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The role of minoxidil on endogenous opioid peptides in the spinal cord: a putative co-agonist relationship between K-ATP openers and opioids

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Abstract

ATP-gated K^+ channel openers produce antinociception that is attenuated by opioid receptor antagonists, indicating K-ATP openers produce antinociception, in part, via the release of endogenous opioid peptides. Utilizing the spinal perfusion method, male Sprague–Dawley rats were administered minoxidil intrathecally (i.t.) at doses ranging from 12.5 to 200 μg/rat for 3 min, tested for antinociception using the tail-flick test, and perfused with artificial cerebrospinal fluid (aCSF) to collect endogenous opioid peptides. Endogenous opioid peptide levels were measured by radioimmunoassay. Naltrindole, a δ-opioid receptor antagonist, at 4 mg/kg, subcutaneously (s.c.), blocked minoxidil-induced antinociception. β-Funaltrexamine, a μ-opioid receptor antagonist, at 100 μg/rat, partially blocked minoxidil, whereas the κ-opioid receptor antagonist nor-binaltorphimine, at a dose of 100 μg/rat, did not attenuate minoxidil. Although antagonists of the μ- and δ-opioid receptor attenuated minoxidil-induced antinociception, there was no increase in β-endorphin, an endogenous ligand with affinity for both μ- and δ-opioid receptors or [Leu^5]enkephalin, an endogenous ligand with affinity for δ-opioid receptors.

Keywords: K^+ channel; Minoxidil; Opioid; (Rat); Antinociception; ATP-gated

1. Introduction

Minoxidil is a vasodilator and has been used for many years to treat hypertension (Triggle, 1990). Minoxidil is also the active ingredient in the product Rogaine®, which increases hair growth due to its ability to stimulate the hair follicle by increasing blood flow to the blood vessels of the scalp (Savin and Atton, 1993). Minoxidil is in a class of drugs called ATP-gated K^+ channel openers, due to their ability to enhance K^+ conductance via channels that are controlled by the ATP:ADP ratio of the cell (DeWeille and Lazdunski, 1990). High ATP:ADP closes the channel and leads to cellular depolarization and neurotransmitter release. Conversely, the opening of the channel leads to hyperpolarization, and a decrease in neurotransmitter release. Oral hypoglycemic agents, such as glyburide, close ATP-gated K^+ channels (Ocaña et al., 1990; Welch and Dunlow, 1993). This channel is opened by levcromakalim and diazoxide, as well as minoxidil (Newgreen et al., 1990). In addition to the above effects, minoxidil produces antinociception in mice when administered centrally (Welch and Dunlow, 1993). It is believed that its antinociceptive effect is due to its ability to open K-ATP channels, and consequently release endogenous opioids. The later is postulated because minoxidil-induced antinociception is attenuated by naloxone, an opioid antagonist (Welch and Dunlow, 1993).

There are three categories of opioid receptors: μ, δ, and κ, which are coupled to inhibitory G proteins (G_i) (Satoh and Minami, 1995). Opioids inhibit adenylate cyclase activity, which leads to a decrease in cAMP, a reduction in Ca^{2+} conductance, and thus an attenuation of neurotransmitter release. It is hypothesized that endogenous opioids are released via a disinhibitory process, and subsequently antinociception is produced (Fields et al., 1983; Depaulin et al., 1987; Heinricher et al., 1994). Studies conducted by North et al. (1987) suggest that opioid receptors may be

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G-protein-coupled to K^+ channels. Therefore, opioids have a duality in their function. In addition to their effects mediated through G-proteins, they share with minoxidil and other openers of the ATP-gated K^+ channel the ability to modulate K^+ efflux (Duty and Weston, 1990). Although K-ATP openers and opioids have similar actions on K-ATP channels, opioids do not bind directly to the K-ATP channel. Receptor binding studies indicate that morphine does not displace [^{3}H]glyburide binding in mouse brain (Welch et al., 1997). There is also no cross-tolerance between opioids and K-ATP openers, further indicating a lack of direct receptor interaction (Welch and Dunlow, 1993).

