden and Wilcox, 1980).

Before intrathecal injection, mice were placed and habituated in a glass cylinder ($\phi 14 \times 18$ cm) with a filter paper at the bottom over 20 minutes. Immediately after intrathecal injection, the mice were placed again in the same glass cylinder, and the number of nociceptive behaviors consisting of licking, biting, and scratching directed toward the caudal part of the body was counted every 1 minute. The cumulative number of events was pooled over 5-minute bins for 30-minute period of observation and analyzed (Shimizu et al., 2004; Ohnou et al., 2016).

Mechanical sensitivity of the hind paw was evaluated with calibrated von Frey hairs (Stoelting Co., Wood Dale, IL) by measuring the tactile stimulus producing a 50% likelihood of hind paw withdrawal response (50% g threshold), which was determined using the up-down paradigm (Chaplan et al., 1994; Yokai et al., 2016).

All behavioral tests were done in double-blinded manner.

Statistical Analysis. Experimental data are expressed as mean ± S.E.M. Single comparisons were made using Student's two-tailed unpaired t test. One- or two-way analysis of variance followed by the Dunnett's test was used for multiple comparisons. For the analyses of mechanical thresholds, we employed the Mann-Whitney U test for single comparisons or the Friedman test followed by the Steel test for multiple comparisons. $P < 0.05$ was considered statistically significant.

Results

In Silico Screening. We performed a multifilter virtual screening protocol to explore candidate compounds for novel PAC1 receptor antagonists, as shown in Fig. 1. In the first stage, we performed three-dimensional (3D) pharmacophore-based virtual screening. We generated 3D pharmacophore models based on Y22, V26, and R30 of PACAP 6–38 in the NMR structure (Fig. 2B), using the Unity module implemented in SYBYL-X Suite (Certara USA Inc., Princeton, NJ). As shown in Supplemental Fig. 1, we constructed two 3D pharmacophore models, both of which possess seven pharmacophore features. Models 1 and 2 differ in features on the hydroxyl group of Y22. Because the hydroxyl group is able to be a hydrogen bond acceptor as well as a donor, we defined hydrogen bond acceptor and donor features on the hydroxyl group of Y22 in models 1 and 2, respectively. As a result, model 1 comprises one aromatic (AR), two hydrophobic (HP), one hydrogen bond acceptor, and three hydrogen bond donor (HBD) features. In contrast, model 2 comprises one AR, two HP, and four HBD features. These two pharmacophore models were used as 3D structural queries for retrieving compounds from the chemical database (201306HTS) of Namiki Shoji Co. Ltd. (Tokyo, Japan) using the Unity module. The 3D coordinates of each molecule were generated with the CONCORD module of SYBYL, and the flex search protocol was performed to screen the database. We used a surface volume constraint based on a MOLCAD surface of the *N*-terminal EC domain of the PAC1 receptor. We extracted compounds satisfying the following criteria as "first hits": 1) matching with at least three of four hydrogen-bond related features (one hydrogen bond acceptor and three HBD features in model 1 and four HBD features in model 2) and 2) matching with at least two of three features consisting of one AR and two HP features. As a result, we obtained 25,737 and 9208 hits from models 1 and 2, respectively. In total, 34,945 hits were subsequently screened using protein structure-based virtual molecular docking (Fig. 1).

In the second stage, molecular docking was performed using the Schrödinger Suite 2013-2 (Schrödinger, LLC, New York, NY). First, the 3D structures of 34,945 hits were constructed using the LigPrep 2.7 program, and their protonation states were predicted using the Epik 2.5 program. The resulting conformers were used in the following docking calculations. The NMR structure of the *N*-terminal EC domain of the PAC1 receptor described above was used as a receptor for docking. We used a virtual screening workflow implemented in the Schrödinger Suite 2013-2. In this workflow, we first filtered hit compounds using Oprea's criteria of lead-likeness (Oprea et al., 2001). Then, first-stage docking was done in HTVS mode using GLIDE version 6.0 of the Schrödinger Suite 2013-2, and

