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ATP-gated K^+ channel openers enhance opioid antinociception: indirect evidence for the release of endogenous opioid peptides

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Abstract

The ATP-gated K^+ channel openers — diazoxide, levcromakalim and morphine — enhance K^+ efflux by opening ATP-gated K^+ channels, thereby inducing cell hyperpolarization. Hyperpolarization decreases intracellular Ca^{2+} levels, which leads to a decrease in neurotransmitter release contributing to the antinociceptive effects of the drugs. Previous findings implicate the release of endogenous opioids as the mediator of the antinociceptive effects of ATP-gated K^+ channel openers. Diazoxide and levcromakalim, administered intracerebroventricularly (i.c.v.), produced dose-dependent antinociception as determined by the tail-flick method {ED_{50} 44 μg/mouse [95% confidence limits (CLs) from 28 to 68 μg/mouse] for diazoxide}. Glyburide (10 μg/mouse), an ATP-gated K^+ channel antagonist, attenuated the effects of diazoxide, levcromakalim and morphine. Diazoxide- and levcromakalim-induced antinociception were both antagonized by CTOP (D-Phe–Cys–Tyr–D-Trp–Orn–Thr–Pen–Thr amide), a μ-opioid receptor selective antagonist, and ICI 174,864 (*N*, *N*-diallyl–Tyr–Aib–Aib–Phe–Leu), a δ-opioid receptor antagonist, but were differentially attenuated by the κ-opioid receptor antagonist, nor-Binaltorphimine. Combinations of inactive doses of the K^+ channel openers and opioid receptor agonists produced significant antinociceptive enhancement. Diazoxide (2 μg/mouse) shifted morphine's dose–response curve 47-fold, while levcromakalim (0.1 μg/mouse) shifted the curve 27-fold. The dose–response curve of κ-opioid receptor agonist U50,488H (*trans*-(±)-3, 4 Dichloro-*N*-[2-(1-pyrrolidinyl)-cyclohexyl] benzeneacetamide methane sulfonate) was shifted 106-fold by diazoxide in a parallel manner, while levcromakalim administration increased the potency of U50,488H by 15-fold. Diazoxide shifted the dose–response curve of the δ-opioid receptor agonist, DPDPE [(D-$Pen^{2,5}$)-enkephalin], leftward in a non-parallel manner, while DPDPE was 6-fold more potent when combined with levcromakalim. We hypothesize that endogenous opioids mediate ATP-gated K^+ channel opener-induced antinociception and enhancement of opioids.

Keywords: K^+ channel, ATP-gated; Opioid; Antinociception; Enhancement; (Mouse)

1. Introduction

The μ-, δ- and κ-opioid receptors have been cloned, are more than 50% homologous and are constructed of seven transmembrane segments (Reisine and Bell, 1993; Satoh and Minami, 1995). In addition to the known homology of the opioid receptors, they are all coupled to inhibitory G proteins (G_i) (Satoh and Minami, 1995). Once the ligand binds to its specific receptor, an increase in K^+ efflux from the cell is observed, which alters the cell's membrane potential. The hyperpolarization of the membrane leads to a decrease in Ca^{2+} entry, subsequently decreasing the release of neurotransmitters. This alteration in neurotransmitter release accounts for the antinociceptive effects of the μ- and δ-opioids receptor agonists (Williams et al., 1982; Werz and MacDonald, 1985; North et al., 1987; Triggle, 1990; Aronsen, 1992). The κ-opioid receptor agonist induces a similar cell hyperpolarization through the closure of Ca^{2+} channels (Cherubini and North, 1985; Gross and MacDonald, 1987; Attali et al., 1989; Xiang et al., 1990) in addition to increasing K^+ efflux from the cell (Grudt and Williams, 1993).

In the central nervous system, there are four general classes of K^+ channels: the voltage-activated K^+ channels, which respond to alterations in membrane electrical activity (Aronsen, 1992; North, 1992); Ca^{2+}-gated K^+

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channels, which enhance cell repolarization (Hughes et al., 1982; Smith et al., 1986; Gimenez-Gallego et al., 1988; Castle et al., 1989; Strong, 1990); ligand-activated K^+ channels, a very diverse group altered by many neurotransmitters and classes of drugs, including opiates. Usually, these channels are G-protein-coupled (Brown, 1990). The final class of K^+ channels is the ATP-gated K^+ channels that open and close in response to changes in intracellular ATP/ADP ratios. Low ATP levels open these channels, allowing K^+ efflux and cell hyperpolarization (Deweille and Lazdunski, 1990).

