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Opioid activity of alkaloids extracted from *Picralima nitida* (fam. Apocynaceae)

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Abstract

Extracts of the seeds of *Picralima nitida* (fam. Apocynaceae) have been reported to have opioid analgesic activity. In this investigation, isolated tissue bioassays and radioligand binding assays have been used to determine the opioid activity of five alkaloids—akuammidine, akuammine, akuammicine, akuammigine and pseudoakuammigine—extracted from the seeds of *P. nitida*. Akuammidine showed a preference for μ-opioid binding sites with K_i values of 0.6, 2.4 and 8.6 μM at μ-, δ- and κ-opioid binding sites, respectively. The agonist actions of akuammidine in the mouse-isolated vas deferens were antagonised by naloxone and the μ-opioid receptor selective antagonist D-Phe–Cys–Tyr–D-Trp–Orn–Thr–Pen–Thr–NH_2 (CTOP) confirming an action at μ-opioid receptors. In contrast, akuammine also showed highest affinity for μ-opioid binding sites (K_i 0.5 μM) but was an antagonist at μ-opioid receptors with a pK_B of 5.7 against the selective μ-opioid receptor agonist [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAMGO). Akuammicine has the highest affinity for κ-opioid binding sites (K_i 0.2 μM) and was a full agonist at κ-opioid receptors in the guinea pig ileum preparation but a partial κ-opioid receptor agonist in the vasa deferentia of the mouse and the rabbit. Akuammigine and pseudoakuammigine showed little or no efficacy in the opioid bioassays. None of the alkaloids had significant activity for opioid receptor-like binding sites (ORL_1-binding sites) with K_i values $\gg 10$ μM. These data show that some alkaloids extracted from the medicinal plant *P. nitida* possess varying degrees of agonist and antagonist activity at opioid receptors but possess neither high affinity nor selectivity for μ-, δ- or κ-opioid receptors or the ORL_1-receptor.

Keywords: Opioid receptor; Bioassay; Binding assay; *Picralima nitida*; Alkaloid; ORL_1-receptor

1. Introduction

It is estimated that 80% of the world's population depend on traditional medicine for their primary health care needs, with higher plant extracts or their active principles forming the major source of these traditional drug therapies (WHO, IUCN, WWF, 1993). *Picralima nitida* (fam. Apocynaceae) is a tall, deciduous tree found growing sparsely in the forests of many West African countries, particularly Ghana, which provides an important source of traditional Ghanaian medicines. Decoctions of the roots and bark are used for the treatment of stomach complaints and as anti-pyretics, and the crushed seeds have been used as a remedy for malarial fever and different types of pain (see the work of Irvine (1961)). It is as a source of pain relief, however, that *P. nitida* has a significant place in contemporary Ghanaian medicine; the dried, powdered seeds of the plant are commercially marketed in Ghana as an analgesic, in the form of 25 mg capsules named 'Picap' (Noamesi Medical Laboratories, Hohoe). Although widely used and trusted, little work has been done to determine the pharmacological properties of either crude seed extracts or the constituent alkaloids.

More than 10 distinct indole alkaloids have so far been isolated from *P. nitida* (Henry, 1949; Henry and Sharp, 1927), their names being derived from the indigenous name of the tree, 'akuamma'. Fig. 1 shows the structures of akuammine, akuammidine, akuammicine, akuammigine and pseudoakuammigine, those alkaloids found in the highest concentrations and the subjects of this investigation. Ansa-Asamoah and Ampofo (1986) demonstrated that

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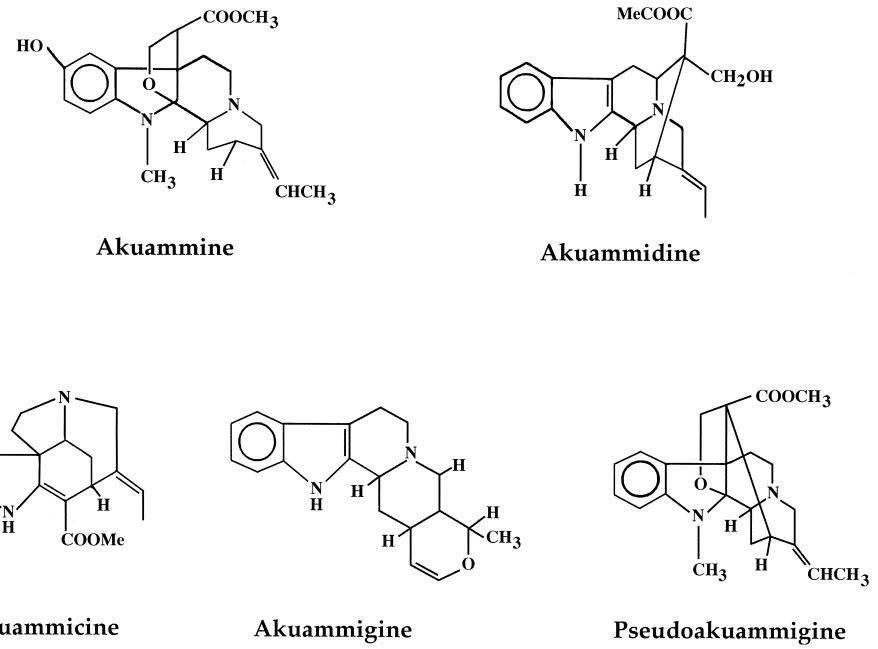