Fig. 4. Effects of PA-8 or PA-9 on the PACAP-induced phosphorylation of CREB in the CHO cells expressing PAC1, VPAC$_1$, or VPAC$_2$ receptors. The cells were incubated with PA-8 or PA-9 compounds (10 pM to 10 nM) for 30 minutes, then the cells were stimulated with PACAP (1 nM). The data represent the mean ± S.E.M. (n = 3–5).
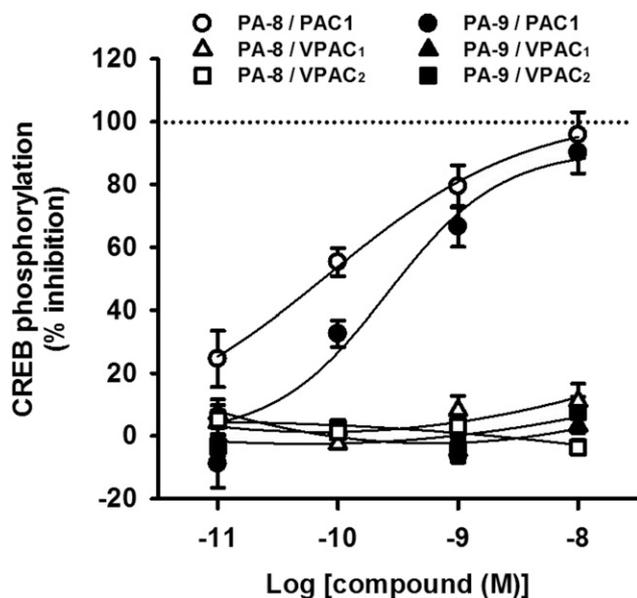

Fig. 5. Dose-dependent inhibitory effects of PA-8, PA-9, or PA-10 on the PACAP-induced cAMP elevation in the CHO cells expressing PAC1 receptors. The cells were incubated with PA-8, PA-9, or PA-10 (1 pM to 100 μM) for 30 minutes, then the cells were stimulated with PACAP (1 nM) for 1 hour and assessed the level of cAMP using cAMP-Glo Assay kit. The data represent the mean ± S.E.M. (n = 3–5).

(Fig. 6A). PA-8 and PA-10 almost completely suppressed PACAP-induced aversive responses. In contrast, a single intrathecal injection of PA-8, PA-9, or PA-10 (100 pmol/5 μl) alone did not induce aversive responses, at least during the 30-minute observation period after injection (Fig. 6B), suggesting that these compounds do not have algesic effects.

Finally, we examined the effects of the three PA compounds on the induction of PACAP-induced mechanical allodynia. In accordance with our previous report (Yokai et al., 2016), a single intrathecal injection of PACAP (100 pmol) induced mechanical allodynia beginning 1 day after injection and persisting for a minimum of 28 days (Fig. 6C). Co-injection of PA-8, PA-9, or PA-10 (100 pmol each) with PACAP (100 pmol) significantly blocked the induction of PACAP-induced mechanical allodynia (Fig. 6C). However, a single intrathecal injection of PA-8, PA-9, or PA-10 (100 pmol) did not induce mechanical allodynia, at least during the 3-day observation period after injection (Fig. 6D).

Models of PA-8 or PA-9 Binding with PAC1 Receptor. Since the identified compounds, particularly PA-8 and PA-9, showed potent and specific antagonistic activity toward PAC1 receptors, we constructed the models of PA-8 or PA-9 binding with the *N*-terminal EC domain of the PAC1 receptor (Fig. 7). Both PA-8 and PA-9 were found to be well accommodated in the subpocket formed by L80, F81, I83, G91, V92, P107, A112, and C113 of the PAC1 receptor, precipitating in hydrophobic interactions. In addition, PA-8 was predicted to form two hydrogen bonds with the backbone carbonyl oxygen of S120 and the oxygen of the carboxylate group of E121 (Fig. 7A). PA-9 was also predicted to form three hydrogen bonds with the backbone carbonyl oxygens of L80, V92, and S120 (Fig. 7B).