Cloning of the ATP-gated K^+ channels has revealed that they are comprised of a sulfonylurea receptor (SUR1) and an inward rectifier K^+ channel subunit (Kir6.2). Both subunits are necessary for proper ion conduction (Inagaki et al., 1995; Philipson, 1995). Glyburide, a potent sulfonylurea, acts at the distinct sulfonylurea binding site to allosterically close ATP-gated K^+ channels (Amoroso et al., 1990). A variety of vasodilators, including diazoxide, have the capability of opening these channels (Newgreen et al., 1990). Additionally, these channels are opened by cromakalim and its active L-isomer, levcromakalim, which are used to treat hypertension and angina (Triggle, 1990). The K^+ channel openers are a class of drugs that share with opioids the ability to open ATP-gated K^+ channels and enhance K^+ efflux from the cell (Duty and Weston, 1990). In addition, central, but not peripheral, administration of these ATP-gated K^+ channels openers has been shown to produce antinociception. This is due to the fact that the openers cannot cross the blood–brain barrier (Edwards and Weston, 1990; Welch and Dunlow, 1993).

Previous findings in our laboratory have demonstrated that opioid receptor antagonists attenuate the antinociceptive effects of the K^+ channel openers administered intrathecally (i.t.) (Welch and Dunlow, 1993). Such data indicate that the ATP-gated K^+ channels openers either interact directly with the opioid receptors or induce the release of an endogenous opioid which can bind to its specific opioid receptor. Lack of cross-tolerance between the ATP-gated K^+ channels openers and morphine, as well as binding studies using [^{3}H]-dihydromorphine and [^{3}H]-glyburide, indicates a lack of direct interaction between the openers and the opioid receptors (Welch and Dunlow, 1993). However, such a lack of interaction directly at the opioid receptor does not preclude interactions between the drug classes at common signal transduction points. The ATP-gated K^+ channels openers most likely induce the release of endogenous opioids. However, it is still unknown which of the endogenous opioids mediate the antinociceptive effects of the ATP-gated K^+ channels openers.

Intracerebroventricular (i.c.v.) administration of cromakalim produces supraspinal antinociception (Narita et al., 1993), as does the administration of other ATP-gated K^+ channels openers (Ocana et al., 1995). It has also been demonstrated that cromakalim enhances μ-opioid receptor antinociception (Narita et al., 1993; Ocana et al., 1996). Administration of cromakalim and diazoxide has also been shown to inhibit the characteristic signs of morphine withdrawal (Robles et al., 1994). Given that previous work has demonstrated that the potency of μ-opioid-receptor-specific agonists is increased by the administration of the δ-opioid receptor agonists, DPDPE [(D-Pen^{2,5})-enkephalin] and [Leu^5]-enkephalin (Vaught and Takemori, 1979; Vanderah et al., 1996), and that μ-opioid receptor agonists potentiate the antinociception produced by the κ-opioid receptor agonist, U50,488H (*trans*-(±)-3, 4 Dichloro-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide methane sulfonate) (Sutters et al., 1990), the ATP-gated K^+ channels openers may release an endogenous opioid, which in turn enhances the antinociceptive effects of the opioid receptor agonists.

Although previous data suggest that the ATP-gated K^+ channels openers release endogenous opioids, no systematic studies have been aimed at identifying which of the endogenous opioids in the brain are being released. Therefore, the goal of this study was to clarify which endogenous opioids mediate the ATP-gated K^+ channel openers' antinociceptive effects by the use of opioid-receptor-selective antagonists.

2. Materials and methods

2.1. The i.c.v. injections

The i.c.v. injections were performed according to the method of Pedigo et al. (1975). Ether-anesthetized male ICR mice, 21–24 g, were injected 2 mm caudal and 2 mm lateral at a 45° angle from the bregma. This area was exposed by a small incision made between the ears of the animal. Injections of 5 μl were made at a depth of 2 mm into the third lateral ventricle. Hamilton 50 μl syringes and 26-gauge 3/8 in. needles were used. A tubing cover was applied to the needle to ensure that injections were made at the proper depth.

K^+ channel openers, diazoxide and levcromakalim, and the antagonist, glyburide, were prepared in 100% DMSO (dimethyl sulfoxide). Opioid-receptor agonists (morphine, U50,488H and DPDPE) and antagonists [CTOP (D-Phe–Cys–Tyr–D-Trp–Orn–Thr–Pen–Thr amide), ICI 174,864 (*N*,*N*-diallyl–Tyr–Aib–Aib–Phe–Leu) and nor-Binaltorphimine] were prepared in distilled water. ICI 174,864 was sonicated briefly to allow dissolution.

Time-course studies and previous findings from our laboratory were used to ascertain peak antinociception as tested by the tail-flick test. Peak time for the K^+ channel openers was 10 min after injection. Peak blockade of the agonists was found to be 5, 10, 15 and 60 min after CTOP (0.1 μg/mouse), ICI 174,864 (10 μg/mouse), glyburide (10 μg/mouse) and nor-Binaltorphimine (70 μg/mouse) administration, respectively. Nor-binaltorphimine pretreat-

ment was consistent with the i.c.v. block of the κ-opioid receptor agonist, U50,488H (Takemori et al., 1988).

Combinations of inactive doses of K^+ channel openers and opioid receptor agonists were utilized to test for greater-than-additive effects. For these studies, opioids were administered i.c.v. 10 min prior to the K^+ channel openers. The animals were tested 10 min later using the tail-flick test. Appropriate vehicle controls were run for each experiment.