Studies conducted in our laboratory seem to indicate that K^+ channel openers produce antinociception via the release of endogenous opioids. Lohmann and Welch (1999a) demonstrated that both diazoxide and levcromakalim, intracerebroventricular (i.c.v.), produced antinociception that was antagonized by D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP), a selective μ-opioid receptor antagonist and ICI 174,864, a δ-opioid receptor antagonist, while diazoxide, but not levcromakalim was also non-selectively blocked by nor-binaltorphimine, a κ-opioid receptor antagonist as well. Lohmann and Welch (1999b) have also shown that antisense oligonucleotides directed against μ-, δ- and κ-opioid receptors attenuate antinociception produced by K-ATP openers, further implicating opioid involvement in their antinociceptive effect. In the present study, male Sprague–Dawley rats were administered minoxidil sulfate via spinal perfusion. Antagonists of the opioid receptor subtypes were administered to determine which endogenous opioid systems were involved in minoxidil-induced antinociception. Finally, endogenous opioid peptide levels were measured directly using radioimmunoassay (RIA).

2. Materials and methods

2.1. Animal husbandry

Male Sprague–Dawley rats weighing between 400 and 450 g obtained from Harlan Laboratories were used in these studies. The rats were housed two per cage and were maintained on a 12-h light/dark cycle. Food and water were provided ad libitum.

2.2. Intrathecal administration of drugs and opioid peptide collection

The intrathecal administration of drugs and the collection of opioid peptides were performed utilizing a modified version of the methods of Yaksh (1981) and Tseng (1989). Rats were anesthetized using sodium pentobarbital at a dose of 65 mg/kg, intraperitoneal (i.p.). An additional injection of atropine methyl nitrate (2 mg/kg, i.p.) was administered. Atropine methyl nitrate and sodium pentobarbital did not alter basal endogenous opioid peptide levels results not shown. The anesthetized rats were placed in a stereotaxis and an incision was made on the atlanto–occipito membrane to expose the cisterna magna. A 9-cm spinal catheter made of PE-10 polyethylene tubing filled with artificial cerebrospinal fluid (aCSF) was inserted into the cisternal cavity, caudally, into the subarachnoid space of the spinal cord. Drugs used in this experiment were pumped through the catheter caudally and entered the lumbar region. Artificial CSF was perfused through the spinal catheter at specified times and then collected cranially at the cisterna magna for evaluation of endogenous opioid peptide release. Artificial CSF was prepared containing: NaCl 146 mM; KCl 2.6 mM; $MgCl_2$ 0.9 mM; $CaCl_2$ 1.2 mM; $NaHCO_3$ 21.0 mM; HPO_4 2.5 mM; 1 mg/ml bovine serum albumin, bacitracin (30 mg/ml), 0.01% Triton X and bubbled with 95% O_2 and 5% CO_2. Baseline tail-flick latency was obtained from the rats following catheter insertion and a 30–45-min acclimation period on a heating pad. Only rats exhibiting a baseline tail-flick less than 4 s were utilized in the study.

During the antagonism studies, a group of rats was administered naltrindole at 0.5–4 mg/kg, subcutaneously (s.c.), immediately following surgery and was allowed to acclimate for 45 min as described above. Other groups of rats were administered nor-binaltorphimine at 100 μg, intrathecally (i.t.) or β-funaltrexamine (10 μg, i.t.) following baseline tail-flick measurements. Ten-minute post-administration of nor-binaltorphimine or β-funaltrexamine, and immediately following the determination of the baseline tail-flick latency of rats administered naltrindole following the 45-min acclimation, a cocktail of endopeptidase inhibitors containing phosphoramidon, bestatin and captopril, all at 25 μg/rat, (i.t.) was administered. Test compounds were administered 5-min post-administration of the endopeptidase inhibitors in a volume of 20 μl at a rate of 30 μl/min. Three-minute post-administration of vehicle dimethyl sulfoxide (DMSO) or drug, aCSF was collected. Utilizing a peristaltic pump, the spinal cavity was rapidly perfused with aCSF, which was collected in a 1.5-ml aliquot from the open cisternal space. The collected fractions were boiled for 10–12 min and centrifuged at a rate of 10,000 rpm for 10 min. The supernatant was collected, frozen at −80°C, and lyophilized. Samples were reconstituted with RIA buffer for opioid peptide measurements.

