Collybolide is a novel biased agonist of κ-opioid receptors with potent antipruritic activity

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Among the opioid receptors, the κ-opioid receptor (κOR) has been gaining considerable attention as a potential therapeutic target for the treatment of complex CNS disorders including depression, visceral pain, and cocaine addiction. With an interest in discovering novel ligands targeting κOR, we searched natural products for unusual scaffolds and identified collybolide (Colly), a nonnitrogenous sesquiterpene from the mushroom *Collybia maculata.* This compound has a furyl-δ-lactone core similar to that of Salvinorin A (Sal A), another natural product from the plant *Salvia divinorum*. Characterization of the molecular pharmacological properties reveals that Colly, like Sal A, is a highly potent and selective κOR agonist. However, the two compounds differ in certain signaling and behavioral properties. Colly exhibits 10- to 50-fold higher potency in activating the mitogen-activated protein kinase pathway compared with Sal A. Taken with the fact that the two compounds are equipotent for inhibiting adenylyl cyclase activity, these results suggest that Colly behaves as a biased agonist of κOR. Behavioral studies also support the biased agonistic activity of Colly in that it exhibits ∼10-fold higher potency in blocking non–histamine-mediated itch compared with Sal A, and this difference is not seen in pain attenuation by these two compounds. These results represent a rare example of functional selectivity by two natural products that act on the same receptor. The biased agonistic activity, along with an easily modifiable structure compared with Sal A, makes Colly an ideal candidate for the development of novel therapeutics targeting κOR with reduced side effects.

G-protein–coupled receptors | natural compounds | salvinorin A | dynorphin | antinociception

Opioid receptors, members of family A of G-protein–coupled receptors (GPCRs), and their ligands have been extensively studied for their involvement in analgesia and sedation (1, 2). Among the different subtypes of opioid receptors, the κ opioid receptor (κOR) displays a repertoire of physiological actions distinct from μ (μOR) and δ (δOR) receptors (3). κOR activation leads to antinociception without the side effects associated with μOR activation such as physical dependence, respiratory depression, or inhibition of gastrointestinal transit (4, 5). In addition, κOR agonists have antipruritic effects and can block the effects of psychostimulants (6–8). Thus, κOR agonists could be potential therapeutics to treat addiction, visceral pain, pruritus, or pain killers with low abuse potential (9–12). However, in vivo κOR activation is associated with side effects like anxiety, stress, diuresis, dysphoria/aversion, and psychomimetic effects (6–8). Also, activation of the κOR system has been implicated in relapse to drugs of abuse; this limits the use of κOR agonists in the treatment of drug addiction (13, 14). Studies correlating the dysphoric effects of κOR agonists to the G-protein–independent activation of the p38 MAPK pathway (8, 13, 15) suggest that identification of κOR agonists biased to G-protein–mediated signaling could lead to the development of therapeutics with reduced side effects. This suggestion has spurred the search for novel κOR ligands or scaffolds that could provide novel treatment strategies for a variety of disorders where κOR involvement has been implicated (16, 17).

Previously identified synthetic κOR ligands were mostly nitrogenous compounds with a tertiary amine group (16). However, about a decade ago, Salvinorin A (Sal A), a nonnitrogenous diterpene extracted from the Mexican mint *Salvia divinorum* and characterized by the presence of a furyl-δ-lactone motif (Fig. 1), was identified as a potent and highly selective κOR agonist (18). The structural novelty of Sal A led to extensive structure–activity relationship (SAR) studies through hemisynthetic approaches (19). The furyl-δ-lactone group of Sal A (Fig. 1; motif highlighted in red) is present in other members of the terpene families (20) and appears as a highly oxygenated motif in the diterpene scaffold that is involved in ligand binding to κOR in conjunction with other oxygenated groups (esters) in the molecule.

In this study, we focused on the sesquiterpene Colly and its diastereoisomer 9-*epi*-Colly, extracted from the fungus *Collybia maculata,* because they represent the first examples, to our knowledge, of sesquiterpene structures with the furyl-δ-lactone motif (21). For molecular pharmacological characterization, we used heterologous cells individually expressing the three human opioid receptor types and show that Colly and 9-*epi*-Colly function as selective and potent κOR agonists. Furthermore, we show that, although Colly and Sal A activate some signal transduction pathways to the same extent and have comparable behavior effects in some assays, they exhibit substantial differences in other pathways and behavioral assays suggesting biased agonism. Finally, taken with our finding that Colly exhibits potent antipruritic

Significance

In recent years, the κ-opioid receptor (κOR) has become an attractive therapeutic target for the treatment of a number of disorders including depression, visceral pain, and drug addiction. A search for natural products with novel scaffolds targeting κOR has been intensive. Here, we report the discovery of a natural product (Colly) from the fungus *Collybia maculata* as a novel scaffold that contains a furyl-δ-lactone core structure similar to that of Salvinorin A, another natural product isolated from the mint *Salvia divinorum.* We show that Colly functions as a κOR agonist with antinociceptive and antipruritic activity. Interestingly, Colly exhibits biased agonistic activity, suggesting that it could be used as a backbone for the generation of novel therapeutics targeting κOR with reduced side effects.