2.2. The tail-flick test

The D'Amour and Smith (1941) tail-flick test was used to assess antinociception. Reaction times of 2–4 s were employed for the control, while a time of 10 s was used as the cutoff. Quantification of effect was done using the percent of maximum possible effect, % MPE, formula:

$$\%\,\mathrm{MPE} = 100 \times \left[\frac{\text{test control}}{10\,\text{control}} \right]$$

(Harris and Pierson, 1964). Using 5–12 mice per dose, a % MPE was calculated for each animal. The mean effect and standard error of the mean (S.E.M.) were calculated for every dose using the % MPE for each mouse. At least four doses of each test drug or combination of drugs were used to generate dose–response curves. Least squares linear regression analysis was performed and ED_{50} values were determined by log-probit analysis. The 95% confidence limits (CLs) were calculated using the methods of Bliss (1967).

2.3. Statistical analysis

Two-tailed unpaired *t*-tests were used to determine significant differences between control and treatment animal groups. *P* values of less than 0.05 were deemed significant. Parallelism of the dose–response curves was determined by the methods of Tallarida and Murray (1987). Potency ratios were determined using the methods of Colquhoun (1971).

2.4. Drugs

U50,488H, CTOP, ICI 174,864 and nor-Binaltorphimine were purchased from Research Biomedicals International (Natick, MA). DPDPE was purchased from Bachem Bioscience (King of Prussia, PA). Glyburide, diazoxide and DMSO were purchased from Sigma (St. Louis, MO). Levcromakalim was kindly provided by Dr. P.G. Treagust at SmithKline Beecham Pharmaceuticals (Worthing, West Sussex, UK). Morphine was obtained from the National Institute of Drug Abuse (NIDA).

3. Results

3.1. The i.c.v. administration of K^+ channel openers produced antinociception as determined by the tail-flick test

Diazoxide produced dose-dependent antinociception with an ED_{50} of 44 μg/mouse (95% CLs from 28 to 68). Unlike diazoxide, levcromakalim was a partial agonist displaying no greater than 47 (±14)% MPE when administered alone (Fig. 1). In the presence of a pretreatment with DMSO vehicle, levcromakalim produced a maximal antinociceptive effect of 76 (±17) % MPE.

3.2. The antinociceptive effects of the K^+ channel openers were blocked by glyburide

The following equally efficacious doses of ATP-gated K^+ channel openers were used for antagonism studies: 200 μg/mouse of diazoxide and 300 μg/mouse of levcromakalim. Glyburide (10 μg/mouse), an ATP-gated K^+ channel antagonist, significantly blocked the effects of levcromakalim-induced antinociception, shifting the % MPE from 76 (±17)% to 12 (±5)%. Similarly, glyburide blocked diazoxide-induced antinociception, from 81 (±12)% to 23 (±11)% MPE, an effect which was also significant ($P < 0.01$). Such studies indicate an interaction of diazoxide and levcromakalim at ATP-gated K^+ channels. The results of all of the antagonism studies performed are summarized in Table 1.

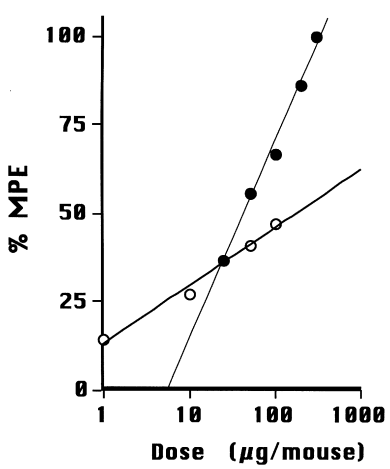

Fig. 1. Dose–response curves of diazoxide and levcromakalim after i.c.v. administration. The i.c.v. administration of both diazoxide (closed circles) and levcromakalim (open circles) produced dose-dependent antinociception with ED_{50}s of 44 (28–68) μg/mouse for diazoxide. Levcromakalim, a partial agonist, did not produce high-enough effects to calculate the ED_{50}. Higher doses of levcromakalim were not utilized due to limited quantities. Antinociceptive effects were measured via the tail-flick test and % MPE was calculated as described in Section 2.

Table 1
Summary of the effects of antagonists on ATP-gated K^+ channel openers and μ-, δ- and κ-opioids
Agonists [diazoxide (DZ), levcromakalim (Lem), morphine (M), DPDPE (D) and U50,488H (U)] were tested for antinociceptive effects after administration of ATP-gated K^+ channel antagonist [glyburide (Glyb)] or opioid-receptor-specific antagonists (CTOP, ICI 174,864 or nor-Binaltorphimine). (+) indicates significant blockade. (−) denotes lack of significant blockade. Significance was determined as discussed in Section 2.