2.3. Measurement of opioid peptides

Opioid peptide levels were measured using specific dynorphin A-(1–17), [Leu^5]enkephalin, and β-endorphin (human) RIA kits. RIA kits were purchased from Peninsula Laboratories. Previous data (results not published) indicate a lack of [Met^5]enkephalin release following minoxidil administration, therefore [Met^5]enkephalin levels were not assayed in these experiments. The samples were analyzed in duplicate.

2.3.1. Measurement of dynorphin A-(1–17)

For the measurement of dynorphin A-(1–17), the lyophilized samples were rehydrated in 250 μl of RIA buffer. Peninsula Laboratories show that there is minimal cross reactivity of dynorphin A-(1–17) to dynorphin A-(1–13) and no cross reactivity to β-endorphin and [Leu^5]enkephalin and [Met^5]enkephalin. Only the linear portion of the RIA standard curve, between 0.1 and 64 pg/ml of standard dynorphin A-(1–17) peptide, was used to calculate dynorphin A-(1–17) concentrations.

2.3.2. Measurement of rat β-endorphin

For the measurement of β-endorphin, samples were reconstituted in 500 μl of RIA buffer. Peninsula Laboratories showed no cross reactivity of the human β-endorphin antibody to [Met^5]enkephalin or [Leu^5]enkephalin. The human β-endorphin antibody is 80% cross-reactive to rat β-endorphin and 100% cross-reactive to equine and porcine β-endorphin. Only the linear portion of the radioimmunoassay standard curve, between 1 and 128 pg/ml of standard β-endorphin peptide, was used to calculate β-endorphin concentrations.

2.3.3. Measurement of [Leu^5]enkephalin

For the measurement of [Leu^5]enkephalin, samples were reconstituted in 500-μl RIA buffer. Peninsula Laboratories indicate that the antibody is 29% cross-reactive to dynorphin A-(1–17), 8% to dynorphin A-(1–8) and 3% to [Met^5]enkephalin. Only the linear portion of the RIA standard curve, between 1 and 128 pg/ml of standard [Leu^5]enkephalin peptide, was used to calculate [Leu^5]enkephalin concentrations.

2.4. Assessment of tail-flick latency

Antinociception was measured using the tail-flick latency method, a modified version of the method described by D'Amour and Smith (1941). Rats were allowed to acclimate in the laboratory for 24 h prior to experimentation. The basal tail-flick latency was not increased by the use of the anesthetic agent sodium pentobarbital. Rats with baseline tail-flick measurements greater than 4 s were not utilized in this study and there was a maximum cut-off time of 10 s. Tail-skin temperatures were not monitored in these experiments due to evidence that the tail-flick response is produced independently from changes in tail-skin or core temperatures (Lichtman et al., 1993). Antinociception as measured by the tail-flick apparatus was transformed into percent maximum possible effect (%MPE) using the following formula:

$$\%\text{MPE} = 100\left[(\text{test} - \text{control})/(10 - \text{control})\right].$$

The control value is the baseline tail-flick obtained after the surgery, but before the administration of drug, while the test value is obtained post-administration of drug and after control values are ascertained (Harris and Pierson, 1964). Using 12 rats/treatment, a %MPE was calculated for each animal. The mean effect and standard error of the mean were calculated for every treatment using the %MPE for each rat.

2.5. Statistical analysis

ED_{50} values were determined using the methods of Tallarida and Murray (1987) for graded data. Both tail-flick data and endogenous opioid peptide concentration data were analyzed using analysis of variance (ANOVA) fol-

