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Effects on Rat Brain κ_1- and κ_2-Opioid Receptors after Chronic Treatment with Non-peptide κ-Agonists

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Abstract

Injection of κ-agonist dynorphins and non-peptide κ-agonists into the hippocampus induces a reduction in blood pressure. It has been postulated that κ-opioid agonists and κ-receptors are important in one mechanism of antihypertension and might have clinical potential for the treatment of hypertension. We have investigated whether chronic treatment with U-50488H and U-62066E, two non-peptide κ-agonists, effects brain κ_1- or κ_2-receptor numbers or affinities in areas that might correlate with changes in blood pressure.

κ_1- and κ_2-Opioid receptor affinities and densities were determined in cortex, hippocampus, hypothalamus, midbrain and pons after 14 days subcutaneous infusion of two non-peptide κ-agonists, U-50488H and U-62066E, 9·6 mg kg day^{-1}, by means of osmotic minipumps, to spontaneously hypertensive rats (SHR) and to Wistar-Kyoto (WKY) rats. This infusion significantly reduced blood pressure. Brains were removed within 48 h of the end of drug infusion and κ-receptor binding studies were performed on homogenates from each brain area using [^{3}H]U-69593 to assay κ_1-receptors and [^{3}H]bremazocine to assay κ_2-receptors. U-62066E treatment seemed to cause a slight decrease in the number of [^{3}H]bremazocine binding sites (κ_2-receptors) from 98·2 ± 9 to 74·9 ± 8 fmol (mg protein)$^{-1}$ in the hippocampus when compared with SHR controls. A small decrease in κ_2-receptor density in the pons of WKY rats was also observed after U-50488H treatment (control, 51·2 ± 5; U-50488H-treated, 24·3 ± 9 fmol (mg protein)$^{-1}$). Although SHR blood pressure values were consistently reduced by treatment with κ-agonists, there was little if any significant change in apparent numbers of κ_1- or κ_2-receptors or their affinities in any of the brain regions examined.

These data indicate that although chronic treatment with κ-agonists reduces blood pressure in SHR, the treatment does not elicit major changes in brain κ-receptors either in SHR or in WKY rats. The potential use of κ-agonists for treating hypertension might not cause receptor changes in the brain and might, therefore, result in fewer side effects or negligible rebound hypertension.

It has been postulated that dynorphins and endogenous opioid agonists of the κ-subtype are involved in the pathophysiology of hypertension (Kunos et al 1981; Hassen et al 1984; Mastrianni & Ingenito 1987; Gulati & Bhargava 1988; Wang & Ingenito 1992). The dynorphin content of the hippocampus of the spontaneously hypertensive rat (SHR) is only approximately 25% of that of the Wistar-Kyoto (WKY) strain (Li et al 1989). Destruction of the dynorphin-producing cells of the hippocampus induces hypertension in normotensive WKY rats and accelerates development in SHR (Privette et al 1994). We have previously shown that injection of κ-agonist dynorphins and non-peptide κ-agonists into the hippocampus induces a reduction in blood pressure (Wang & Ingenito 1994; Zhai & Ingenito 1997). κ-Agonists might have clinical potential for the treatment of hypertension. Although dynorphin peptides are unlikely to penetrate the brain on systemic

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administration and are subject to rapid biodegradation (Numata et al 1988), non-peptide κ-opioid agonists such as U-50488H and U-62066E do penetrate the CNS when administered systemically (Vonvoightlander et al 1983; Vonvoightlander & Lewis 1988). Administration of these two non-peptide κ-agonists to male SHR and WKY rats over a two-week period reduced blood pressure and heart rate by 10–20% in SHR, but not in WKY rats (Zhai & Ingenito 1997). Because tolerance to the analgesic effects of these agents has been demonstrated (Vonvoightlander et al 1983; Vonvoightlander & Lewis 1988) one might expect tolerance to the antihypertensive effects reflected in receptor number or affinity changes after chronic treatment. In this study we wished to determine whether chronic treatment with the κ-agonists U-50488H and U-62066E would have any effect on brain κ_1- or κ_2-receptor numbers or affinities in various areas that might correlate with blood-pressure changes.