Antagonists	Agonists				
	DZ	Lem	M	D	U50
Of K_{ATP}					
Glyb (10 μg/mouse)	+	+	+	−	−
Of opioid receptors					
CTOP (0.1 μg/mouse)	+	+	+	−	−
ICI 174,864 (10 μg/mouse)	+	+	−	+	−
Nor-Binaltorphimine (70 μg/mouse)	+	−	−	−	+

3.3. The i.c.v.-administered opioid receptor antagonists blocked the antinociceptive effects of the K^+ channel openers (i.c.v.)

CTOP (0.1 μg/mouse, a μ-opioid receptor antagonist), partially, yet significantly, blocked the effects of diazoxide ($P < 0.05$) (Fig. 2, Panel A). A shift in % MPE from 88 (±12)% to 39 (±14)% was produced with CTOP administration. In addition, pretreatment with ICI 174,864 (10 μg/mouse), a δ-opioid-receptor-specific antagonist, significantly and totally attenuated the antinociceptive effects of diazoxide. The % MPE was shifted from 74 (±16)% MPE with vehicle to 21 (±16)% MPE with ICI 174,864. The antinociceptive effects of diazoxide were blocked partially and significantly by the κ-opioid receptor antagonist, nor-Binaltorphimine (70 μg/mouse). Nor-Binaltorphimine pretreatment shifted the % MPE from 81 (±11)% MPE to 41 (±12)% MPE. All doses of antagonists were chosen based upon the selectivity for one specific opioid receptor as illustrated in Table 1.

Levcromakalim-induced (300 μg/mouse) antinociception was significantly attenuated by CTOP (0.1 μg/mouse) and ICI 174,864 (10 μg/mouse) administration. CTOP shifted the % MPE from 78 (±12)% to 15 (±9)%, while ICI 174,864 shifted the % MPE from 86 (±14)% to 14 (±)%. A similar dose of nor-Binaltorphimine had no effect on levcromakalim-induced antinociception (Fig. 2, Panel B). Thus, levcromakalim-induced antinociception did not appear to involve the release of a κ- opioid, whereas that of diazoxide appeared to involve μ-, κ- and δ-opioid release.

3.4. K^+ channel openers enhanced opioid-induced antinociception

If ATP-gated K^+ channel openers release endogenous opioids, one might expect an enhancement of opioid-induced antinociception by the openers. Diazoxide, at an antinociceptively inactive dose of 2 μg/mouse, was administered following i.c.v. injection of morphine and produced a significant leftward shift in the morphine dose–response curve. The ED_{50} value shifted from 0.05 (0.03–0.11) to 0.0017 (0.001–0.003) μg/mouse, yielding a 47-fold shift of the dose–response curve of morphine (Fig. 3). Similarly, an inactive dose of levcromakalim (0.1 μg/mouse) produced a significant 27-fold leftward shift of morphine's dose–response curve and resulted in an ED_{50} of 0.0021 (0.001–0.004) μg/mouse (Fig. 3).

Diazoxide shifted the dose–response curve of DPDPE leftward in a non-parallel manner, making potency comparisons invalid (Fig. 4). Levcromakalim significantly lowered the ED_{50} of DPDPE from 0.98 (0.5–2.0) to 0.15 (0.08–0.27) μg/mouse and produced a 6 (2–16)-fold shift in potency (Fig. 4).

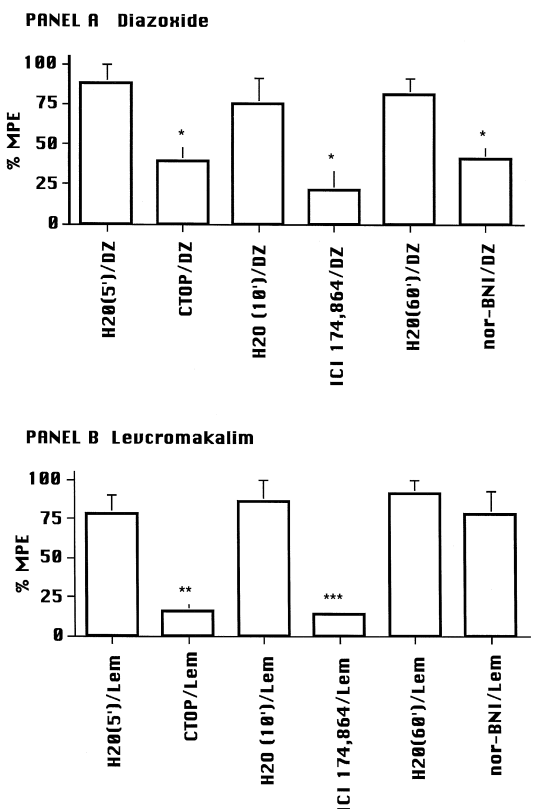

Fig. 2. Attenuation of ATP-gated K^+ channel openers by opioid receptor antagonists. Panel A: Diazoxide-induced antinociception was significantly attenuated by administration of CTOP (a μ-opioid-receptor-specific antagonist), ICI 174,864 (a δ-opioid-receptor-specific antagonist) and by nor-Binaltorphimine (a κ-opioid-specific antagonist). Asterisk (*) denotes $P < 0.05$ from vehicle followed by K^+ channel opener administration. Panel B: Levcromakalim-induced antinociception was significantly attenuated by administration of CTOP (a μ-opioid-receptor-specific antagonist) and ICI 174,864 (δ) but not by nor-Binaltorphimine (κ). % MPE and significant blockade were calculated as described in Section 2. $^{**}P < 0.01$ from vehicle/K^+ channel opener and $^{***}P < 0.001$.