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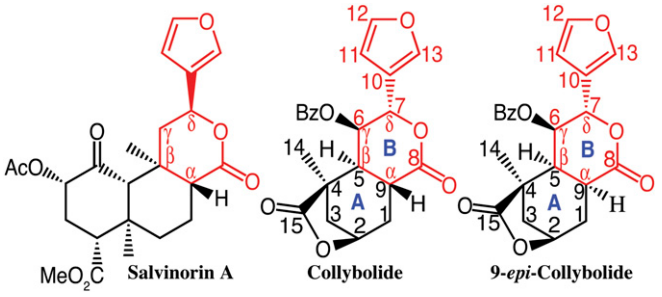

Fig. 1. Structures of salvinorin A, collybolide, and 9-*epi*-collybolide. Chemical structures of the naturally occurring Sal A (from the Mexican mint *S. divinorum*) and of collybolide and 9-*epi*-collybolide (from the fungus *C. maculata*) shown with the common furyl-δ-lactone motif highlighted in red. The absolute configurations of collybolide (2*R*,4*R*,5*S*,6*R*,7*S*,9*R*) and 9-*epi*-collybolide (2*R*,4*R*,5*S*,6*R*,7*S*,9*S*) were determined by the Bijvoet's method using the oxygen atoms as anomalous scattering centers (*SI Materials and Methods*).

activities, these results demonstrate that Collys represent a novel class of κOR-selective agonists that could be used for further explorations of the molecular pharmacology and functioning of this important therapeutic target.

Results

Collybolides Exhibit Structural Similarity to Sal A. Colly and 9-*epi*-Colly share the common furyl-δ-lactone motif (i.e., a δ-lactone with its Cδ carbon substituted by the furyl group) present in Sal A (Fig. 1). A bicyclic core with rings A (cyclohexane) and B (δ-lactone) is a common feature of these terpene compounds. Colly has a *trans*-ring A/B junction, as in Sal A, whereas 9-*epi*-Colly has a *cis* junction. Besides the δ-lactone, the central ring A is substituted by an additional ring, a highly oxygenated cyclohexane with two esters and a ketone in Sal A, and a γ-lactone in the Collys. In contrast to Sal A, Collys have a bulky C-6 benzoyl ester in ring B (Fig. 1). In this study, we report the absolute configurations of both sesquiterpenes using single crystal X-ray diffraction analysis in the absence of any chemical transformation (*SI Materials and Methods*) and establish the epimeric nature of Colly and 9-*epi*-Colly at the level of C-9 (Fig. 1). Interestingly, the absolute configuration of the asymmetric Cδ carbon (furyl-bearing carbon) in the δ-lactone (Fig. 1) is inverse in Sal A and Collys. The presence of the furyl-δ-lactone motif in Collys, as well as their novel stereochemical features led us to examine whether they could function as κOR selective agonists.

Collybolides Exhibit High Affinity Binding to Human κOR. Ligand binding assays using whole cells or membrane preparations expressing human κOR (hκOR) and [^{3}H]Naloxone, a nonselective opioid receptor antagonist, as the radiolabeled ligand show that Colly exhibits a dose-dependent displacement profile with a K_i ~10^{-10} M in membrane preparations (Fig. 2 and Table S1) and ~10^{-7} M in whole cells (Fig. S1). Sal A, the synthetic κOR agonist U69,593, and the peptidic κOR agonist dynorphin A8 (Dyn A8) displace [^{3}H]Naloxone binding with affinities in the nanomolar range (K_i ~ 1–7 × 10^{-9} M) in membrane preparations (Table S1), and 10^{-8}–10^{-9} M in whole cells (Table S2). Interestingly, epimerization at position 9 (9-*epi*-Colly) decreases the ability of the compound to displace [^{3}H]Naloxone binding to hκOR (Fig. 2, Table S1, and Fig. S1). The displacement profile of receptor-bound [^{3}H]Naloxone by Colly and 9-*epi*-Colly is not probe specific or dependent on the assay buffer conditions used, because a similar profile is seen with [^{3}H]Diprenorphine or [^{3}H]U69,593 or when using different assay buffer conditions (Fig. 2, Table S1, and Fig. S1). Together these results indicate that Collys bind to hκOR.

Collybolides Exhibit Selectivity to hκOR. We examined the selectivity of Collys for hκOR by comparing their ability to displace [^{3}H]Naloxone binding to hμOR or hδOR and to signal through these receptors. We find that neither Colly nor 9-*epi*-Colly displace [^{3}H]Naloxone binding to hμOR or hδOR (Fig. 3). Also, Collys do not elicit changes in [^{35}S]GTPγS binding or ERK1/2 phosphorylation in cells expressing hμOR or hδOR (Fig. S2) nor do they block DAMGO (μOR agonist) or deltorphin II (δOR)-mediated increases in phospho ERK1/2 (Fig. S2). These results indicate that Collys exhibit selectivity toward hκOR. This selectivity is supported by the ability of Colly to displace [^{3}H]Naloxone binding to membranes from WT mice but not from mice lacking κOR (Fig. 3 and Fig. S3).