Materials and Methods

Materials

The benzene acetamide non-peptide κ-agonists, U-50488H and U-62066E (spiradoline) were gifts from Pharmacia and Upjohn, Kalamazoo, Michigan. [^{3}H]Bremazocine and [^{3}H]U-69593 were purchased from New England Nuclear. D-Penicillamine^2, D-penicillamine^5-enkephalin and D-ala^2,(Me)phe^4,glyol^5 enkephalin were purchased from Sigma.

Membrane preparations and radioligand-binding assays

Male spontaneously hypertensive rats, 10 weeks, and Wistar-Kyoto rats, 200–270 g (Harlan, Indianapolis, IN), 72 in all, were administered U-50488H, U-62066E (200 mg mL^{-1}, 9·6 mg kg day^{-1}) or equivalent volumes of normal saline subcutaneously for two weeks by means of Alzet #2002 osmotic mini-pumps. When administration was complete the pumps were removed and blood pressure and heart rate were measured for two more days at which time the rats were killed and the brains rapidly removed and dissected on ice. The various brain regions were then frozen at −70°C until assay.

For each assay the brain sections were thawed and then homogenized for 30 s in ice-cold buffer (50 mM Tris-HCl, 2 mM $MgCl_2$, 200 mM sucrose, pH 7·4; 10 mL) by means of a Brinkmann Polytron PT-10 tissue homogenizer at setting 6. The homogenate was then centrifuged at 18 000 *g* for 15 min and the pellet resuspended in 2–4 mL buffer. Protein determination was by the method of Lowry et al (1951) and ranged from 5 to 7 mg mL^{-1}.

Binding studies were performed with [^{3}H]bremazocine and [^{3}H]U-69593 to assay κ_1- and κ_2-receptors, respectively. Single-point analyses, using a saturating concentration of [^{3}H]ligand, and Scatchard plot analyses (Scatchard 1949), using a concentration range of 0·2 to 10 nM [^{3}H]ligand, were used to assay total apparent κ-receptor numbers. K_i values (inhibition constants) were calculated from displacement curves resulting from a fixed concentration of [^{3}H]ligand and increasing concentrations of displacing drugs. Briefly, for measurement of κ_1-binding the membrane preparation (50–75 μL) was added to buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 100 nM D-pencillamine^2,D-penicillamine^5-enkephallin, 100 nM D-ala^2,(Me)-phe^4,glyol^5, pH 7·4; 400 μL) in 12 × 75 polypropylene test tubes with 1·1 nM [^{3}H]U-69593 and incubated at 30°C for 45 min. Non-specific binding was determined in the presence of 1 μM U-62066E. κ_2-Receptor binding was assessed in a similar manner except that incubation was at 25°C for 60 min with [^{3}H]bremazocine, 100 nM U-62066E was added to the incubation buffer to block κ_1-binding sites, and non-specific binding was determined by use of 1 μM bremazocine. All assays were performed in triplicate or duplicate. The bound radioligand was separated from free ligand by rapid vacuum filtration through Whatman GFB glass-fibre filters and rinsed twice with ice-cold incubation buffer (5 mL). Radioactivity remaining on the filters was determined by liquid scintillation spectroscopy. Specific binding ranged from 60 to 85% of total binding.

Data analysis

Values were determined as means ± s.e.m. (standard error of the mean) and subjected to analysis of variance. $P < 0.05$ was regarded as indicative of statistical significance of the data.

Results

As shown in Table 1, very few changes were seen in densities of κ-receptors in the various brain regions assessed. No significant differences were seen between the densities of κ_1- or κ_2-receptors in any of the brain regions tested (pons, cortex, hippocampus, hypothalamus, midbrain) after any of the drug treatments, with the exception of a slight decrease in [^{3}H]bremazocine binding in the pons after U-50488H treatment of WKY rats and in the hippocampus after U-62066E treatment of SHR, when compared with saline controls. We also found a slight but significant increase in total κ_2-receptor binding sites in the hippocampus of SHR controls when compared with WKY controls.