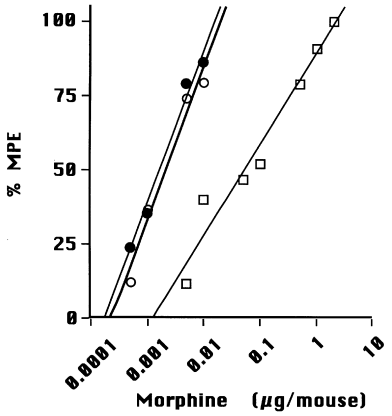

Fig. 3. The i.c.v. administration of the ATP-gated K^+ channel openers enhanced morphine-induced antinociception. Diazoxide (closed circles), at an inactive dose of 2 μg/mouse, shifted the dose–response curve of morphine (with vehicle, open squares) to the left. Levcromakalim (open circles), at an inactive dose of 0.1 μg/mouse, shifted the dose–response curve of morphine (with vehicle) to the left. ED_{50} doses with 95% CL and parallelism of the dose–response curves were determined as described in Section 2.

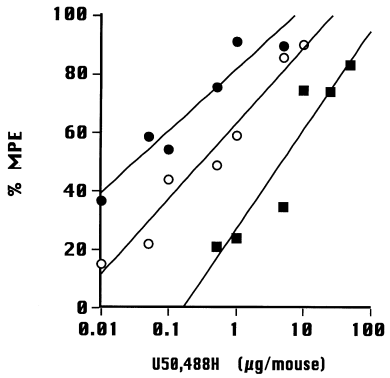

Fig. 5. The i.c.v. administration of the ATP-gated K^+ channel openers enhanced U50,488H-induced antinociception. Diazoxide (dark circles), at an inactive dose of 2 μg/mouse, shifted the dose–response curve of U50,488H (with vehicle, dark squares) to the left. Levcromakalim (open circles), at an inactive dose of 0.1 μg/mouse, increased the potency of U50,488H (with vehicle). ED_{50} doses with 95% CL, parallelism of the dose–response curves and potency ratios were determined as described in Section 2.

Diazoxide also enhanced the antinociceptive effects of U50,488H, significantly shifting its dose–response curve to the left, from an ED_{50} of 4.9 (1.4–10) μg/mouse to an ED_{50} value of 0.03 (0.02–0.05) μg/mouse (Fig. 5). The potency ratio calculated for the diazoxide/U50,488H interaction was 106 (29–555). A significant leftward shift in the dose–response curve for U50,488H was generated with levcromakalim administration. An ED_{50} of 0.30 (0.1–0.7) μg/mouse was determined, producing a 15-fold (4–59) shift of the U50,488H dose–response curve (Fig. 5).

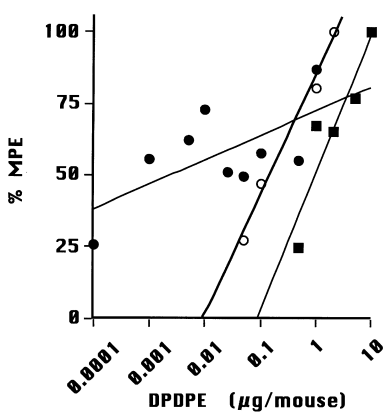

Fig. 4. The i.c.v. administration of the ATP-gated K^+ channel openers enhanced DPDPE-induced antinociception. Diazoxide (dark circles), at an inactive dose of 2 μg/mouse, shifted the dose–response curve of DPDPE (with vehicle, dark squares) in a non-parallel manner. Levcromakalim (open circles), at an inactive dose of 0.1 μg/mouse, shifted the dose–response curve of DPDPE (with vehicle) to the left. ED_{50} doses with 95% CL and parallelism of the dose–response curves were determined as described in Section 2.

3.5. Since combinations of the K^+ channel openers with opioid receptor agonists resulted in greater-than-additive effects, opioid receptor antagonists were employed to further characterize such interactions

