The peripheral antinociceptive effect induced by morphine is associated with ATP-sensitive K⁺ channels

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1 The effect of several K⁺ channel blockers such as glibenclamide, tolbutamide, charybdotoxin (ChTX), apamin, tetraethylammonium (TEA), 4-aminopyridine (4-AP) and cesium on the peripheral antinociceptive effect of morphine was evaluated by the paw pressure test in Wistar rats.

2 The intraplantar administration of a carrageenan suspension (250 µg) resulted in an acute inflammatory response and a decreased threshold to noxious pressure. Morphine administered locally into the paw (25, 50, 100 and 200 µg) elicited a dose-dependent antinociceptive effect which was demonstrated to be mediated by a peripheral site up to the 100 µg dose.

3 The selective blockers of ATP-sensitive K⁺ channels glibenclamide (20, 40 and 80 µg paw⁻¹) and tolbutamide (40, 80 and 160 µg paw⁻¹) antagonized the peripheral antinociception induced by morphine (100 µg paw⁻¹).

4 This effect was unaffected by ChTX (0.5, 1.0 and 2.0 µg paw⁻¹), a large conductance Ca²⁺-activated K⁺ channel blocker, or by apamin (2.5, 5.0 and 10.0 µg paw⁻¹), a selective blocker of a small conductance Ca²⁺-activated K⁺ channel.

5 Intraplantar administration of the non-specific K⁺ channel blockers TEA (160, 320 and 640 µg), 4-AP (10, 50 and 100 µg) and cesium (125, 250 and 500 µg) also did not modify the peripheral antinociceptive effect of morphine.

6 These results suggest that the peripheral antinociceptive effect of morphine may result from activation of ATP-sensitive K⁺ channels, which may cause a hyperpolarization of peripheral terminals of primary afferents, leading to a decrease in action potential generation. In contrast, large conductance Ca²⁺-activated K⁺ channels, small conductance Ca²⁺-activated K⁺ channels as well as voltage-dependent K⁺ channels appear not to be involved in this transduction pathway.

British Journal of Pharmacology (2000) **129**, 110–114

Keywords: Analgesia; morphine; K⁺ channel blockers; μ -opioid receptor agonists; glibenclamide; tolbutamide; apamin; charybdotoxin

Abbreviations: 4-AP, 4-aminopyridine; ATP, adenosine 5'-triphosphate; ChTX, Charybdotoxin; K_{ATP}, ATP-sensitive K⁺ channel; L paw, left paw; R paw, right paw; TEA, Tetraethylammonium

Introduction

Experiments at the cellular level have shown that agonists at the μ opioid receptor increase potassium conductance (North, 1989). In the central nervous system, the opening of K⁺ channels seems to play a role in opioid-mediated antinociception, since the ATP-sensitive K⁺ channel blockers (sulphonylureas) antagonize the antinociceptive effect of opioids (Ocaña *et al.*, 1990; 1995; Wild *et al.*, 1991; Roane & Boyd, 1993). The antinociceptive effect of opioid agonists was also demonstrated to be enhanced by ATP-sensitive K⁺ channel openers such as pinacidil (Vergoni *et al.*, 1992) and cromakalim (Ocaña *et al.*, 1996). Evidence that some opioids induce opening of calcium-activated K⁺ channels has also been obtained (Stretton *et al.*, 1992). In addition, the diversity of K⁺ channels (Halliwell, 1990) with different electrophysiological and pharmacological characteristics described in neurones, suggests that other types of K⁺ channels may be involved in this effect.

Opioids can produce analgesia by inhibiting nociceptive input at supraspinal and spinal sites (Herz & Teschemacher, 1971; Yaksh & Rudy, 1976). Several studies have also

indicated that exogenous as well as endogenous opioids can act on peripheral nociceptors to produce an antinociceptive effect against the hyperalgesia induced by local inflammation (Ferreira & Nakamura, 1979; Bentley *et al.*, 1981; Stein *et al.*, 1990). Stein *et al.* (1990) demonstrated the presence of opioid receptors on the peripheral terminals of primary afferents, however, the mechanism by which opioid agonists induce peripheral antinociception is unclear. It was previously shown that opioid receptors at peripheral sites are coupled with inhibitory G proteins since pertussis toxin inhibits the peripheral antinociception induced by morphine (Levine & Taiwo, 1989) and are also coupled with L-arginine-NO-cGMP pathway (Ferreira *et al.*, 1991; Duarte *et al.*, 1992).

The present study was undertaken to determine whether specific and non-specific K⁺ channel blockers have any effect on the peripheral antinociception induced by morphine. For this purpose we tested the effects of glibenclamide and tolbutamide, sulphonylureas that specifically block ATP-sensitive K⁺ channels (Edwards & Weston, 1993), apamin, a selective blocker of small conductance Ca²⁺-activated K⁺ channels (Romey *et al.*, 1984), charybdotoxin (ChTX), a blocker of large conductance Ca²⁺-activated K⁺ channels (Miller *et al.*, 1985), and non-selective K⁺ channel blockers, 4-aminopyridine (4-AP), tetraethylammonium (TEA) and cesium (Cook & Quast, 1990).

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Methods

Animals

The experiments were performed on 180–250 g male Wistar rats (from CEBIO-UFGM). The animals were housed in a temperature-controlled room ($23 \pm 1^\circ\text{C}$) on an automatic 12 h light/dark cycle (06.00–18.00 h). All testing was conducted during the light phase (12.00–17.00 h). Food and water were freely available until the beginning of the experiments. Naïve animals were used throughout.