Collybolide Is a High Affinity Agonist of hκOR. Next we examined signaling by Colly using [^{35}S]GTPγS binding assays. Colly dose-dependently increases [^{35}S]GTPγS binding with an EC_{50} of ~10^{-9} M (Fig. 4*A*), a potency similar to that of 9-*epi*-Colly, Sal A, U69,593, and Dyn A8 (Fig. 4*A* and Table 1). Interestingly, 9-*epi*-Colly exhibits substantial lower efficacy suggesting partial agonism (Fig. 4*A*). The increase in [^{35}S]GTPγS binding mediated by both Collys is blocked by a κOR antagonist, nor-BNI (Fig. 4*B*). Moreover, Colly dose-dependently increases [^{35}S]GTPγS binding to WT but not κOR KO membranes (Fig. 4*C*), supporting Collys as κOR selective agonists.

Because κOR activation leads to inhibition of adenylyl cyclase activity and consequently decreases in cAMP levels (22), we examined effects of Colly on adenylyl cyclase activity. We find that Colly dose-dependently decreases adenylyl cyclase activity with a nanomolar potency comparable to that of Sal A (Fig. 4*D* and Table 1) but markedly lower than that of 9-*epi*-Colly that exhibits a potency in the micromolar range (Table 1). Together these results indicate that Collys exhibit agonistic activity at hκOR.

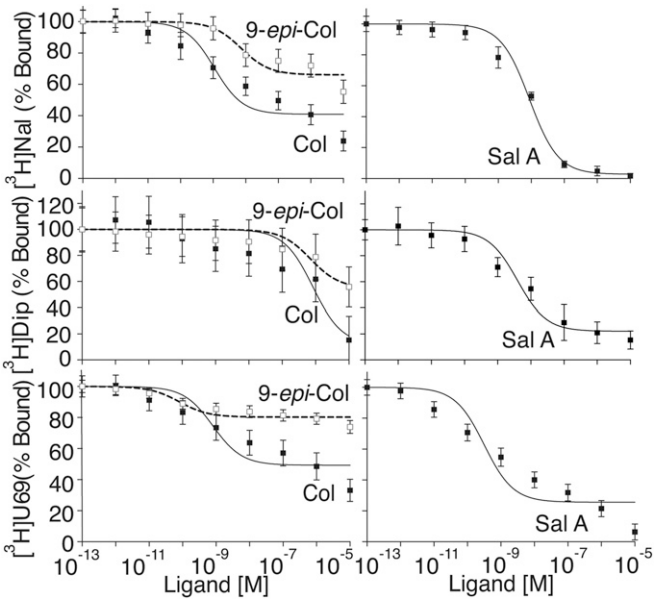

Fig. 2. Displacement of radiolabeled ligand binding to human κOR. Membranes (200 μg) from HEK-293 cells expressing hκOR were incubated with [^{3}H]Naloxone (Nal), [^{3}H]Diprenorphine (Dip), or [^{3}H]U69,493 (U69) (3 nM) in 50 mM Tris·Cl, pH 7.8, containing 1 mM EGTA, 5 mM $MgCl_2$, and protease inhibitor mixture in the absence or presence of Colly (Col), 9-*epi*-Colly (9-*epi*-Col), or Sal A (10^{-13}–10^{-5} M). Values at 10^{-13} M cold ligand were taken as 100%. Data represent mean ± SEM; n = 6.

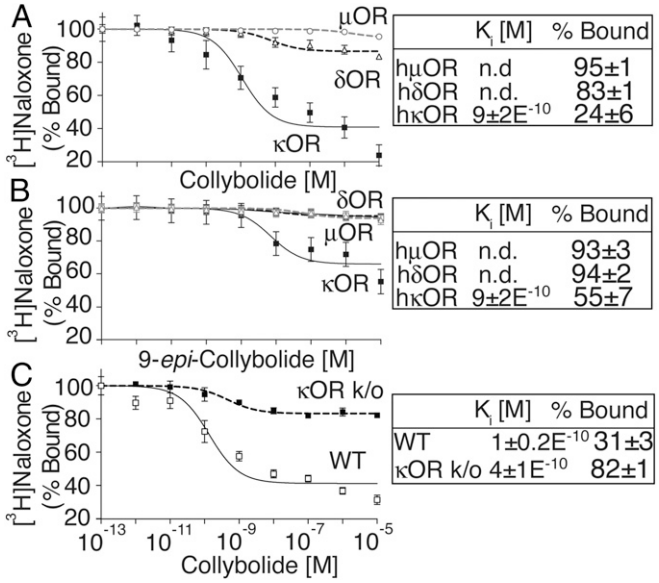

Fig. 3. Collybolides bind to human κOR but not μOR or δOR. Membranes (200 μg) from HEK-293 cells expressing hκOR, hδOR, or hμOR were incubated with [^{3}H]Naloxone (3 nM) in 50 mM Tris·Cl, pH 7.8, containing 1 mM EGTA, 5 mM $MgCl_2$, and protease inhibitor mixture in the presence of collybolide (*A*) or 9-*epi*-collybolide (*B*) (10^{-13}–10^{-5} M). Data represent mean ± SEM; $n = 6$. (*C*) Cerebral cortex membranes (100 μg) from WT or κOR KO mice (κOR k/o) were incubated with [^{3}H]Naloxone (3 nM) in the presence of collybolide (10^{-13}–10^{-5} M). Data represent mean ± SEM; $n = 3$ individual animals. Values at 10^{-13} M cold ligand were taken as 100%. n.d., not detected.