Drug combinations (opioid + K^+ channel opener) that resulted in a combined effect of approximately 80% MPE were tested in the presence of the antagonists. Glyburide (10 μg/mouse) totally attenuated the enhancement of morphine (0.01 μg/mouse) by diazoxide and levcromakalim but had no effect on any of the other combinations tested (Table 2). Glyburide s1hifted the morphine/diazoxide enhancement from 83 (±17) % MPE to 10 (±7)% ($P < 0.01$), while the morphine/levcromakalim interaction shifted from 70 (±11)% to 25 (±15)% MPE ($P < 0.05$). Opioid receptor antagonists, CTOP and ICI 174,864, which blocked the antinociceptive effects of both diazoxide and levcromakalim, significantly attenuated the antinociceptive effects generated by all the drug combinations tested (Figs. 6 and 7). Of significance is the fact that nor-Binaltorphimine antagonized the effects of only the diazoxide-induced enhancements. Nor-Binaltorphimine, which failed to block the effects of levcromakalim, also failed to block any interaction between the opioids and levcromakalim. The morphine/diazoxide combination, which produced 98 (±1)% MPE, was attenuated to 12 (±5)% MPE by the administration of nor-Binaltorphimine ($P < 0.001$) (Fig. 6, Panel A). In addition, the DPDPE (1 μg/mouse)/diazoxide combination, which produced 82 (±12)% MPE, was attenuated to 37 (±10)% MPE with nor-Binaltorphimine administration ($P < 0.05$) (Fig. 6, Panel B). Nor-Binaltorphimine significantly attenuated the

Table 2
Summary of effects of antagonists on the greater-than-additive effects of ATP-gated K^+ channel blockers and opioids
Combinations of ATP-gated K^+ channel openers [diazoxide (DZ), levcromakalim (Lem) and opioids (morphine (M), DPDPE (D) and U50,488H (U))] were tested for antinociceptive effects after administration of the ATP-gated K^+ channel antagonist, glyburide (Glyb) or opioid-receptor-selective antagonists (CTOP, ICI 174,864 or nor-Binaltorphimine). (+) indicates significant blockade. (−) denotes lack of significant blockade. Significance was determined as described in Section 2.

Antagonists	Combinations					
	M/DZ	M/Lem	D/DZ	D/Lem	U/DZ	U/Lem
Of K_{ATP}						
Glyb (10 μg/mouse)	+	+	−	−	−	−
Of opioid receptors						
CTOP (0.1 μg/mouse)	+	+	+	+	+	+
ICI 174,864 (10 μg/mouse)	+	+	+	+	+	+
Nor-Binaltorphimine (70 μg/mouse)	+	−	+	−	+	−

U50,488H (1 μg/mouse)/diazoxide enhancement decreasing the % MPE from 88 (±13)% to 22 (±16)% ($P < 0.01$) (Fig. 6, Panel C). Table 2 summarizes the above results.

4. Discussion

The ATP-gated K^+ channel openers, diazoxide and levcromakalim, produce dose-dependent antinociception which is attenuated by opioid receptor antagonists. Based upon earlier observations, this result was to be expected (Narita et al., 1993; Welch and Dunlow, 1993; Ocana et al., 1995). Previous studies in our laboratory demonstrated that opioid-receptor-selective antagonists significantly attenuate the antinociception produced by ATP-gated K^+ channel openers administered i.t. (Welch and Dunlow, 1993). Similarly in this study, i.c.v.-administered opioid-receptor-selective antagonists attenuated the effects of diazoxide- and levcromakalim-induced antinociception. CTOP, a μ-opioid-receptor-specific antagonist, attenuated the effects of both diazoxide and levcromakalim, implicating the involvement of an endogenous μ-opioid in their antinociceptive effects. The δ-opioid-receptor-specific antagonist, ICI 174,864, attenuated both diazoxide- and levcromakalim-induced antinociception, which suggests that the enkephalins are involved in producing the antinociceptive effects of the ATP-gated K^+ channel openers. Unlike diazoxide, levcromakalim-induced antinociception was unaffected by the κ-opioid-receptor-specific antagonist, nor-Binaltorphimine. These results suggest that diazoxide-induced antinociception involves all three opioid receptors while levcromakalim-induced antinociception involves only the μ- and δ-opioid receptors. Such findings, following administration of the drugs i.c.v., are comparable to our previous findings in the spinal cord (Welch and Dunlow, 1993) and are suggestive of differential effects of the openers at either the sulfonylurea site to which they bind, differences in localization of binding sites, or non-specific, non-sulfonylurea-receptor-mediated effects particularly of diazoxide. Such differences may explain why diazoxide is a full agonist, while levcromakalim is a partial agonist.

If the ATP-gated K^+ channel openers are releasing an endogenous opioid, they may be expected to enhance the effects of morphine or endogenous opioids based upon previous studies of opioid interactions with other opioids and with the K^+ channel openers (Vaught and Takemori, 1979; Sutters et al., 1990; Vergoni et al., 1992; Narita et al., 1993; Robles et al., 1994; Ocana et al., 1995; Vanderah et al., 1996). It has been shown that δ-opioid receptor agonists potentiate morphine-induced antinociception. The ATP-gated K^+ channel openers tested were antagonized by the δ-opioid-receptor-specific antagonist; therefore, it seemed logical that the combination of the ATP-gated K^+ channel openers with morphine would produce greater-than-additive effects. Indeed, this was the case. Both diazoxide and levcromakalim, at inactive doses, significantly shifted the dose–response curve of morphine to the left; however, neither shift was parallel, indicating that some non-opioid components might also be involved in these interactions. Earlier works which employed cromakalim, the racemate of levcromakalim, indicate enhancement of morphine-induced antinociception, but not to as great an extent as that observed in our study (Narita et al., 1993; Robles et al., 1994; Ocana et al., 1995). Perhaps this can be attributed to the racemic cromakalim which may not be as potent an ATP-gated K^+ channel opener as its active L-isomer, levcromakalim. In addition to the ATP-gated K^+ channel openers utilized, there are other differences between our study and those studies previously performed. In previous studies, the majority of the work compared cromakalim- and vehicle-treated groups using a single dose study, whereas we generated and compared complete dose–response curves (Ocana et al., 1995). From this, we were able to determine the significance and parallelism in the shifts in values of ED_{50}.