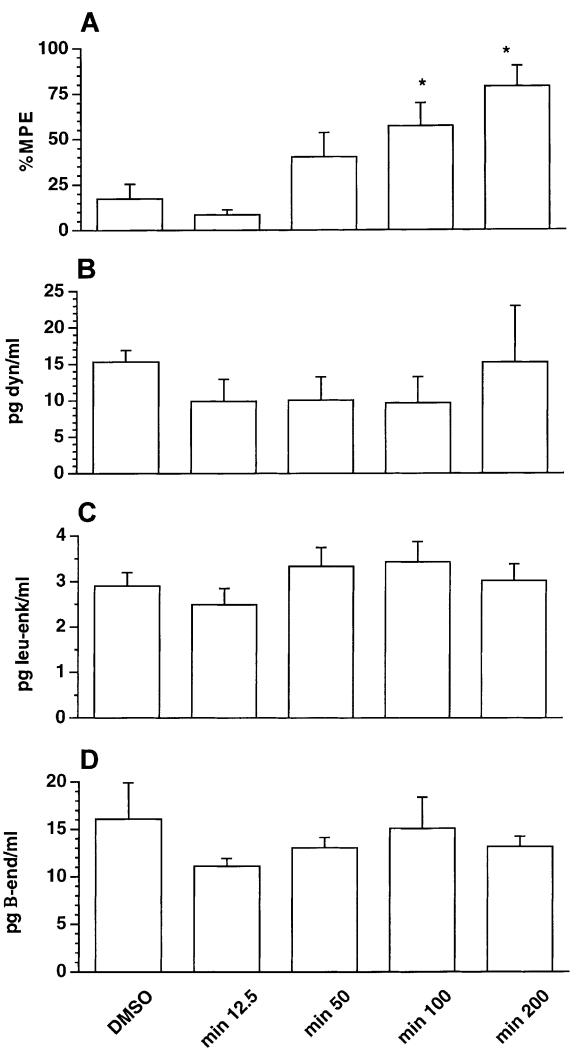

Fig. 1. Comparison of spinal endogenous opioid peptide levels after the administration of minoxidil at doses of 12.5, 50, 100 and 200 μg/rat, i.t. and the DMSO vehicle. Panel A indicates the %MPE of minoxidil and DMSO, at the above doses. Panels B, C and D show the levels of dynorphin A-(1–17), [leu^5]enkephalin and β-endorphin (pg/ml of peptide) post-administration of the above doses of minoxidil and for the DMSO vehicle, respectively. Mean concentrations of endogenous opioid peptides ± S.E. are presented. The (*) represents significant values $p < 0.05$ in comparison to control animals receiving DMSO vehicle (i.t.).

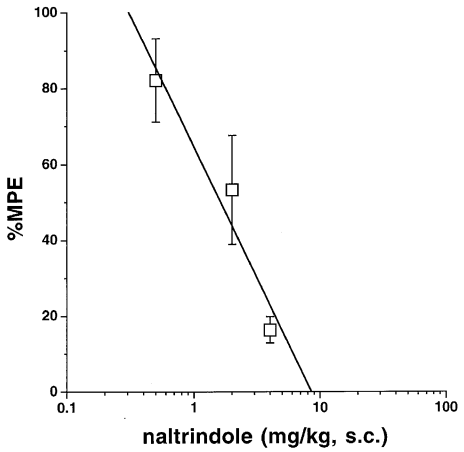

Fig. 2. The s.c. administration of naltrindole prior to an ED_{80} dose of minoxidil at 200 μg/rat, (i.t.). The AD_{50} of naltrindole was 1.64 (1.1–2.5) mg/kg.

lowed by post-hoc Fisher (PLSD) test. *P* values less than 0.05 were considered significant denoted with an (*).

2.6. Drugs

Minoxidil sulfate, nor-binaltorphimine, β-funaltrexamine, naltrindole, phosphoramidon, bestatin, and captopril were all purchased from Sigma (St. Louis, MO).

3. Results

3.1. Minoxidil-induced antinociception and endogenous opioid peptide release

To determine if minoxidil sulfate (i.t.) produced antinociception in male Sprague–Dawley rats, a dose–response curve was generated. Minoxidil produced a dose-dependent antinociceptive effect with an ED_{50} value of 71 (45–110) μg/rat, i.t. (Fig. 1). The %MPE of minoxidil at a dose of 100 μg/rat was 57 ± 13% and 200 μg/rat was 78 ± 11%. Both doses produced an effect greater than the DMSO control 17 ± 5% (Fig. 1). Endogenous opioid peptide levels were measured 3-min post-administration of minoxidil at doses of 12.5, 50, 100 and 200 μg/rat. These doses did not produce alterations in the immunoreactive concentrations of dynorphin A-(1–17), β-endorphin, or [Leu^5]enkephalin. Peptide concentrations at the above doses of minoxidil were no different than that of the DMSO control (Fig. 1).