Collybolide Induces Robust Endocytosis of hκOR. Because treatment with agonists can induce rapid and robust internalization of hκOR (23), we examined the ability of Colly to induce κOR endocytosis. A time course analysis reveals rapid and robust endocytosis (Fig. S4), and the dose–response analysis reveals an EC_{50} for Colly ~5×10^{-10} M that is comparable to that of Sal A and much greater than that of 9-*epi*-Colly (Fig. 4*E* and Table 1). These results show that Colly, like other high potency hκOR agonists, induces rapid and robust receptor internalization.

Collybolide Exhibits Biased Agonism at hκOR. Next, we examined the ability of Collys to induce ERK1/2 phosphorylation. We find that Collys exhibit peak ERK1/2 phosphorylation at ~3–5 min (Fig. S5). Interestingly, Colly exhibits an inverted "U-shape" profile with maximal stimulation at ~10^{-9} M; at higher concentrations, Colly-mediated signaling is rapidly desensitizing both in HEK or CHO cells expressing hκOR (Fig. 4*F* and Fig. S5). In contrast, Sal A (like 9-*epi*-Colly and U69,593) exhibits a sigmoidal dose–response curve with maximal ERK1/2 phosphorylation at ~10^{-7}–10^{-6} M (Fig. 4*F* and Table 1). To ascertain whether the inverted U-shaped profile for ERK1/2 phosphorylation by Colly is also seen with other signaling pathways, we examined the profile for phosphorylation of Akt at S473 and at T308. We find that Colly exhibits a sigmoidal dose (and not an inverted U-shaped) response curve for Akt both at S473 and T308 (Fig. 4 *G* and *H*). Interestingly, Colly appears to be more efficacious than Sal A at phosphorylating Akt at both sites (Table 1). These observations suggest that Colly exhibits differences in signaling from Sal A at hκOR.

Collybolide Exhibits κOR Agonist Activity in Mice. Next, we focused on Colly and evaluated its behavioral effects in mice. We find that the antinociceptive activity of Colly is similar to that of Sal A with peak activity at 20 min following drug administration (Fig. 5*A*). We did not find significant differences between Colly and vehicle-treated controls in the forced swim test, although Colly-treated mice tended to spend less time being immobile (Fig. 5*B*). In the elevated plus maze test, Colly and Sal A did not exhibit significant differences, although Colly-treated animals showed a higher tendency to spend less time in the open arms (i.e., tend to exhibit a higher level of anxiety), and this was blocked in animals treated with nor-BNI (Fig. 5*C*). The effect of Colly in the conditioned place preference/aversion assay shows that single daily injections for 4 d leads to significant ($^*P < 0.05$) conditioned place aversion (Fig. 5*D*); this has been shown for κOR agonists including Sal A (3, 24). In contrast to these assays where Colly behaves in a manner similar to Sal A, Colly differs remarkably from Sal A when examined for its ability to block pruritus (non–histamine-mediated itch). Colly (2 mg/kg) significantly attenuates chloroquine-mediated scratching behavior; this is not seen with the same dose of Sal A (Fig. 5*E*). In addition, Colly exhibits dose-dependent inhibition of pruritus with a high potency