Discussion

In the present study, we attempted to identify small-molecule antagonists of the PAC1 receptor by using in silico screening. The 10 compounds identified were further
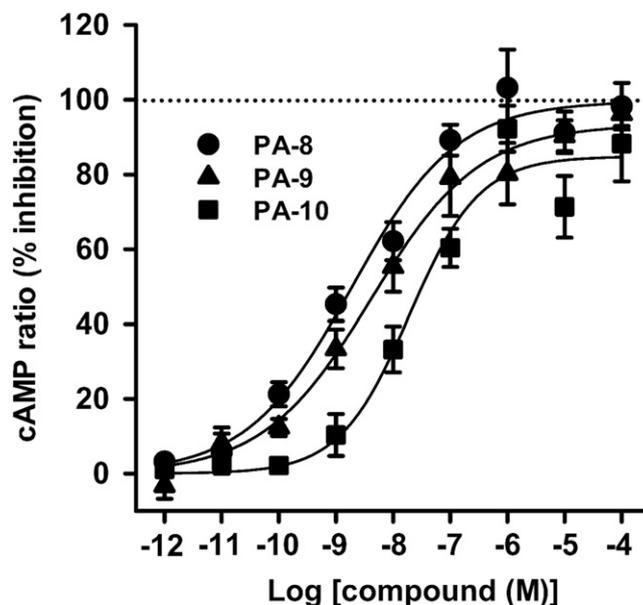
characterized using cell-based functional assays: PACAP-induced CREB phosphorylation and cAMP elevation. We finally identified three small-molecules (named PA-8, PA-9, and PA-10) as candidate antagonists of the PAC1 receptor from 10 virtual hits, corresponding to a hit rate of 30%. This high hit rate suggested that our multifilter virtual screening protocol was quite useful for exploring novel PAC1 receptor antagonists. The identified compounds, particularly PA-8 and PA-9, showed potent and specific antagonistic activity toward PAC1 receptors, but not VPAC$_1$ or VPAC$_2$ receptors, and no detectable agonistic activity toward PAC1 receptors. These compounds also blocked intrathecal PACAP-induced aversive responses and mechanical allodynia without any algesic effects. To the best of our knowledge, this is the first report on the discovery of nonpeptide small-molecule antagonists of the PAC1 receptor.

Studies of PACAP and PACAP receptors have been hampered due to a lack of specific PAC1 receptor antagonists. Although several PAC1 receptor antagonists, such as PACAP 6-38, M65, and max.d.4 (Robberecht et al., 1992; Uchida et al., 1998; Moro et al., 1999), have been often used as pharmacological tools, limitations exist in their use in vivo because all of them are peptide compounds. In particular, PACAP 6–38, a peptide fragment lacking the first five residues of PACAP, has been used in numerous studies as a PAC1 specific antagonist; however, the IC$_{50}$ value of binding of PACAP 6–38 for PAC1 and VPAC$_2$ receptors are 30 and 40 nM, respectively (Gourlet et al., 1995). Therefore, PACAP 6–38 should be recognized as a potent dual PAC1/VPAC$_2$ antagonist (Laburthe et al., 2007). It has also been reported that PACAP 6–38 acts as an agonist in certain cells/tissues, including isolated rat trachea and trigeminal ganglia neurons (Nemeth et al., 2006; Reglodi et al., 2008; Sághy et al., 2015). Maxadilan, a peptide isolated from salivary gland extracts of the hematophagous sand fly, is a specific
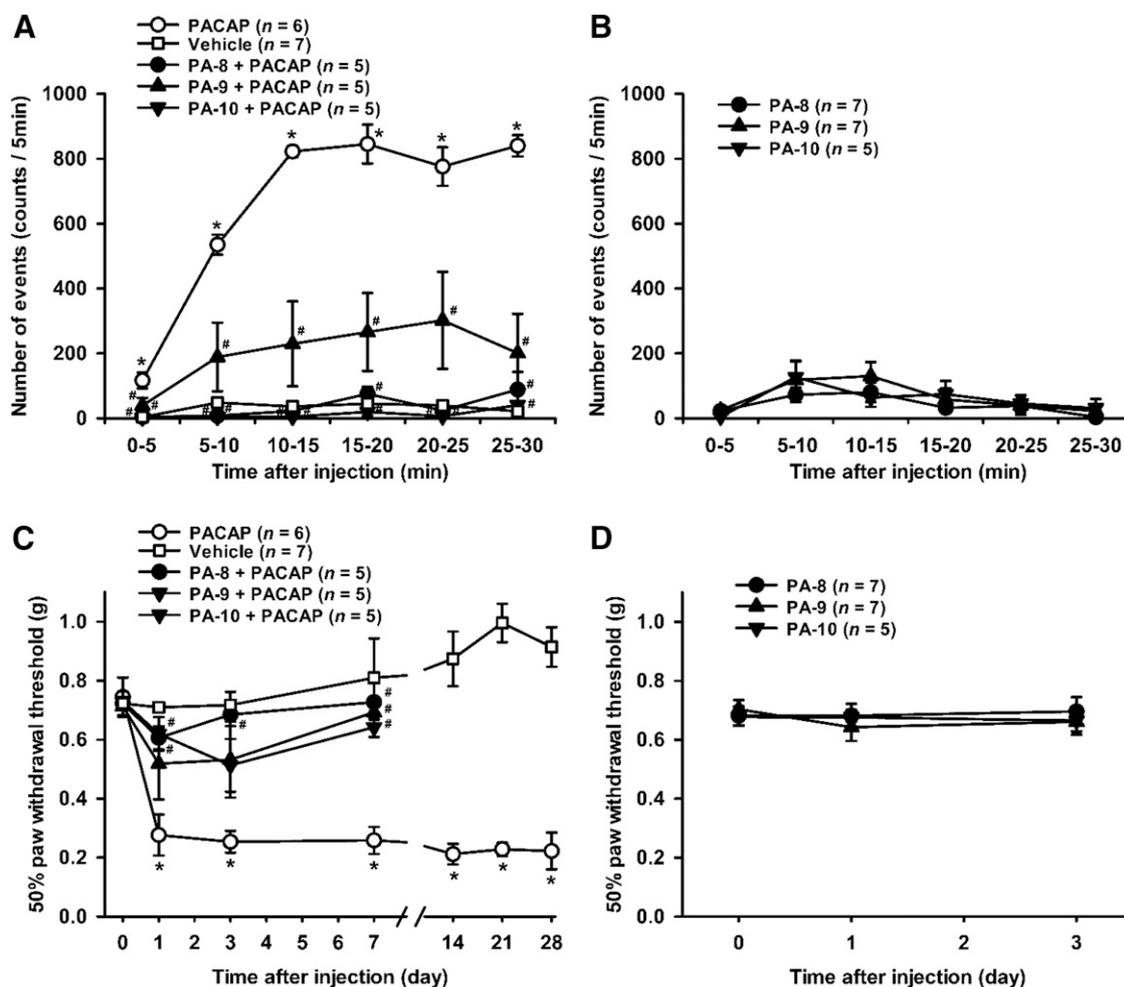
Fig. 6. Blockade of PACAP-induced aversive responses and mechanical allodynia by the identified PA compounds. (A) A single intrathecal injection of PACAP (100 pmol) induced transient aversive responses, and the PACAP-induced behaviors were ameliorated by co-injection of PA-8, PA-9, or PA-10 (100 pmol each). (B) PA-8, PA-9, or PA-10 (100 pmol each intrathecally) in itself did not induce aversive responses. (C) A single intrathecal injection of PACAP (100 pmol) induced long-lasting mechanical allodynia, and the induction of allodynia was prevented by co-injection of PA-8, PA-9, or PA-10 (100 pmol each). (D) PA-8, PA-9, or PA-10 (100 pmol each intrathecally) per se did not induce mechanical allodynia. *$P < 0.05$ compared with vehicle. #$P < 0.05$ compared with PACAP.