3.2. Naltrindole block of minoxidil and endogenous opioid peptide release

The antinociceptive effect of an ED_{80} dose of minoxidil at 200 μg/rat, i.t. was attenuated by the δ-opioid receptor antagonist naltrindole with an AD_{50} of 1.6 (1.1–2.5) mg/kg, s.c. (Fig. 2). There was a complete attenuation of minoxidil-induced antinociception following the administration of naltrindole at a dose of 4 mg/kg, (s.c.). The %MPE of minoxidil decreased from 78 ± 11% to 16 ± 4% in the presence of naltrindole at 4 mg/kg, s.c. (Fig. 3). The administration of naltrindole had no effect on the immunoreactive concentrations of dynorphin A-(1–17), [Leu^5]enkephalin, or β-endorphin as demonstrated in Fig. 3, panels B, C and D.

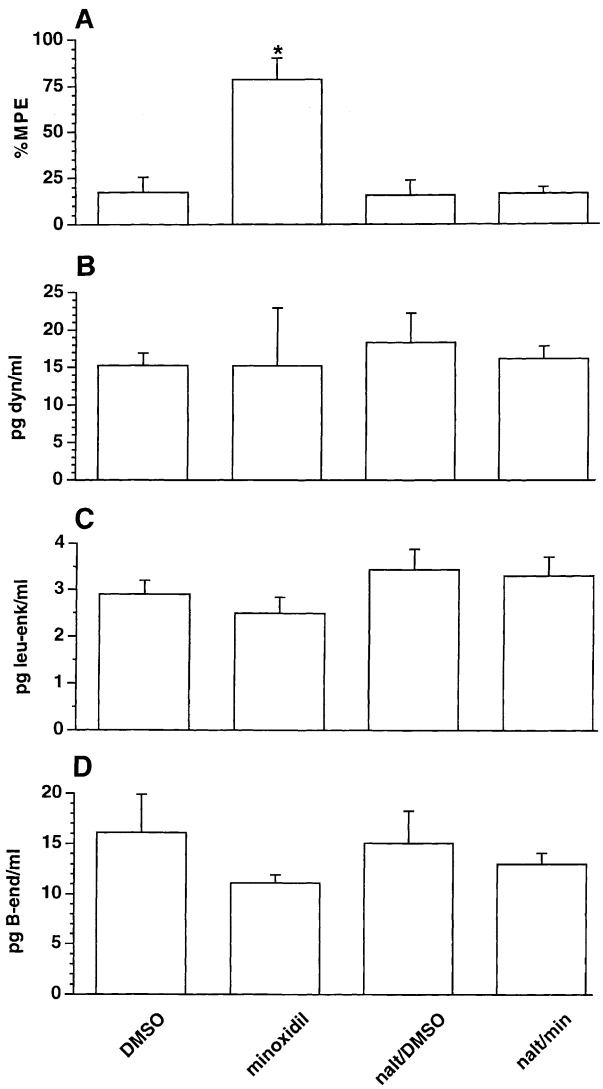

Fig. 3. Comparison of spinal endogenous opioid peptide levels after the administration of the DMSO vehicle; minoxidil at 200 μg/rat, i.t.; naltrindole 4 mg/kg followed by DMSO; and naltrindole 4 mg/kg followed by minoxidil at 200 μg/rat, i.t. Panel A indicates the %MPE post-administration of the drugs mentioned above. Panels B, C and D show the levels of dynorphin A-(1–17), [leu^5]enkephalin and β-endorphin (pg/ml of peptide) following the treatments described above, respectively. Mean concentrations of endogenous opioid peptides ± S.E. are presented. The (*) represents significant values $p < 0.05$ in comparison to control animals receiving DMSO vehicle (i.t.).