Table 1. Total apparent κ_2- and κ_1-receptor densities after chronic treatment for two weeks with κ-agonists U-50488H or U-62066E.

Brain region	Wistar-Kyoto rats			Spontaneously hypertensive rats		
	Saline	U-50488H	U-62066E	Saline	U-50488H	U-62066E
			κ_2-Receptors			
Pons	51·2±5	24·3±9*	38·9±6	44·0±7	43·9±5	45·3±7
Cortex	122·6±13	137·3±16	139·4±9	118·2±12	117·6±14	110·9±11
Hippocampus	77·1±6	71·4±5	77·9±4	98·2±9**	91·2±11	74·9±8*
Hypothalamus	98·2±21	79·2±36	111·1±2	132·0±45	160·5±35	102·9±12
Midbrain	135·3±24	94·0±13	99·0±19	85·5±15	153·1±17	93·6±13
			κ_1-Receptors			
Pons†	50·0±4	57·1±5	45·0±4	49·1±7	48·1±3	41·6±4
Cortex†	38·5±2	32·4±3	34·9±1	35·5±4	29·2±5	31·6±3
Hippocampus	28·4±4	26·3±6	21·4±4	19·5±3	18·6±5	19·7±3
Hypothalamus	33·1±3	36·9±4	26·7±8	28·1±7	22·3±3	28·5±4
Midbrain†	39·8±4	35·4±2	40·6±6	40·1±8	42·3±4	37·8±2

κ_2- and κ_1-Receptors were measured by use of [^{3}H]bremazocine and [^{3}H]U-69593, respectively. Values are means±s.e.m. of results from 3–6 separate preparations each assayed in triplicate and expressed as fmol [^{3}H]ligand bound (mg protein)$^{-1}$. n=6 unless noted by † (n=3). *$P<0.05$, significantly different from result for saline controls of the same strain. **$P<0.05$, significantly different from result for saline controls of the WKY strain.

κ_1-Receptor affinities were determined by use of displacement curves and Scatchard analyses to assess K_i values. No significant differences were found in any of the brain areas after any of the agonist treatments for κ_1-receptors, (Table 2). Although predominately interested in κ_1-receptor affinity changes we also performed limited studies on the affinities of the κ_2-receptor but also found no changes in any of the brain areas as a result of the drug treatment (data not shown).

Discussion

In this study we have demonstrated that chronic treatment of SHR and WKY rats with the κ-agonists U-50488H or U-62066E resulted in few, if any, significant changes in κ_1- or κ_2-receptor affinities or densities in the different areas of the brain. In a previous study we demonstrated a sustained decrease in blood pressure and heart rate when each non-peptide κ-agonist was similarly administered (Zhai & Ingenito 1997). No tolerance to the cardiovascular effects were observed despite sustained administration of the drugs for 14 days. Our findings of lack of receptor changes with chronic agonist treatment is consistent with the lack of tolerance of the cardiovascular effects. With chronic treatment, if tolerance were developing we might expect to see a down-regulation of receptors. These findings are in agreement with those of Fujibayashi et al (1994) who demonstrated that no tolerance developed to the antinociceptive effect during chronic treatment with a novel κ-opioid agonist. Although our previous findings suggest the potential use of chronic κ-agonist treatment for hypertension, few studies have addressed the

Table 2. Effect on rat-brain κ_1-receptor K_i of chronic two-week treatment with κ-agonists U-50488H or U-62066E.

Brain region	Wistar–Kyoto rats			Spontaneously hypertensive rats		
	Saline	U-50488H	U-62066E	Saline	U-50488H	U-62066E
Pons	5·5±0·9	4·0±0·8	4·1±0·9	4·2±1	5·0±0·8	4·2±1·0
Cortex*	6·3	3·8	5·0	5·1	4·1	6·2
Hippocampus	5·1±0·2	6·5±0·3	5·3±0·2	5·2±0·6	5·0±0·6	6·5±0·9
Hypothalamus	4·0±0·5	5·1±0·3	5·1±0·4	4·0±0·4	4·9±0·8	6·2±0·9
Midbrain*	5·0	6·0	5·9	5·3	5·8	6·9