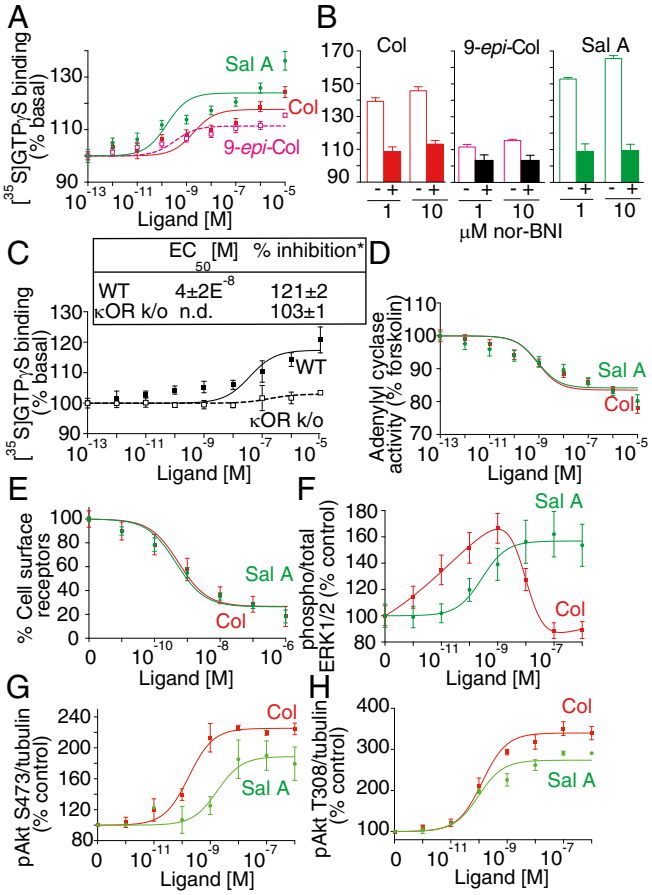

Fig. 4. Signaling by collybolides in membranes expressing hκOR. Membranes (20 μg) from HEK-293 cells expressing hκOR were subjected to a [^{35}S]GTPγS binding assay in the presence of Colly (Col), 9-*epi*-Colly (9-*epi*-Col), or Sal A (10^{-13}–10^{-5} M) (*A*) without or with preincubation (30 min) with nor-BNI (*B*). (*C*) [^{35}S]GTPγS binding using cerebral cortex membranes from WT and κOR KO mice and Col (10^{-13}–10^{-5} M). (*D*) Membranes (2 μg) from HEK-293 cells expressing hκOR were treated with Col or Sal A (10^{-13}–10^{-5} M), and cAMP levels were measured. Values (*A*–*D*) at 10^{-13} M ligand were taken as 100%. (*E*) Receptor internalization in HEK-293 cells expressing hκOR treated with Col or Sal A (0–1 μM) for 30 min at 37 °C; percent internalized receptors were calculated as described in *SI Materials and Methods*. (*F*–*H*) HEK-293 cells expressing hκOR were treated for 3 min at 37 °C with either Col or Sal A (10^{-13}–10^{-6} M) and phosphorylated levels of ERK1/2 (*F*), Akt at S473 (*G*), or Akt at T308 (*H*) measured. Data (*A*–*H*) represent mean ± SEM; $n = 3$–6. *Values obtained with 10 μM ligand. n.d., not detected.

Table 1. Signaling with collybolides

	[^{35}S]GTPγS binding		AC activity		phospho ERK1/2		phospho Akt S473		phospho Akt T308		Internalization	
Ligands	EC_{50} [M]	% E_{max}*	EC_{50} [M]	% Inhibition*	EC_{50} [M]	% E_{max}	EC_{50} [M]	% E_{max}	EC_{50} [M]	% E_{max}	EC_{50} [M]	% E_{max}*
Colly	$2 \pm 1\ E^{-9}$	124 ± 2	$9 \pm 2\ E^{-10}$	22 ± 2	$9 \pm 2\ E^{-12}$	$167 \pm 11^{\dagger}$	$2 \pm 1\ E^{-10}$	225 ± 8	$1 \pm 1\ E^{-10}$	$350 \pm 9^{\ddagger}$	$5 \pm 1\ E^{-10}$	18 ± 8
9-*epi*-Colly	$3 \pm 2\ E^{-10}$	115 ± 1	$4 \pm 2\ E^{-6}$	10 ± 3	$4 \pm 1\ E^{-9}$	141 ± 7*	$4 \pm 2\ E^{-10}$	160 ± 17	$6 \pm 2\ E^{-11}$	190 ± 31	$8 \pm 3\ E^{-11}$	79 ± 10
Sal A	$2 \pm 1\ E^{-10}$	136 ± 4	$9 \pm 1E^{-10}$	20 ± 2	$3 \pm 1\ E^{-10}$	$162 \pm 17^{\ddagger}$	$3 \pm 2\ E^{-9}$	$190 \pm 19^{\ddagger}$	$1 \pm 1\ E^{-10}$	$291 \pm 3^{\ddagger}$	$5 \pm 2\ E^{-10}$	18 ± 5
U69,593	$6 \pm 2\ E^{-8}$	132 ± 1	$2 \pm 1\ E^{-9}$	30 ± 2	$2 \pm 1\ E^{-10}$	$274 \pm 39^{\ddagger}$	$2 \pm 1\ E^{-10}$	156 ± 9	$3 \pm 2\ E^{-10}$	271 ± 8	$2 \pm 1\ E^{-9}$	15 ± 5
DynA8	$2 \pm 1\ E^{-10}$	133 ± 3	$2 \pm 1\ E^{-9}$	16 ± 1	ND	ND	ND	ND	ND	ND	$1 \pm 1\ E^{-9}$	26 ± 5

HEK-293 membranes expressing hκOR were subjected to a [^{35}S]GTPγS binding assay (20 μg) or to an adenylyl cyclase (AC) activity assay (2 μg) with κOR ligands (10^{-13}–10^{-5} M). HEK-293 cells expressing hκOR (2×10^5 cells per well) were treated with κOR ligands, phosphorylation of ERK1/2, Akt S473, or T308 measured after 3 min or receptor internalized after 30 min. Values obtained at 10^{-13} M ligand were taken as 100%. Data are mean ± SEM; $n = 6$. ND, not done.

*Values obtained with 10 μM ligand concentration.

†Values obtained with 1 nM ligand concentration.

‡Values obtained with 100 nM ligand concentration.

(IC_{50} = 0.08 mg/kg), and this effect is desensitized at higher doses (Fig. 5*E*). Also, Colly-mediated attenuation of pruritus can be reversed by the κOR antagonist nor-BNI (Fig. 5*F*). We also find that Colly treatment has no effect on animal movement in an open field test (Fig. 5*G*). These results indicate that Colly is a novel, high-potency hκOR agonist with behavioral properties distinct from that of Sal A that taken with its biased agonistic activity makes it an attractive candidate for further studies exploring the molecular pharmacology of κOR.