Fig. 1. The structures of the five alkaloids extracted from *P. nitida* (fam. Apocynaceae) used in this investigation.

a crude extract of the seeds had anti-nociceptive activity 'comparable' to morphine; this action was partially antagonised by the prototypical opioid receptor antagonist naloxone implying an action at opioid receptors. It has been reported (Ansa-Asamoah, personal communication) that the active opioid principle in the crude extract is akuammine, the most abundant alkaloid in the seeds (0.56% dry weight of total crude extract) which was isolated and partially characterised in the 1920s (Henry and Sharp, 1927). Akuammidine and akuammigine are found in much smaller amounts, 0.034% and 0.01% dry weight of total crude extract, respectively. Akuammigine has been reported to be a weak antagonist at both pre- and post-synaptic α-adrenoceptors in the isolated vas deferens of the rat (Demichel and Roquebert, 1984) but little is known of the pharmacological properties of akuammidine. Akuammicine and pseudoakuammigine are minor alkaloids of *P. nitida* ($<0.006\%$ dry weight of total crude extract) and to our knowledge nothing has appeared in the literature describing their pharmacological actions. Recently, Duwiejua et al. (1995) reported that crude extracts of *P. nitida* have anti-inflammatory effects in the rat paw.

In light of the reported opioid actions of the crude seed extract and of akuammine, the similarity in structure of the five alkaloids and their common origin, the purpose of this investigation was to characterise fully the opioid activity of the alkaloids and the crude extract using classical opioid receptor bioassays and radioligand binding assays. The objective was to determine if any of the alkaloids were either highly selective or had high affinity for opioid receptors, possibly providing a novel source of potentially useful experimental or clinical opioid receptor ligands. In addition, binding assays were used to determine if the alkaloids had affinity for the orphan opioid receptor (ORL_1) (Mollereau et al., 1994).

2. Materials and methods

2.1. Isolation of alkaloids

Ripe fruits of *P. nitida* were collected from the botanical gardens of the University of Science and Technology in Kumasi, Ghana in early spring. The dried and powdered seeds were treated first with *n*-hexane (extracting mainly terpenoids and some alkaloids), then with ethyl acetate. The ethyl acetate fractions were applied to alumina columns (Al_2O_3, Spence type H) from which the alkaloids were isolated by elution in *n*-hexane:ethyl acetate (1:1). The identities and purities of the dried, isolated alkaloids were confirmed by H-Nuclear Magnetic Resonance spectroscopy. By this method each alkaloid sample was shown to have purity $>98\%$.

2.2. Binding assays

Competition binding assays were carried out in membrane suspensions prepared from the brains of Dunkin–Hartley guinea pigs as described by Kosterlitz et al. (1981). [^{3}H][D-Ala^2,MePhe^4,Gly-ol^5]enkephalin ([^{3}H]DAMGO, 1 nM) was used to label selectively μ-opioid binding sites, [^{3}H]naltrindole (0.25 nM) to label δ-opioid binding sites, [^{3}H]CI-977 (0.25 nM) to label κ-opioid binding sites and [^{3}H]nociceptin (0.1 nM) to label ORL_1-binding sites. Spe-

cific binding was determined with 1 μM diprenorphine for the opioid binding sites and with 100 nM unlabelled nociceptin for the ORL_1-binding sites. Samples were incubated at 25°C for 60 min and bound ligand was separated from free by filtration over glass fibre filters pre-soaked in 1% polyethylineimine. The inhibition constants (K_i, nM) were calculated by the method of Cheng and Prusoff (1973).