Effects on κ_2-receptors were measured by use of [^{3}H]U-69593. Displacement curves were generated in the presence of increasing concentrations of U-50488H and K_i values determined by Scatchard analysis. Values are the means±s.d. of two determinations of six pooled preparations each. *Only one determination of pooled brain regions. Values expressed $\times 10^{-7}$M.

potential adverse effects of such treatment. It is well known that treatment with opioid antagonists causes up-regulation of opioid receptors (Zukin et al 1993; Yoburn et al 1995). Results with chronic agonist treatment are not so clear and very few studies have been performed with κ-agonists (Bhargava et al 1989a; Morris & Herz 1989). Numerous groups have studied the possibility of agonist down-regulation of opioid receptors with varied results, most showing no significant changes in receptor density (Cox 1990). An interesting finding was reported by Holiday et al (1986), who demonstrated an increase in opioid receptor number after chronic agonist treatment with morphine. Bhargava et al (1989a, b) have shown that tolerance will develop to analgesia in rats treated chronically with κ-agonists, which, in the case of U-50488H, led to a down-regulation of non-selective opioid binding consisting of mostly μ- and some δ- and κ-receptors. Bremazocine, a κ_2-agonist, has been shown elicit a decrease in κ_2-receptor binding only when μ- and δ-receptor-binding have been suppressed (Morris & Herz 1989). In our study there seemed to be no tolerance to the blood-pressure-reducing effects over the 14 days of treatment with either of the κ-agonists. We were also unable to demonstrate changes in receptor numbers, suggesting that tolerance might not develop during chronic use of κ-agonists in-vivo. Because down-regulation can easily be shown in-vitro (Chang et al 1982; Zukin et al 1993; Raynor et al 1994) but is difficult to demonstrate in-vivo with κ-agonists, it is possible that compensatory mechanisms are involved and prevent observation of any tolerance of the antihypertensive effects or down-regulation in the whole animal.

Part of the problem in determining down-regulation of κ-receptors in-vivo is possible residual agonist remaining in the tissue and on receptors after chronic treatment (Morris & Herz 1989). Remaining agonist might be difficult to remove and might lead to false assessments of receptor numbers if a sufficient wash-out time is not observed. By waiting 48 h after removal of the drugs before killing the rats and measuring the receptors we hoped for removal of all residual ligand which might artifactually cause a reduction in the apparent number of κ-receptors. At this time although blood pressures were beginning to return to control values, they were still significantly depressed (Zhai & Ingenito 1997). It is difficult to determine the optimum time for brain removal after chronic agonist treatment but by 48 h most residual drug should have been cleared. Although it is certainly possible that after this time receptors might have recovered to control levels, we still observed a significant and sustained decrease in blood pressure, suggesting that if receptor changes were indeed involved in the reduction of blood pressure, these changes would, in all likelihood, still be present. This is also supported by previous studies in which Pasternak (1993) demonstrated an in-vivo turnover rate of opioid receptors of three to four days. Therefore, if receptors have been changed and down-regulated they would not have time to return to normal values two days post-infusion. A possible follow-up to this study would be to investigate potential receptor changes immediately after drug discontinuation and to follow these until blood-pressure-reduction effects were no longer observed. Such blood-pressure-reduction effects without receptor down-regulation might have important clinical implications.

A primary focus of this study centred on the hippocampus and hypothalamus because we have previously shown that dynorphin A (1–8) in these areas is lower in SHR, implying a role in hypertension (Li et al 1989, 1990, 1992). These findings led us to investigate the possible effects of chronic treatment with κ-agonists on κ-receptor numbers and affinities in these areas. Because reduction of blood pressure was seen in hypertensive but not normotensive rats as a result of chronic treatment with κ-agonists, and no changes were seen in receptor numbers or affinities, tolerance related to changes in κ-receptors in the brain does not seem to occur. Accordingly, receptor-density changes (and therefore certain side effects) might not be a problem during long-term use of these drugs for treatment of hypertension.

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