Discussion

Sal A was the first described naturally occurring nonnitrogenous furyl-δ-lactone diterpene agonist of κOR (18). In this study, we show that Collys are potent and selective hκOR agonists, thereby extending the repertoire of naturally occurring furyl-δ-lactone terpenes that function as κOR ligands. It is interesting to note that most κOR ligands, particularly Sal A, exhibit nanomolar affinity for κOR. Interestingly, docking studies using the crystal structure of hκOR bound to the antagonist JDTic suggest that different chemotypes of κOR ligands bind to the same pocket in the receptor (25). We find that epimerization of Colly at C9 reduces agonist binding and signaling. These results suggest that the C9 position makes significant contributions to full binding and signaling at hκOR. The generation of novel ligands based on the Colly structure that could be used in cocrystallization studies with hκOR would help elucidate how Collys bind to κOR.

An observation in this study is that, although Sal A completely displaces radiolabeled binding to hκOR Collys, in particular 9-*epi*-Colly, do not. This partial displacement of radiolabeled binding could be because (*i*) Collys exhibit poor solubility in aqueous solutions such that at high concentrations lesser amounts are available for displacement of bound radiolabel; (*ii*) at high concentrations, Collys bind to a second site in hκOR functioning as an allosteric modulator of bound radiolabeled ligand; (*iii*) the radiolabeled ligands used in this study stabilize different conformations of the receptor some of which are not accessible for displacement by Collys; and (*iv*) it is possible that hκOR is complexed with different proteins, and Collys are able to recognize and bind to hκOR only in some of these complexes. Among these possibilities the most probable one particularly in the case of 9-*epi*-Colly would be poor solubility in aqueous solution. Thus, further studies are needed to explore in detail how Collys bind to hκOR in comparison with naloxone, Dip, and U69 and generation of water-soluble analogs of Collys could help address some of these questions.

Behavioral studies report that Sal A exhibits antinociceptive activity (26), is aversive in rodents (24), dose-dependently increases immobility in the forced swim test (27), is anxiolytic in the elevated plus maze test (28), and can attenuate cocaine-induced drug seeking behavior (29). In this study, we find that Colly, like Sal A, exhibits antinociception and is aversive in mice. However, unlike Sal A, a 2-mg/kg dose of Colly tends to decrease immobility time in the forced swim test, suggesting that Colly may exhibit antidepressant activity. Interestingly, evaluation of the effects of Colly in the open field test suggests that at a dose of 2 mg/kg it tends to be anxiogenic, whereas Sal A has been reported to be anxiolytic (28). Studies examining the effects of Sal A on compound 48/80-induced itch behavior (30) reported low and inconsistent effects in attenuation of scratching behavior. We find that the Sal A-mediated antipruritic effect on chloroquine-mediated itch is low and inconsistent (Fig. 5), whereas Colly-mediated antipruritic effect is consistent and robust (Fig. 5). These properties make Colly an ideal candidate for further studies exploring molecular and behavioral properties of κOR, as well as for the development of novel therapeutics for the treatment of itch and other κOR-mediated pathologies.

A hallmark characteristic of Sal A is that it is a potent hallucinogen. Thus, administration of vaporized Sal A to human volunteers leads to intense psychotropic effects including hallucinations, disconnection from external reality, and altered interoceptive abilities (31). To date, it is not known if Collys exhibit hallucinogenic effects. An extensive web search for information about the fungus *C. maculata* did not reveal any reports of hallucinogenic properties: only that the fungus is bitter to taste. If it holds true that Collys are not hallucinogenic, this would rule out the involvement of the furyl-δ-lactone motif in the hallucinogenic effects of Sal A. Structure–activity studies comparing modifications in Sal A and Colly structures could help elucidate the motifs required for the hallucinogenic properties. In addition, it would be important to examine the contribution of biased signaling toward the differences in the behavioral effects of Sal A and Colly. Such studies are particularly important given reports that biased signaling at κOR can distinguish the aversive effects of κOR agonists from the antinociceptive effects (8). Consistent with this, studies have shown that, whereas Sal A appears to be an unbiased ligand based on G-protein activation and β-arrestin recruitment assays, a structural derivative, RB-64, appears to exhibit G-protein–biased activity (32). Studies with this compound argue for a contribution of the G-protein pathway in κOR-mediated analgesia and aversion and of other pathways on κOR-mediated motor coordination, sedation, and anhedonia (33). In the present study, we show that Colly exhibits biased agonistic activity and differs from Sal A in blocking chloroquine-mediated itch. This observation makes Colly an ideal compound for further studies. The amenability of the structure of Colly to modifications provides a platform for generation of analogs, thus expanding the repertoire of biased κOR ligands; this could help delineate the signaling pathways in κOR-mediated behaviors and