2.3. Bioassays

Male, albino guinea pigs (Dunkin–Hartley strain, weighing 400–500 g) were killed by cervical dislocation and exsanguination, the small intestine was removed and the first 10–12 cm of the distal section was discarded. Myenteric plexus–longitudinal muscle preparations were made from 12–20-cm segments of the whole intestine by the method of Kosterlitz et al. (1970). Briefly, a segment of small intestine was slipped onto a glass rod and the longitudinal muscle, along with the adherent myenteric plexus, was separated from the circular muscle layer by means of tangential strokes with fine cotton wool dampened with Krebs solution. Adult, male mice (strain DBA/2, weighing 25–30 g) were killed by cervical dislocation and adult, male New Zealand white rabbits (2–2.5 kg) were killed by an overdose of pentobarbitone; the vasa deferentia were rapidly removed and trimmed of any fatty and vascular tissue. Tissue preparations were mounted under tension (mouse and rabbit isolated vasa deferentia, 1 g; guinea pig myenteric plexus–longitudinal muscle preparation, 2 g) in 3 ml organ baths containing Krebs solution maintained at 37°C and bubbled with 95% O_2 and 5% CO_2.

Neurogenic twitch contractions were induced electrically using a Grass S88 stimulator via platinum electrodes at the top and bottom of the organ baths (Corbett et al., 1982). The guinea pig preparations were stimulated at 0.1 Hz (supramaximal voltage, 0.5 ms pulse duration) and the vas deferens preparations stimulated with trains of 3 pulses delivered at 5 Hz every 10 s (supramaximal voltage, 0.5 ms pulse duration). Isometric responses were recorded using Grass FT03C force displacement transducers linked to a Grass four channel pen recorder.

Preparations were allowed to equilibrate for 30–60 min prior to addition of concentrations of a selective opioid agonist to standardise sensitivity; DAMGO (3–100 nM) for guinea pig myenteric plexus–longitudinal muscle preparations, [D-Pen^2,D-Pen^5]enkephalin (DPDPE, 0.3–10 nM) for the mouse isolated vasa deferentia and CI-977 (0.01–100 nM) for the rabbit isolated vasa. Opioid activity of the alkaloids was assessed by their ability to inhibit the electrically-evoked contractions of the isolated tissue bioassays; only one alkaloid was tested in each individual preparation. Opioid actions were confirmed, and opioid receptor selectivity determined, by the use of naloxone and selective opioid antagonists. The opioid agonist potencies of the alkaloids are expressed as IC_{50} (μM): the concentration of agonist, estimated from cumulative log concentration–response curves, that reduced the height of the stimulus-evoked contractions by 50%. The affinities of antagonists were measured where possible by the method of Arunlakshana and Schild (1959) and are expressed as the equilibrium constant, pK_B. In some cases the apparent pK_B was measured using the method of Kosterlitz and Watt (1968).

2.4. Drugs

The composition of the Krebs solution was as follows: (in mM) NaCl 118, KCl 4.74, $CaCl_2$ 2.54, KH_2PO_4 1.19, $MgSO_4 \cdot 7H_2O$ 1.20, $NaHCO_3$ 25, glucose 11; pH was 7.4 when gassed with 95% O_2 and 5% CO_2. When the vas deferens of the mouse was used, Mg^{2+} was omitted from the Krebs solution (Hughes et al., 1975).

The drugs used were carbamylcholine chloride (carbachol), DPDPE, DAMGO and clonidine (all from Sigma), D-Phe–Cys–Tyr–D-Trp–Orn–Thr–Pen–Thr–NH_2 (CTOP), norbinaltorphimine and naltrindole (all from Research Biochemicals International), naloxone hydrochloride (Endo Laboratories), U69,593 ([5α,7α,8β-(−)-*N*-methyl-*N*-[74-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]-benzene acetamide; Upjohn), nociceptin (Bachem), diprenorphine (Reckitt & Colman) and CI-977 ((5R)-(5α,7α,8α)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4,-5]dec-8-yl]-4-benzofuranacetamide monohydride; Dr A.T. McKnight, Parke-Davis).

Stock solutions of akuammidine, akuammicine, akuammigine and pseudoakuammigine were made up in 0.01 M hydrochloric acid whereas akuammine and the crude extract from the seeds were dissolved in acidified 50% ethanol. Peptides were dissolved in methanol:0.01 M CH_3COOH (50:50; v:v) containing 1 mg/ml bovine serum albumin to reduce adsorptive losses. Stock solutions of the other drugs were prepared in de-ionised water. All drugs solutions were stored at −20°C and fresh dilutions were made daily in Krebs solution. When diluted by 1:10, none of the vehicles had any effect on electrically-evoked responses in any of the bioassay preparations used.

2.5. Labelled ligands

The labelled ligands used were [^{3}H]DAMGO (48–66 Ci/mmol), [^{3}H]CI-977 (21 Ci/mmol) and [^{3}H]nociceptin (168 Ci/mmol) from Amersham International and [^{3}H]naltrindole (19.4 Ci/mmol) from the Institute of Isotopes, Budapest.