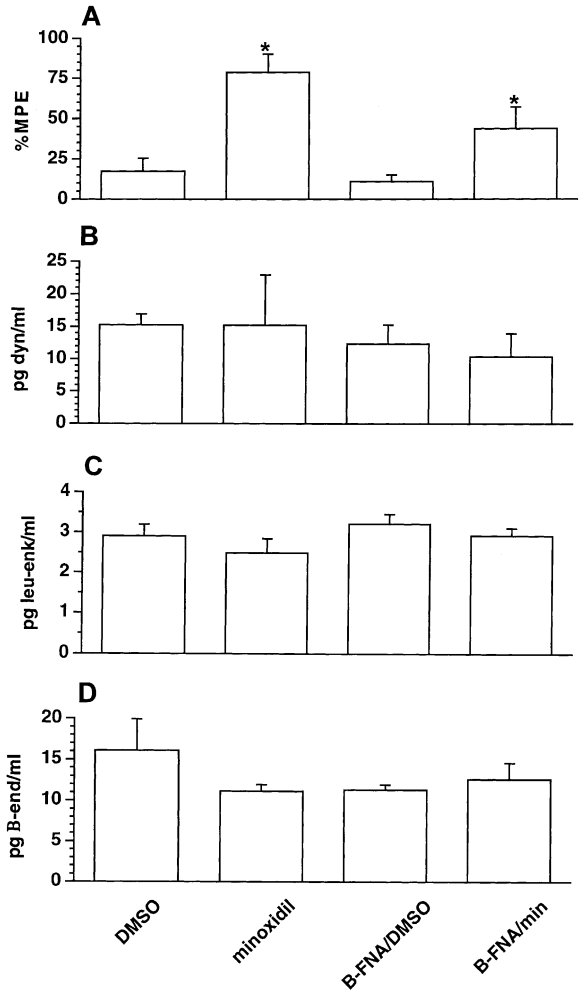

Fig. 4. Comparison of spinal endogenous opioid peptide levels after the administration of the DMSO vehicle; minoxidil at 200 μg/rat, i.t.; β-funaltrexamine at 100 μg/rat, i.t. followed by DMSO; and β-funaltrexamine at 100 μg/rat, i.t. followed by minoxidil at 200 μg/rat, i.t. Panel A indicates the %MPE post-administration of the drugs mentioned above. Panels B, C and D show the levels of dynorphin A-(1–17), [leu^5]enkephalin and β-endorphin (pg/ml of peptide) following the treatments described above, respectively. Mean concentrations of endogenous opioid peptides ± S.E. are presented. The (*) represents significant values $p < 0.05$ in comparison to control animals receiving DMSO vehicle (i.t.).

3.3. β-Funaltrexamine antagonism of minoxidil and endogenous opioid peptide release

Minoxidil-induced antinociception was partially blocked by the irreversible μ-opioid receptor antagonist β-funaltrexamine. The administration of β-funaltrexamine at 100 μg/rat, i.t. decreased the %MPE of minoxidil to 43 ± 13%, but this was significantly greater than the control group administered β-funaltrexamine at 100-μg/rat, i.t. and DMSO, %MPE of 11 ± 4.0%. Like naltrindole, β-funaltrexamine antagonized minoxidil, but did not alter the levels of endogenous opioid peptides in comparison to the control (Fig. 4, panels B, C and D).

3.4. Nor-binaltorphimine block of minoxidil and endogenous opioid peptide release

Minoxidil-induced antinociception was not attenuated by the κ-opioid receptor antagonist, nor-binaltorphimine at 100 μg/rat, (i.t.). There was no difference between rats pretreated with nor-binaltorphimine and administered minoxidil at 200 μg/rat, i.t., %MPE of 83 ± 11%, and rats administered minoxidil alone, %MPE of 78 ± 11%. The antinociception produced by both of these treatments was greater than the control of nor-binaltorphimine followed by