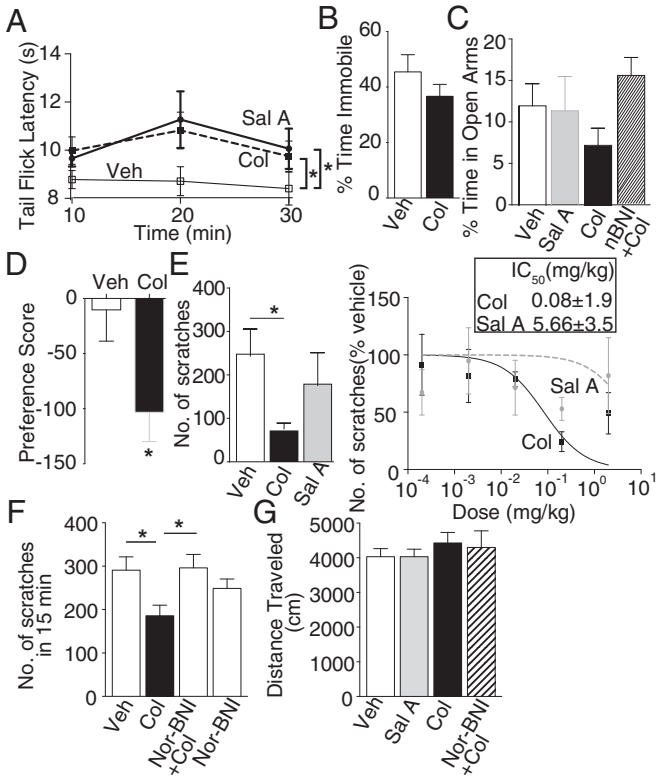

Fig. 5. Behavioral effects of collybolide. (*A*) Mice were administered with vehicle (Veh), Sal A (Sal), or Colly (Col) (2 mg/kg), and antinociception was measured at different time intervals using the tail flick assay. Two-way ANOVA: time, $F(2,42) = 1.486$, $P = 0.238$; drug, $F(2,21) = 4.199$, $P = 0.029$; Bonferroni post hoc: $^*P < 0.05$ vehicle vs. Sal A/Col. (*B*) Forced swim test with mice administered with Veh or Col (2 mg/kg). (*C*) Mice administered with Veh, Sal, Col (2 mg/kg), or nor-BNI (10 mg/kg) + Col were placed in the elevated plus maze, and time spent in the open arms was measured. (*D*) Mice were administered with Veh or Col (2 mg/kg) for 4 d, and place preference was measured. *t* test: $^*P < 0.05$. (*E*) Mice were administered with chloroquine-phosphate (20 mg/kg) in the absence or presence of Sal or Col (2–2,000 μg/kg), and number of scratches was measured for 15 min. Veh vs. Col 2,000 μg/kg, unpaired *t* test: $^*P < 0.05$. (*F*) Assessment of the effect of nor-BNI (10 mg/kg) on Col-mediated attenuation of itch. $^*P < 0.05$; one-way ANOVA. (*G*) Mice were injected i.p. with Veh, Col (2 mg/kg), Sal A (2 mg/kg), or nor-BNI (10 mg/kg) + Col (2 mg/kg) 20 min before subjecting them to the open field test. Data (*A*–*G*) represent mean ± SEM; $n = 7$–10 mice per group.

side effects. In sum, our studies show that collybolides represent novel κOR selective agonists. These compounds could be used to further investigate the molecular pharmacology of κOR, explore the role of κOR in vivo, and develop novel therapeutics (agonists, antagonists, and partial agonists) that could be clinically used to treat disorders involving the κOR system.

Materials and Methods

Natural Compounds, Materials, and Analytical Data. Sal A was from In Solve Scientific, Colly and 9-*epi*-Colly were from the collection of two of the coauthors (A.C. and J.P.) after their discovery (21) (analytical details in *SI Materials and Methods*); 10 mM stock solutions of Sal A, Colly, and 9-*epi*-Colly in DMSO were used in all assays. U69,593 (34), Dyn A8, and protease inhibitor mixture were from Sigma. [^{3}H]Diprenorphine, [^{3}H]Naloxone, [^{3}H]U69,593, and [^{35}S]GTPγS with specific radioactivities of 42.3, 61.1, 37.4, and 1250 Ci/mmol, respectively, were from Perkin-Elmer. κOR KO mice brains were a gift from Charles Chavkin (University of Washington, Seattle).

Cell Culture. HEK-293 or CHO cells were grown in DMEM or F12 media, respectively, that contained 10% (vol/vol) FBS and penicillin-streptomycin. Cells were transfected with h*μOR*, h*δOR*, or h*κOR* cDNAs using lipofectamine as per the manufacturer's protocol (Invitrogen). Saturation binding assays using [^{3}H]Naloxone (0–10 nM) show that the cells express similar amounts of receptors (874 ± 240 fmol/mg protein for hμOR, 837 ± 171 for hδOR, and 816 ± 253 for hκOR).

Membrane Preparation. Membranes were prepared from HEK-293 cells expressing hκOR, hδOR, and hμOR, from cerebral cortex or whole brains of WT or κOR KO mice as described previously (35).