2.6. Statistical analysis

Data are expressed as mean ± S.E.M. or mean and range. Student's unpaired *t*-test was used to establish

Table 1
The inhibitory effects of alkaloids from *P. nitida* and of some highly selective opioid ligands on binding at μ-, δ- and κ-opioid binding sites in homogenates of guinea pig brain

	K_i (μM)		
	μ-site	δ-site	κ-site
Akuammidine	0.60 ± 0.20	2.37 ± 0.09	8.65 ± 1.27
Akuammine	0.48 ± 0.09	2.56 ± 0.92	1.89 ± 0.71
Akuammicine	1.71 ± 0.51	3.25 ± 0.85	0.19 ± 0.04
Pseudoakuammigine	0.89 ± 0.30	0.92 ± 0.20	5.95 ± 0.67
Akuammigine	n.a.	n.a.	n.a.
DAMGO (μ)	0.0015 ± 0.0002	0.21 ± 0.015	0.93 ± 0.17
DPDPE (δ)	0.90 ± 0.11	0.00027 ± 0.00012	> 100
CI-977 (κ)	0.046 ± 0.004	0.98 ± 0.24	0.00024 ± 0.00006

The values are expressed as the means ± S.E.M. of three to six observations. The μ-binding sites were labelled with [^{3}H]DAMGO (1 nM), δ-sites with [^{3}H]naltrindole (0.25 nM) and the κ-sites with [^{3}H]CI-977 (0.25 nM). Incubations were performed for 60 min at 25°C. DAMGO is [D-Ala^2,MePhe^4,Gly-ol^5]-enkephalin and DPDPE is [D-Pen^2,D-Pen^5]enkephalin, n.a.: not active.

statistical significance; a probability level of $P < 0.05$ was considered to be significant.

3. Results

3.1. Opioid binding assays

Competition binding assays revealed that four of the five alkaloids extracted from *P. nitida* bound to opioid sites in homogenates of guinea pig brain (Table 1). Akuammidine and akuammine interacted with all three opioid sites but bound preferentially to μ-opioid binding sites with K_i values of 0.60 and 0.48 μM, respectively. These values correspond to affinities of 1.7 μM^{-1} for akuammidine and 2.1 μM^{-1} for akuammine which are almost 400-fold lower than the affinity of the highly selective μ-opioid receptor ligand DAMGO (Table 2). The high selectivity of DAMGO is reflected in a relative μ-affinity of 0.993 whereas the relative affinity of the μ-preferring alkaloids is much lower at ~0.7 (Table 2). Akuammicine bound preferentially to κ-opioid binding sites with a K_i value of 0.19 μM. The affinity of akuammicine for κ-opioid binding sites, 5.3 μM^{-1}, was 10-fold greater than for either μ- or δ-sites reflected in a relative κ-affinity of 0.86. Although akuammicine showed the highest relative affinity of any of the alkaloids tested, the affinity and selectivity for κ-opioid binding sites was still much lower than that of the highly selective κ-opioid receptor ligand CI-977 (Table 2). Pseudoakuammigine bound equally to both μ- and δ-opioid binding sites but the affinity of 1.1 μM^{-1} at δ-sites was some 3400 times less than that of the δ-opioid receptor selective agonist DPDPE (Table 2). Akuammigine showed no affinity for any of the opioid binding sites.

3.2. ORL_1-binding assays

Akuammine, akuammidine and pseudoakuammigine displayed little affinity for ORL_1-binding sites, since at the high concentration of 10 μM these alkaloids inhibited the binding of [^{3}H]nociceptin by only 20–30% (Table 3). Nociceptin binding was unaffected by either 10 μM akuammicine or 10 μM akuammigine. None of the ligands selective for classical opioid receptors affected [^{3}H]nociceptin binding; K_i values for DAMGO, naltrindole and CI-977 were all > 10 μM ($n = 4$–6). In contrast, unlabelled nociceptin readily inhibited the binding of [^{3}H]nociceptin with a K_i value of 0.16 ± 0.04 nM ($n = 8$).

Table 2
The relative opioid affinity of alkaloids from *P. nitida* and of some highly selective opioid ligands

	Affinity at preferred site, K_i ($\mu M)^{-1}$	Relative affinity at		
		μ-site	δ-site	κ-site
Akuammidine	1.7	0.76	0.19	0.05
Akuammine	2.1	0.69	0.13	0.18
Akuammicine	5.3	0.09	0.05	0.86
Pseudoakuammigine	1.1	0.47	0.46	0.07
DAMGO (μ)	680	0.993	0.007	< 0.001
DPDPE (δ)	3700	< 0.003	> 0.997	< 0.001
CI-977 (κ)	4200	0.005	0.0002	0.995

Using the data from Table 1, the relative affinities were calculated from: K_i^{-1} for μ, δ or κ/(K_i^{-1} for $\mu + K_i^{-1}$ for $\delta + K_i^{-1}$ for κ). DAMGO is [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin and DPDPE is [D-Pen^2,D-Pen^5]enkephalin.