agonist of the PAC1 receptor, and a truncated peptide (#25–41) of maxadilan (termed M65) has been considered a specific PAC1 receptor antagonist (Uchida et al., 1998; Moro et al., 1999). However, M65 also showed similar stimulatory effects as maxadilan on trigeminal ganglia neurons (Sághy et al., 2015). The compounds we identified (PA-8, PA-9, and PA-10)

antagonized PACAP-induced intracellular events in PAC1-expressing cells but not in VPAC$_1$- or VPAC$_2$-expressing cells at very low doses (nanomolar order), and they had no agonistic actions. These small-molecules are likely to contribute to advancing our understanding of the function of PACAP-PAC1 receptor systems.
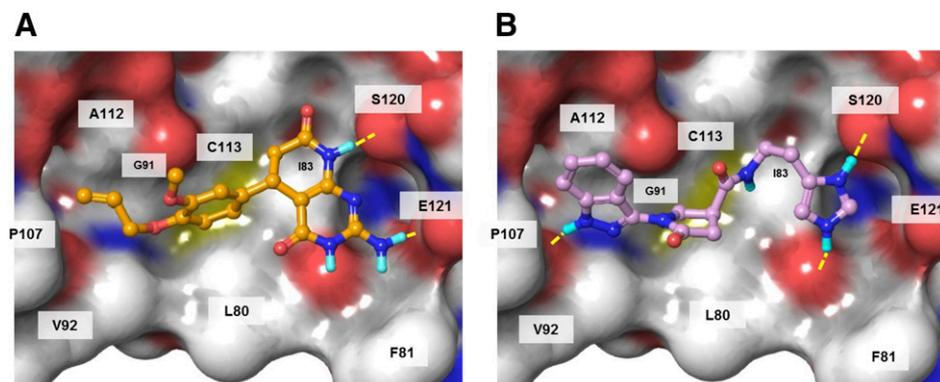
Fig. 7. Docking models of PA-8 (A) or PA-9 (B) with PAC1 receptor. Hydrogen-bonding interactions are indicated by yellow dashed lines.