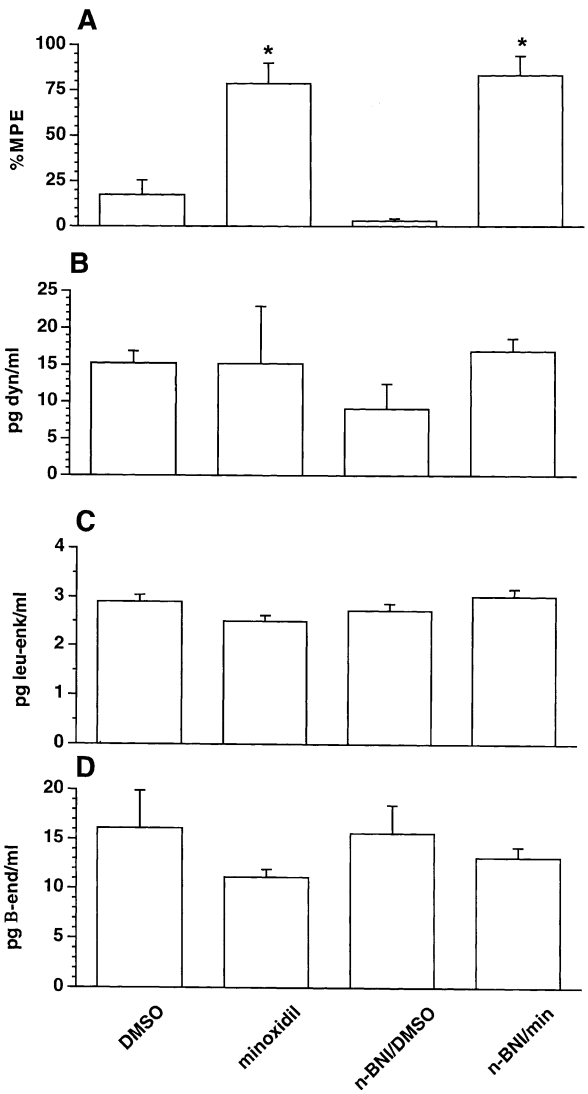

Fig. 5. Comparison of spinal endogenous opioid peptide levels after the administration of the DMSO vehicle; minoxidil at 200 μg/rat, i.t.; nor-binaltorphimine at 100 μg/rat, i.t. followed by DMSO; and nor-binaltorphimine at 100 μg/rat, i.t. followed by minoxidil at 200 μg/rat, i.t. Panel A indicates the %MPE of the drugs mentioned above. Panels B, C and D show the levels of dynorphin A-(1–17), [leu^5]enkephalin and β-endorphin (pg/ml of peptide) following the treatments described above, respectively. Mean concentrations of endogenous opioid peptides ± S.E. are presented. The (*) represents significant values $p < 0.05$ in comparison to control animals receiving DMSO vehicle (i.t.).

DMSO 3.0 ± 1.2% (Fig. 5). Similar to the results with μ- and δ-blockers, there was no change in endogenous opioid peptide levels (Fig. 5, panels B, C, and D).

4. Discussion

Our data indicate that the ATP-gated K^+ channel opener minoxidil produced dose-dependent antinociception as demonstrated by the tail-flick latency test, which was antagonized by the δ-opioid receptor antagonist, naltrindole. This indicates potential involvement of enkephalins, endogenous ligands with affinity for the δ-opioid receptor, in minoxidil-induced antinociception (Mansour et al., 1995; Noble et al., 1996). In addition, minoxidil was partially antagonized by β-funaltrexamine, a μ-opioid receptor antagonist, implicating the involvement of endorphins (Yaksh and Henry, 1978; Yaksh et al., 1982) or endomorphins (Zadina et al., 1997), endogenous ligands with affinity for the μ-opioid receptor. However, minoxidil-induced antinociception was not antagonized by the κ-opioid receptor antagonist, nor-binaltorphimine. This indicates the lack of involvement of dynorphins, endogenous ligands with affinity for the κ-opioid receptor in minoxidil-induced antinociception. These data suggest that minoxidil-induced antinociception involves both μ- and δ-, but not the κ-opioid system. Our data is in accordance with previous studies conducted by Welch and Dunlow (1993). Our laboratory, as well as that of others, has shown that both the i.t. (Welch and Dunlow, 1993) and i.c.v. (Narita et al., 1993; Ocaña et al., 1995) administration of K-ATP openers produces antinociception that is attenuated by opioid receptor antagonists. In this study, we attempted to determine the mechanism by which minoxidil produces its antinociceptive effect and the role of endogenous opioid peptides in the antinociception produced by minoxidil via direct quantification of opioid peptide release.