Table 3
The inhibitory effects of alkaloids (10 μM) from *P. nitida* on binding at ORL_1-binding sites in homogenates of guinea pig brain

	% Inhibition of [^{3}H]-nociceptin binding
Akuammidine	18.8 ± 8.4
Akuammine	33.2 ± 3.6
Akuammicine	0
Pseudoakuammigine	21.3 ± 6.1
Akuammigine	0

The values are expressed as the means ± S.E.M. of three observations. The ORL_1-binding sites were selectively labelled with [^{3}H]nociceptin (0.1 nM) and incubations were performed for 60 min at 25°C.

3.3. Opioid bioassays

Akuammidine caused concentration-dependent, reversible inhibitions of the twitch contractions of the guinea pig myenteric plexus–longitudinal muscle preparation (μ- and κ-opioid receptors) and the mouse vas deferens (μ-, δ- and κ-opioid receptors), with IC_{50} values of 6.8 μM (range 4.5–9.5 μM, $n = 6$) and 5.1 μM (range 2.8–7.0 μM, $n = 6$), respectively (Fig. 2). In the guinea pig preparation, the inhibitions caused by the alkaloid were antagonised by naloxone (10 nM) and by the selective μ-opioid receptor antagonist CTOP (100 nM) but not by the selective κ-opioid receptor antagonist norbinaltorphimine (30 nM) (Fig. 3A). The agonist actions of akuammidine in the mouse vas deferens were also antagonised by naloxone and CTOP with pK_B values of 7.33 ± 0.25 ($n = 4$) and 8.61 ± 0.22 ($n = 4$), respectively. In contrast, akuammidine at concentrations up to 100 μM had no agonist effect ($n = 4$) in the rabbit isolated vas deferens (κ-opioid receptors only).

No opioid agonist action of akuammine was seen in either the mouse or guinea pig bioassays; concentrations up to 30 μM caused no inhibition of electrically-evoked contractions (Fig. 2). Instead, this compound antagonised the inhibitory effects of the selective μ-opioid receptor agonist DAMGO with a pK_B of 5.68 ± 0.07 ($n = 4$) in the mouse vas deferens (Fig. 3B) and 5.00 ± 0.16 ($n = 4$) in the myenteric plexus preparation; these pK_B values are significantly different ($P < 0.05$). The antagonism by akuammine was not restricted to μ-opioid receptors, however, since in the mouse preparation, akuammine antagonised the selective δ-opioid receptor agonist DPDPE with a pK_B value of 5.27 ± 0.03 ($n = 3$). In the mouse vas deferens, akuammine antagonised the selective κ-opioid agonist U69,593 with a pK_B value of 6.23 ± 0.02 ($n = 3$) and in the rabbit vas deferens the alkaloid antagonised CI-977 with a pK_B of 5.27 ± 0.24 ($n = 3$; $P < 0.05$ vs. pK_B in mouse vas deferens).

Akuammicine is a full agonist in the guinea pig preparation with an IC_{50} value of 8.14 μM (range 5.5–14.5 μM, $n = 6$). In the mouse and rabbit vasa deferentia, however, it appears to be a partial agonist causing a maximal inhibition of the twitch of only $68 \pm 23\%$ ($n = 6$) and $65 \pm 9\%$ ($n = 5$), respectively (Fig. 2). In the guinea pig preparation, naloxone antagonised akuammicine with an apparent pK_B of 7.35 ± 0.01 ($n = 3$), as did the κ-opioid receptor selective antagonist norbinaltorphimine (apparent $pK_B = 6.05 \pm 0.26$, $n = 3$). The agonist actions of akuammicine and CI-977 in the rabbit vas deferens were antagonised fully by 30 nM norbinaltorphimine.