A growing body of evidence demonstrates that the PACAP-PAC1 receptor signaling system in the spinal dorsal horn plays a crucial role in pain transmission. Recently, we showed that a single intrathecal administration of PACAP or maxadilan induced short-lasting aversive responses, such as licking, biting, and scratching, followed by a long-lasting mechanical allodynia (Ohnou et al., 2016; Yokai et al., 2016). Such aversive responses and mechanical allodynia were attenuated by intrathecal pretreatment with max.d.4, a specific PAC1 receptor antagonist (Ohnou et al., 2016; Yokai et al., 2016). In contrast to maxadilan, VIP, a selective agonist of VPAC$_1$ and VPAC$_2$ receptors but not PAC1 receptor, induced neither nociceptive behaviors nor mechanical allodynia (Ohnou et al., 2016; Yokai et al., 2016). Consistent with our previous findings, it has been reported that PAC1 receptor knockout mice have markedly reduced late-phase nociceptive responses in the formalin test (Jongsma et al., 2001). These findings strongly suggest that spinal PACAP-induced nociceptive behaviors and mechanical allodynia are predominantly mediated by the PAC1 receptor and that the PAC1 receptor is a useful target for this type of pain. In fact, in the present study, PACAP-induced aversive behaviors and mechanical allodynia were significantly inhibited by PA-8, PA-9, and PA-10. The results thus indicate that the identified compounds may be effective against pain in which the PACAP-PAC1 receptor system is implicated. It has been reported that transection of the sciatic nerve induces a rapid and strong upregulation of PACAP mRNA levels in mice and rats (Zhang et al., 1995, 1996; Mabuchi et al., 2004). Upregulation of PACAP in the dorsal root ganglia was also observed after peripheral inflammation induced by complete Freund's adjuvant (Zhang et al., 1998). Development of both inflammatory and neuropathic pain was shown to be abrogated in PACAP-deficient mice (Mabuchi et al., 2004). These results indicate that the spinal PACAP-PAC1 receptor system plays a central role in the regulation of these types of inflammatory and neuropathic pain, and potentially other types, and that the drugs targeting the PAC1 receptor may provide a new approach to treat PACAP-PAC1-mediated pain. Whether the novel PAC1 receptor antagonists identified here are effective against inflammatory and/or neuropathic pain needs to be examined in future studies.

Recent studies also point to the involvement of the PACAP-PAC1 receptor system in migraine pain and neuropsychiatric disorders. It has been reported that the infusion of PACAP, but not VIP, elicits a migraine-like attack in some healthy volunteers and migraine patients (Hansen et al., 2006; Rahmann et al., 2008; Schytz et al., 2009). In rodents, nitroglycerin-induced light-aversive behaviors (photophobia), one of the most common symptoms of migraine, were significantly reduced in PACAP knockout mice (Markovics et al., 2012). Thus, current evidence suggests the involvement of PACAP-PAC1 receptor signaling in migraine pathophysiology and the PAC1 receptor as a target for migraine treatment. A role of the PACAP-PAC1 receptor system in anxiety- and stress-related behaviors has also been proposed. It has been reported that chronic stress in mice leads to the upregulation of PACAP mRNA in the bed nucleus of the stria terminalis (BNST) (Hammack et al., 2009; Roman et al., 2014). Microinjection of PACAP or maxadilan into the BNST induced anxiety-like behaviors, and PACAP 6–38 injection into the BNST blocked stress-induced psychiatric behaviors

(Roman et al., 2014). Furthermore, mice lacking PACAP or the PAC1 receptor have been found to demonstrate reduced anxiety-like behavior (Hashimoto et al., 2001; Otto et al., 2001; Hattori et al., 2012). Whether the new PAC1 receptor antagonists we identified suppress migraine-like and/or stress-associated behaviors in mice is an interesting question that should be examined in future studies.

In conclusion, we identified novel small-molecule antagonists of the PAC1 receptor using in silico screening and in vitro/vivo pharmacological assays. These compounds will be useful for elucidating new functional roles of PAC1 receptor systems in the peripheral and central nervous systems and for further development of novel drugs against neuropathic and/or inflammatory pain, migraine, and psychiatric disorders.

Authorship Contributions

Participated in research design: Takasaki, Toyooka, Miyata, Kurihara.

Conducted experiments: Takasaki, A. Watanabe, Yokai, Nagashima, Okada, Fukuchi, Kurihara.

Contributed new reagents or analytic tools: Y. Watanabe, Hayakawa, Gouda.

Performed data analysis: Takasaki, A. Watanabe, Yokai, Y. Watanabe, Hayakawa, Gouda, Kurihara.

Wrote or contributed to the writing of the manuscript: Takasaki, Toyooka, Miyata, Gouda, Kurihara.

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