Receptor Displacement Binding Assays. Glass tubes treated for 10 min with 0.1% (vol/vol) aquaSil-siliconizing fluid in double distilled water were used. Membranes from HEK-cells expressing hμOR, hδOR, or hκOR (200 μg/tube), cerebral cortex, or whole brains (100 μg/tube) of WT or κOR KO mice were incubated with [^{3}H]Diprenorphine, [^{3}H]Naloxone, or [^{3}H]U69,593 (3 nM final concentration) in the presence of different concentrations (10^{-13}–10^{-5} M) of κOR ligands for 2 h at 30 °C as described previously (35, 36). Details of buffers used are given in *SI Materials and Methods*.

[^{35}S]GTPγS Binding Assays. Assays were carried out in glass tubes treated as described above. Membranes (20 μg) were subjected to a [^{35}S]GTPγS binding assay with κOR ligands (10^{-13}–10^{-5} M) as described previously (35, 36). nor-BNI (10 μM) was added 30 min before the assay.

Measurement of Adenylyl Cyclase Activity. Membranes (2 μg) were treated with 20 μM forskolin in the absence or presence of κOR ligands (10^{-13}–10^{-5} M) for 30 min at 37 °C. cAMP levels were determined using the HitHunter cAMP MEM chemiluminescence detection kit from DiscoveRx.

Measurement of ERK1/2 and Akt Phosphorylation. CHO or HEK-293 cells expressing hκOR, hμOR, or hδOR (2×10^5 cells per well) were treated with vehicle or κOR ligands (10^{-13}–10^{-6} M) for 3 min at 37 °C. ERK1/2 phosphorylation and Akt phosphorylation at S473 or T308 were measured by Western blot as previously described (36, 37).

Receptor Internalization. HEK-293 cells expressing hκOR (2×10^5 cells per well) were incubated at 4 °C for 1 h with 1:1,000 anti-HA antibody to label cell surface κOR. Cells were treated without or with 100 nM of κOR ligands for different time intervals (0–120 min) or with different doses (0–1 μM) of κOR ligands for 30 min at 37 °C. Cells were fixed and cell surface receptors determined by ELISA using 1:1,000 dilution (in PBS containing 1% BSA) of anti-rabbit IgG coupled to HRP (Vector Laboratories).

Animal Studies. Male C57BL/6J mice, 10–12 wk of age (Jackson Laboratories) were housed in groups of three to five per cage in a humidity- and temperature-controlled room with a 12-h light/dark cycle (lights on 07:00–19:00 hours) and given free access to food and water throughout the experiment. Behavioral procedures were according to ethical guidelines/regulations approved by the Icahn School of Medicine Animal Care and Use Committee. Animal care and all experimental procedures were in accordance with guidelines from the National Institutes of Health.

Tail Flick Assay. Mice (7–10/group) were injected i.p. with vehicle [6% (vol/vol) DMSO in saline], Sal A, or Colly (2 mg/kg), and antinociception was measured using the tail-flick assay (38).

Conditioned Place Preference/Aversion. Animals (7–10/group) were administered i.p. with vehicle in one chamber and 8 h later with vehicle/Colly (2 mg/kg, i.p.) in the other chamber. These injections were repeated for 4 d using an unbiased, counterbalanced design. On the test day (day 6), animals were placed in the central white chamber, and place preference/aversion was measured for 20 min. Change in preference (seconds) = time spent in drug-paired compartment on test day − time spent in this compartment in the preconditioning session.

Forced Swim Test. Mice (7–10/group) were injected i.p. with vehicle or Colly (2 mg/kg) and placed inside a glass beaker (height 44 cm, diameter 22 cm) filled with water at a depth of 30 cm, at 25 ± 2 °C, and behavior was recorded for 6 min. Mice were scored blind for the treatment conditions for time spent immobile.

Elevated Plus Maze. Mice (7–10/group) were injected i.p. with vehicle, Sal A, or Colly (2 mg/kg) and placed onto the center of the elevated plus maze, and the number of open- and closed-arm entries and time spent in open arms

were recorded. Percentage time spent in open arms was used as a measure of anxiety. A separate set of animals was given nor-BNI (10 mg/kg, i.p.) 20 min before Colly administration.

Open Field. Mice were injected with i.p. with vehicle, Colly (2 mg/kg), Sal A (2 mg/kg), or nor-BNI (10 mg/kg) + Colly (2 mg/kg) 20 min before the open field test. Distance traveled (cm) over 10 min was quantified using Ethovision XT tracking system (Noldus).

Chloroquine-Mediated Itch Behavior. Mice (7–10/group) were injected i.p. with vehicle (0.9% saline + 2% DMSO), Colly, or Sal A (2–2,000 μg/kg) 10 min before inducing itch. To induce itch, mice were injected s.c. with chloroquine-phosphate (20 mg/kg) into the base of the neck, placed into the recording chamber, and videotaped for 15 min. Total number of scratches in 5-min intervals were scored by experimenters blinded to the drug treatments. A scratch was defined as hind paw movements directed to the site of injection (30, 39). Another set of animals was evaluated for the effect of nor-BNI on collybolide-mediated itch attenuation.

Statistical Analysis. Data were analyzed using Prism 6.0 (Graph Pad), and statistical analysis was carried out using *t* test or one-way ANOVA.

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