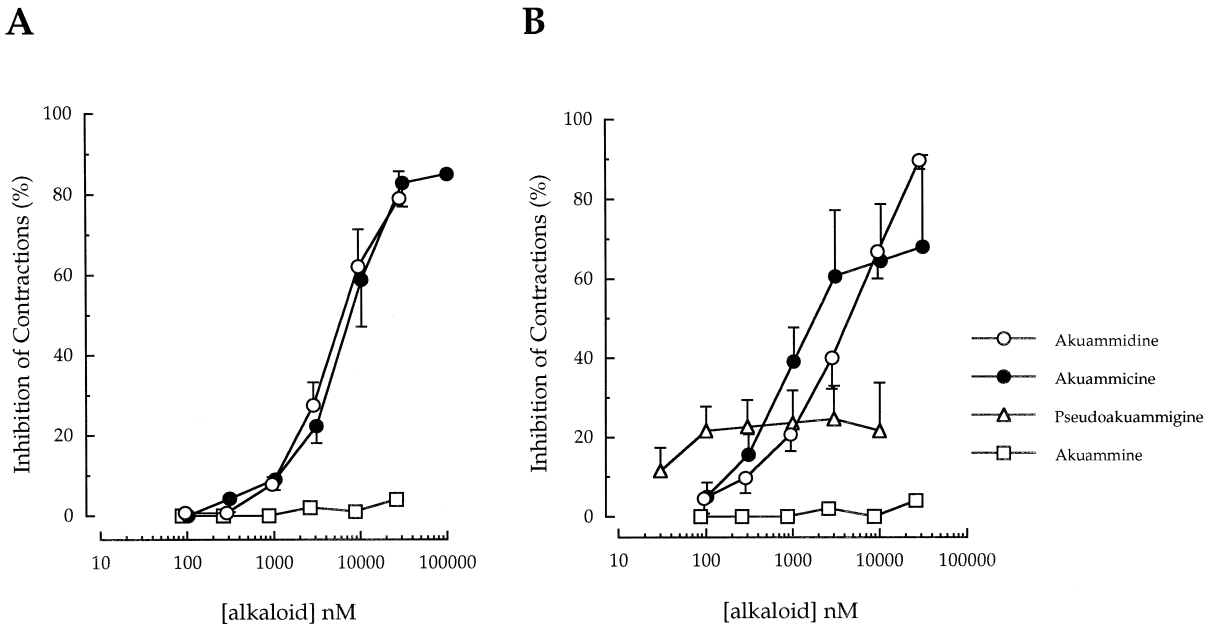

Fig. 2. The effects of alkaloids extracted from *P. nitida* on the electrically-evoked contractions of (A) the guinea pig myenteric plexus–longitudinal muscle preparation and of (B) the mouse isolated vas deferens. (○) Akuammidine, (●) akuammicine, (△) pseudoakuammigine, (□) akuammine. Each point is the mean ± S.E.M. of four to six observations.

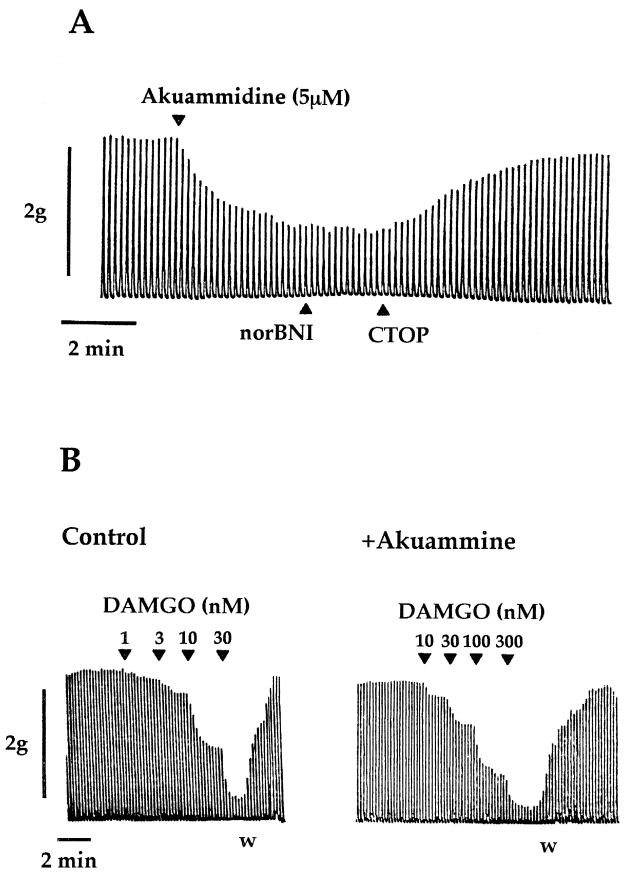

Fig. 3. The agonist activity of akuammidine and antagonist activity of akuammine at opioid receptors in the myenteric plexus–longitudinal muscle preparation of guinea pig small intestine. (A) The inhibition of the electrically-evoked contractions produced by akuammidine are antagonised by the selective μ-opioid receptor antagonist CTOP (100 nM) but not by the selective κ-opioid receptor antagonist norbinaltorphimine (30 nM). (B) The inhibition of the electrically-evoked contractions produced by the selective μ-opioid receptor antagonist DAMGO are antagonised by akuammine (3 μM).