Hollt et al. (1978) demonstrated that morphine administration (i.v.) increases plasma β-endorphin levels in the rat. Natsuki and Dewey (1993) showed an increase in the levels of both [Met^5]enkephalin and [Leu^5]enkephalin, β-endorphin and dynorphin A-(1–13) following the administration of morphine (s.c.) in dog CSF. The blockade of K-ATP openers by opioid antagonists led us to hypothesize that K-ATP openers might also increase the release of endogenous opioid peptides, in a manner similar to that of morphine. RIA results indicate at 3- and 10-min (data not shown) post-administration of minoxidil, there was no increase in the levels of [Leu^5]enkephalin, β-endorphin or dynorphin A-(1–17), although minoxidil produced antinociception that was attenuated by opioid receptor antagonists. There was also no change in endogenous opioid levels when minoxidil was administered in combination with the opioid antagonists. Similarly, the antagonist administered with the DMSO vehicle did not alter endogenous opioid levels.

Thus, the interaction between K-ATP openers and opioids remains unknown. K-ATP openers are antagonized by opioid receptor antagonists and opiates are attenuated by K-ATP blockers such as glyburide (Ocaña et al., 1990; Rodrigues and Duarte, 2000; Lohmann and Welch, 1999a; Welch and Dunlow, 1993). However, most of the data suggest that the interaction between the drug classes is indirect. There is no cross-tolerance between K-ATP openers and morphine, thus K-ATP openers produce antinociception in morphine tolerant mice (Welch and Dunlow, 1993). However, there are a lack of studies that evaluate the effect of morphine on mice or rats tolerant to K-ATP openers, since K-ATP openers do not cross the blood–brain barrier and can only be administered centrally (Edwards and Weston, 1990). Receptor binding studies show that morphine does not displace the K-ATP blocker, [^{3}H]glyburide, indicating morphine does not bind to the sulfonylurea receptor, the binding site of oral sulfonyureas and K-ATP openers (Welch et al., 1997). Conversely, glyburide does not displace [^{3}H]DAMGO ([D-Ala^2, NMePhe^4,Gly-ol]-enkephalin), [^{3}H]DPDPE ([D-Pen^2, D-Pen^5]-enkephalin)or [^{3}H]U-69,593 ((+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide), which are μ-, δ- and κ-opioid receptor agonists, respectively (Raffa and Codd, 1994). This indicates that ligands of the sulfonylurea receptor site of the K-ATP channel do not bind to opioid receptors.

In conclusion, minoxidil-induced antinociception at 3 min does not increase the release of endogenous opioid peptides, but is sensitive to opioid antagonists. There are several potential explanations for our results. This study was limited in that only three endogenous opioid peptides were evaluated, [Met^5]enkephalin was previously tested, but there was no significant increase in release following the administration of minoxidil. It is possible that minoxidil may increase the release of other endogenous opioid peptides such as endomorphins, which are also attenuated by μ-antagonists such as β-funaltrexamine (Dun et al., 2000) or nociceptin, an atypical opioid peptide that is not antagonized by classical opioid antagonists (Xu et al., 1996), but is blocked by J-113397 (1-[(3*R*,4*R*)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1, 3-dihydro-2*H*-benzimidazol-2-one), a novel non-peptide antagonist (Bigoni et al., 2000), two peptides that were not evaluated in this study. Alternatively, it is possible, but unlikely, that the opioid antagonists might directly antagonize the antinociceptive effects of minoxidil via non-opioid receptors. However, we hypothesize that tonic endogenous opioid release may in some manner modulate the ATP-gated K^+ channel. We further hypothesize that blockade of that tonic opioid control may in turn alter the efficacy of minoxidil. The antagonists administered alone did not produce antinociception, hyperalgesia or alter endogenous opioid peptide levels. Taken together, our data strongly indicate that basal levels of endogenous opioid peptides are needed at opioid receptors in order for minoxidil to

produce an effect. This could explain why opioid receptor antagonists attenuate minoxidil-induced antinociception. In addition, such data are the first putative evidence of a tonic interaction between the endogenous opioid and K-ATP system.

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