Previous studies have demonstrated that μ-opioid receptor agonists potentiate U50,488H-induced antinociception (Sutters et al., 1990). The attenuation of diazoxide and levcromakalim by CTOP suggests that both drugs may

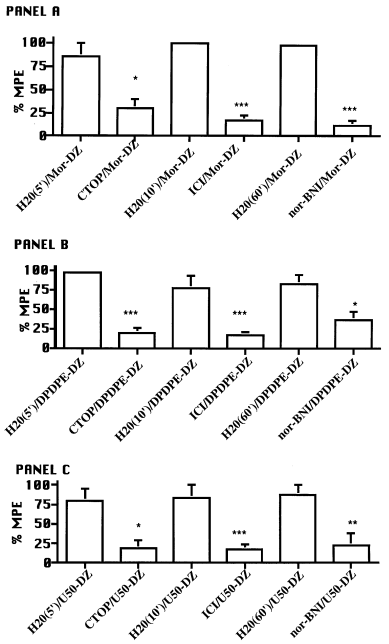

Fig. 6. Attenuation of diazoxide and opioid combinations by opioid receptor antagonists. Panel A: The diazoxide (at an inactive dose of 2 μg/mouse) enhancement of morphine (0.01 μg) was significantly attenuated by administration of CTOP (0.1 μg/mouse, a μ-opioid-receptor-specific antagonist), ICI 174,864 (10 μg/mouse) a δ-opioid-receptor-specific antagonist) and by nor-Binaltorphimine (a κ-opioid-receptor-specific antagonist). Panel B: The diazoxide enhancement of DPDPE (1 μg) was significantly attenuated by administration of CTOP (0.1 μg/mouse, a μ-opioid-receptor-specific antagonist), ICI 174,864 (10 μg/mouse, δ) and by nor-Binaltorphimine (70 μg/mouse, κ). Panel C. The diazoxide enhancement of U50,488H (1 μg) was significantly attenuated by administration of CTOP (0.1 μg/mouse, a μ-opioid-receptor-specific antagonist), ICI 174,864 (10 μg/mouse, δ) and by nor-Binaltorphimine (70 μg/mouse, κ). % MPE and significant blockade were calculated as described in Section 2. $^{*}P < 0.05$ from distilled water/opioid/diazoxide; $^{**}P < 0.01$ from distilled water/opioid/diazoxide; $^{***}P < 0.001$ from distilled water/opioid/diazoxide.

release μ-opioids. As such, the enhancement of U50,488H was expected. Both ATP-gated K^+ channel openers produced significant and parallel leftward shifts of the dose–response curve of U50,488H, implicating the release of μ-opioids. Our study differs from that of Ocana et al. (1995), who found no enhancement of U50,488H by cromakalim. Such a difference may lie in the use of the racemic form, cromakalim, vs. that of levcromakalim or other species and methodological differences. There is no reason to assume that the lack of release of κ-opioids by levcromakalim would preclude an enhancement of morphine by levcromakalim if it were releasing μ-opioids. The data suggest that levcromakalim does release μ-opioids or interact with μ-opioid systems.

The δ-opioid-receptor-specific agonist, DPDPE, was also tested in conjunction with diazoxide and levcromakalim. Only levcromakalim, in combination with DPDPE, produced a significant and parallel leftward shift of the DPDPE dose–response curve. Diazoxide administration altered the antinociceptive effects of DPDPE in a non-parallel manner. Diazoxide-induced antinociception has been shown to involve not only the ATP-gated K^+ channels, but also both the small and large conductance Ca^{2+}-gated K^+ channels (Welch and Dunlow, 1993). It is possible that these non-opioid mechanisms of action of diazoxide provide an explanation for the finding of non-parallelism. Such an effect of diazoxide is also consistent with the differences between levcromakalim and diazoxide observed in terms of opioid receptor antagonists. In all of our studies, diazoxide has always proven to have multiple mechanisms of action distinct from the ATP-gated K^+ channels (Welch and Dunlow, 1993). We have only evaluated the interactions with K^+ channels and opioids, but clearly, diazoxide is not exclusively an ATP-gated K^+ channel opener.