High concentrations of akuammicine (10–30 μM) caused a potentiation of the size of the electrically-evoked contractions of the mouse vasa deferentia, without an increase in tone. No potentiation of the twitch response was observed in either the vas deferens of the rabbit or the guinea pig myenteric plexus preparation. Since the ability of akuammicine to antagonise α_2-adrenoceptors (Demichel and Roquebert, 1984) may be the cause of the potentiation in the mouse vas deferens, the effects of this alkaloid were examined on the ability of clonidine (α_2-adrenoceptor agonist) to inhibit contractions in guinea pig small intestine. Clonidine (1–300 nM) caused a concentration-dependent inhibition of the electrically-evoked contraction in two guinea pig preparations; the inhibitory actions of clonidine were unaffected by the presence of akuammicine (30 μM). No further attempt was made to determine the mechanism underlying the potentiating action of akuammicine.

Although pseudoakuammigine bound to both μ- and δ-opioid binding sites with equal affinity, this compound was found to have little efficacy in the bioassays (Fig. 2). Pseudoakuammigine, at the high concentration of 10 μM, inhibited the electrically-evoked contractions of the mouse vas deferens by only $21.6 \pm 12.1\%$ ($n = 4$). In addition, there was no evidence for pseudoakuammigine having any opioid antagonist activity in this preparation ($n = 4$). Akuammigine caused concentration-dependent inhibitions of the twitch responses of the vasa deferentia of the mouse and the rabbit but these were not antagonised by either naloxone (1 μM; $n = 3$) or norbinaltorphimine (1 μM; $n = 3$).

A range of concentrations of the crude extract diluted serially in Krebs solution (100 ng/ml–10 μg/ml) showed no activity in either preparation.

3.4. Site of opioid action of the alkaloids in bioassays

In order to determine if the opioid activity of the alkaloids in the guinea pig myenteric plexus–longitudinal muscle preparation was due to a pre- or post-junctional site of action, concentration–response curves to carbachol (0.01–3 μM) were constructed in the absence and presence of a supramaximal concentration of akuammicine. The log concentration–response curve to carbachol (IC_{50} value of 172 nM, range 147–201 nM, $n = 3$) was unchanged by the presence of 30 μM akuammicine (IC_{50} value of 151 nM, range 130–173 nM, $n = 3$); the maximum response was similarly unaffected. In single experiments, none of the other alkaloids had any affect on the sensitivity of the guinea pig preparation to carbachol.

3.5. ORL_1-receptor bioassays

Experiments in our laboratory have shown that neurotransmission in the guinea pig myenteric plexus–longitudinal muscle preparation is sensitive to nociceptin, the endogenous ligand of the ORL_1-receptor (Meunier et al., 1995). Nociceptin (1–3000 nM) produces a concentration-dependent inhibition of the electrically-evoked twitch response although the maximum inhibition is only $45.0 \pm 7.1\%$ ($n = 4$). In four preparations of guinea pig small intestine, neither high concentrations of akuammine (30 μM) nor naloxone (1 μM) had any effect on the agonist activity of nociceptin.

4. Discussion

For thousands of years, natural plant sources have provided man with a host of pharmacologically active compounds such as morphine (poppy, *Papaver somniferum*) and digitalis (foxglove, Digitalis family). Plant extracts still provide useful, effective remedies (often

through unknown mechanisms) and the active ingredients from these plant sources may provide leads to novel pharmacological compounds. The results from this investigation show that alkaloids from *P. nitida* possess varying degrees of affinity, preference and efficacy for opioid receptors.

Akuammidine, akuammine, akuammicine and pseudoakuammigine bind with low affinity to μ-, δ- and κ-opioid binding sites; akuammigine showed no opioid binding. The opioid affinities of these alkaloids are at least two orders of magnitude less than those of the selective μ-opioid receptor ligand DAMGO, the selective δ-opioid receptor ligand DPDPE and the selective κ-opioid receptor ligand CI-977. For practical purposes, a selective opioid compound is at least 100 times more active at its preferred site than at other opioid binding sites, i.e., it has a relative affinity of > 0.98 (see the work of Corbett et al. (1993)). None of the alkaloids from *P. nitida* is selective. Although akuammidine and akuammine show a preference for μ-opioid binding sites, and akuammicine for κ-opioid binding sites, the affinity for their preferred site is less than 10-fold greater than that for another opioid site.