In an effort to further evaluate the enhancement of the opioids by ATP-gated K^+ channel openers occurring via the release of endogenous opioids, the opioid receptor antagonists were tested vs. the combinations of opioids and openers. CTOP significantly attenuated the effects of all the combinations of opioid agonists with diazoxide or

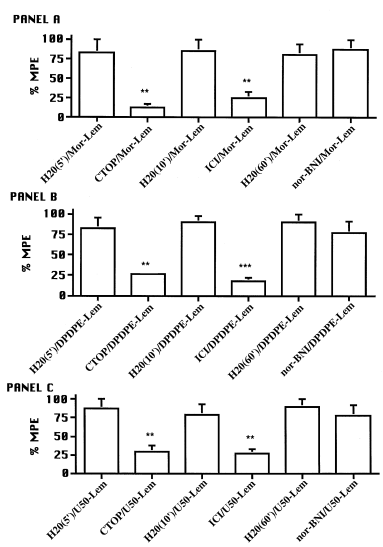

Fig. 7. Attenuation of levcromakalim and opioid combinations by opioid receptor antagonists. Panel A: The levcromakalim (at an inactive dose of 0.1 μg/mouse) enhancement of morphine (0.01 μg) was significantly attenuated by administration of CTOP (0.1 μg/mouse, a μ-opioid-receptor-specific antagonist) and ICI 174,864 (10 μg/mouse, δ), but not by nor-Binaltorphimine (70 μg/mouse, κ). Panel B: The levcromakalim enhancement of DPDPE (1 μg) was significantly attenuated by administration of CTOP (0.1 μg/mouse, a μ-opioid-receptor-specific antagonist) and ICI 174,864 (10 μg/mouse, δ), but not by nor-Binaltorphimine (70 μg/mouse, κ). Panel C: The levcromakalim enhancement of U50,488H (1 μg) was significantly attenuated by administration of CTOP (0.1 μg/mouse, a μ-opioid-receptor-specific antagonist) and ICI 174,864 (10 μg/mouse, δ), but not by nor-Binaltorphimine (70 μg/mouse, κ). % MPE and significant blockade were calculated as described in Section 2. $^{*}P < 0.05$ from distilled water/opioid/levcromakalim; $^{**}P < 0.01$ from distilled water/opioid/levcromakalim; $^{***}P < 0.001$ from distilled water/opioid/levcromakalim.

levcromakalim. These data are a significant indication of the involvement of μ-opioids in the production of the greater-than-additive antinociceptive effects seen with the drug combinations. Alternatively, CTOP blockade of the combination could be attributed to its block of morphine- and the K^+-channel-openers-induced antinociceptive effects. To further determine which endogenous opioids were involved, δ- and κ-specific antagonists were tested vs. the combinations. ICI 174,864 significantly antagonized all μ-, δ- and κ-opioid combinations with the openers even though it failed to block morphine or U50,488H, suggesting that the opioids are enhanced by diazoxide and levcromakalim via the release of enkephalins or β-endorphin acting at the δ-opioid receptor. Nor-Binaltorphimine, which was κ-opioid-selective at the dose used, once again differentiated between diazoxide and levcromakalim by only attenuating the diazoxide-induced enhancements of opioids. Therefore, we hypothesize from the antagonism studies that diazoxide-induced enhancements occur through the release of β-endorphin, enkephalins and/or dynorphins, while levcromakalim enhances the opioids via the release of β-endorphin and/or enkephalins, but not the κ-opioid agonists such as the dynorphins.

In summary, the data presented here demonstrate that the ATP-gated K^+ channel openers, diazoxide and levcromakalim, produce antinociceptive effects that are blocked by opioid receptor antagonists, albeit in a differential manner. These same openers enhance opioid-induced antinociception again in a differential manner. Our results support the hypothesis that the ATP-gated K^+ channel openers may mediate their antinociceptive effects via the release of endogenous opioids and that their distinct release is reflected in differences in enhancement of the opioids. Thus, the critical component in K^+ channel opener enhancement of opioids is the opioid that the opener releases.

It is known that the ATP-gated K^+ channel openers cause an increase in K^+ efflux from the cell. This efflux hyperpolarizes the cell and decreases Ca^{2+} entry into the cell. The decrease in intracellular Ca^{2+} levels lessens the amount of neurotransmitters released by the cell. We hypothesize that the ATP-gated K^+ channel openers may mediate a disinhibition of inhibitory processes tonically involved with the release of endogenous opioids in either a descending or ascending pathway in the central nervous system. It is possible that this disinhibition allows for the release of endogenous opioids and contributes, at least in part, to ATP-gated K^+ channel opener-induced antinociception.

There is a great amount of clinical relevance to this study. If a combination of lower, inactive doses of the opioids and ATP-gated K^+ channel openers can be used to achieve similar analgesic results to that of an opioid alone, the amount of opioid necessary for analgesia will be decreased. This is beneficial because less opioid in the system can potentially lessen the negative side effects associated with opioid use: nausea, vomiting, constipation, respiratory depression, tolerance and dependence. In order for such drug interaction studies to proceed, it is critical to determine the mechanisms of interaction of the drugs. This study represents indirect evidence indicating an interaction of the drug classes via central release of endogenous opioid peptides. Direct quantitation of such release is yet to be performed.

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