It is well-recognised that neurotransmission in some bioassay preparations is sensitive to inhibition by opioids (see the work of Leslie (1987)). Akuammicine, akuammidine and pseudoakuammigine each inhibited the electrically-induced contractions of guinea pig myenteric plexus–longitudinal muscle preparations (μ- and κ-opioid receptors, Lord et al., 1977; Chavkin et al., 1982); these inhibitions were antagonised by naloxone confirming an action at opioid receptors. It seems likely, that the alkaloids are acting pre-junctionally on opioid receptors to inhibit neurotransmitter release as they did not affect contractions caused by carbachol stimulating post-junctional muscarinic cholinoceptors.

The opioid agonist actions of akuammidine in the guinea pig myenteric plexus were primarily at μ-opioid receptors since they were antagonised by a low concentration of the non-selective opioid receptor antagonist naloxone and by CTOP, a highly selective μ-antagonist (Hawkins et al., 1989). The data from the mouse vasa deferentia (μ-, δ- and κ-opioid receptors, Hughes et al., 1975; Lord et al., 1977; Cox and Chavkin, 1983) support this μ-agonist action, as CTOP and naloxone antagonised akuammidine with pK_B values of 7.3 and 8.6, respectively, characteristic of an action at μ-opioid receptors (see the work of Corbett et al. (1993)). In addition, akuammidine was inactive in the rabbit vas deferens in which only κ-agonists are active (Oka et al., 1980). Akuammicine, on the other hand, is an agonist in the rabbit preparation and its actions are blocked by norbinaltorphimine, a selective κ-opioid receptor antagonist (Birch et al., 1987). The agonist actions of this alkaloid in the guinea pig small intestine were antagonised by norbinaltorphimine (apparent pK_B 6.05) and high concentrations of naloxone (pK_B 7.35), typical of an action at κ-receptors (Corbett et al., 1993). Thus, the bioassays confirmed the binding data that akuammicine has a preference for μ-opioid receptors and akuammidine for κ-opioid receptors.

One of the primary stimuli for this investigation was the observation that the analgesic actions of akuammine were attenuated by naloxone (Ansa-Asamoah, personal communication). Since these data implied that akuammine was causing pain relief by an agonist action at opioid (most likely μ-opioid) receptors, our findings that akuammine is an opioid antagonist were somewhat surprising. Although akuammine is not an agonist at opioid receptors a metabolite may well be, and it is the metabolite which exerts the analgesic action. Such an explanation has been proposed to account for the anti-nociceptive activity of the indole alkaloid mitragynine which has a structural similarity to the alkaloids from *P. nitida* (Macko et al., 1972; Matsumoto et al., 1996).

Another explanation for the analgesic activity of akuammine is that it blocks the action of a pronociceptive/hyperalgesic endogenous substance, i.e., one which produces a hypersensitivity to pain. Nociceptin/orphanin FQ acting at the ORL_1-receptor (an 'orphan opioid receptor'; Mollereau et al., 1994) has been proposed to be such a substance (Meunier et al., 1995; Reinscheid et al., 1995). The analgesic actions of akuammine, therefore, could result from antagonism of the hyperalgesic activity of nociceptin/orphanin FQ. In binding assays however, neither akuammine nor any of the other alkaloids showed appreciable affinity for ORL_1-binding sites. In addition, the bioassay data indicate that akuammine does not antagonise nociceptin at functional ORL_1-receptors in the guinea pig small intestine. Thus, akuammine does not produce analgesia by antagonising the actions of nociceptin/orphanin FQ at ORL_1-receptors.

In clinical practice, it is not akuammine which is taken for pain relief but rather extracts of the seeds. In the concentrations which we were able to use in this investigation, the crude extract was without opioid agonist activity in the bioassay systems. The extract contains a mixture of akuammine, akuammidine, akuammicine and other alkaloids with the possibility that opioid receptor antagonism by akuammine cancels out the agonist activity of the other two alkaloids resulting in no activity in the bioassays. This is especially likely since akuammine is the most abundant alkaloid in the seeds of *P. nitida*. Although a similar situation could arise following ingestion of seed extracts, the situation in vivo is more complicated as the alkaloids may be differentially absorbed and/or metabolised. In addition, different extraction procedures are likely to result in different proportions of the alkaloids.

Thus, it remains unclear by what mechanism(s) akuammine and the dried powdered seeds of *P. nitida* bring about pain relief, but the many receptors and transmitters involved in processing nociceptive information (see the works of McMahon et al. (1993) and Yaksh and Malmberg (1994)) allow for many possible sites of intervention.

Although none of the alkaloids examined in this investigation are either highly selective or high affinity opioid ligands they display varied efficacy and relative affinity for μ-, δ-and κ-opioid receptors. Since the alkaloids share a very similar structure, unlike those of opioid receptor ligands currently in use, perhaps changes in the structure of alkaloids from *P. nitida* could lead to more selective opioid compounds with higher affinities.

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