Measurement of hyperalgesia

Hyperalgesia was induced in the hindpaw by intraplantar administration of carrageenan suspension (250 μg) and measured according to the paw pressure test described by Randall & Selitto (1957). An analgesy-meter (Ugo-Basile, Italy) with a cone-shaped paw-presser with a rounded tip, which applies a linearly increasing force to the plantar surface of the paw, was used. The weight in gram (g) required eliciting nociceptive responses such as paw flexion or struggling was defined as the nociceptive threshold. A cut-off value of 300 g was used to prevent damage to the paws. The nociceptive threshold was always (except when indicated) measured in the right hindpaw and determined by the average of three consecutive trials recorded before and 3 h after carrageenan injection. The results were calculated by the difference between these two averages (Δ of nociceptive threshold) and expressed as grams.

Experimental protocol

Morphine was administered subcutaneously into the right hindpaw 2 h after local injection of the carrageenan suspension. In the protocol used to determine whether morphine was acting at central sites carrageenan was injected into both hindpaws while morphine was administered 2 h later into the left or right paw. All the K⁺ channel blockers were injected subcutaneously into the right hindpaw. The sulphonylureas (glibenclamide and tolbutamide) were administered 5 min before morphine while all the other K⁺ channel blockers were injected 45 min after morphine (Wild *et al.*, 1991; Ocaña & Baeyens, 1993; Yonehara & Takiuchi, 1997).

Chemicals

The drug used as hyperalgesic agent was lambda carrageenan (Sigma) and the μ -opioid receptor agonist was morphine hydrochloride (Merck). The K⁺ channel blockers and their suppliers were: glibenclamide (Sigma), tolbutamide (ICN Biomedicals), charybdotoxin (Sigma), apamin (Sigma), tetraethylammonium chloride (Sigma), 4-aminopyridine (Sigma) and cesium (Mitsuwa's Pure Chemicals). Morphine and carrageenan were dissolved in isotonic saline and injected in a volume of 100 μl per paw. The K⁺ channel blockers were dissolved in demineralized water with exception of sulphonylureas that were dissolved in saline and tween (2%) vehicle, immediately before use and injected in a volume of 50 μl per paw. For acidic or alkaline solutions the pH was adjusted closer to 7.

Statistical analysis

The statistical analyses were carried out by one-way analysis of variance (ANOVA) followed by Bonferroni's test for multiple comparisons. Probabilities less than 5% ($P < 0.05$) were considered to be statistically significant.

Results

Antinociceptive effect of morphine

The administration of morphine (25, 50, 100, 200 μg) into the right hind paw produced an antinociceptive response against the hyperalgesia induced by prior local injection of carrageenan (Figure 1). Morphine at the dose of 100 μg , when administered into the left paw, did not produce an antinociceptive effect in the right paw, whereas morphine at the dose of 200 μg when injected into the left paw induced a potent antinociceptive effect in the contra-lateral paw (Figure 2).

Antagonism of morphine-induced antinociception by glibenclamide and tolbutamide

Glibenclamide (20, 40, 80 μg paw⁻¹) significantly reduced the morphine-induced antinociception (100 μg paw⁻¹) in a dose-dependent manner (Figure 3). As shown in Figure 4, the other sulphonylurea tested, tolbutamide (40, 80 and 160 μg paw⁻¹) also significantly inhibited the morphine-induced antinociceptive effect. None of the sulphonylureas tested significantly modified the nociceptive threshold in control animals (data not shown), or induced any overt behavioural effect at the doses used. Furthermore, the maximum dose of glibenclamide (80 μg), by the same route, did not alter significantly the plasma glucose level (results not shown).

Effect of apamin and ChTX on morphine-induced antinociception

Intraplantar injection of apamin (2.5, 5.0 and 10 μg) had no significant effect on morphine-induced antinociception, even at the highest dose tested. Charybdotoxin (0.5, 1.0 and 2.0 μg paw⁻¹) also failed to significantly counteract the antinociception induced by morphine (Figure 5).

Figure 1 Effect of morphine on the nociceptive threshold in carrageenan-induced hyperalgesia in rats. Morphine (μg) was administered intraplantarly 2 h after the local administration of 100 μl of a carrageenan suspension (250 μg). Each column represents the mean $\pm$ s.e. mean ($n = 10$). * $P < 0.01$ vs carrageenan + vehicle-injected control (Bonferroni's test).

Figure 2 Exclusion of a central antinociceptive effect of morphine. Morphine (μg) was administered into the right (R) or left (L) paw 2 h after carrageenan administration into both hind paws. Each column represents the mean $\pm$ s.e.mean ($n=10$). * $P<0.01$ vs carrageenan + vehicle-injected control (Bonferroni's test).

Figure 3 Antagonism induced by intraplantar administration of glibenclamide of the peripheral antinociception produced by morphine in hyperalgesic paws. Glibenclamide (μg) was administered 5 min before morphine (100 μg). Each column represents the mean $\pm$ s.e.mean ($n=5$). * and # $P<0.01$ as compared to (Carrageenan + vehicles) and (Carrageenan + morphine + vehicle)-injected controls, respectively (Bonferroni's test).

Effect of 4-AP, TEA and cesium on morphine-induced antinociception

As shown in Figure 6, 4-AP (10, 50 and 100 $\mu\text{g paw}^{-1}$), TEA (160, 320 and 640 $\mu\text{g paw}^{-1}$) and Cesium (125, 250 and 500 $\mu\text{g paw}^{-1}$) did not significantly modify the antinociception induced by morphine.

Figure 4 Antagonism induced by intraplantar administration of tolbutamide of the peripheral antinociception produced by morphine in hyperalgesic paws. Tolbutamide (μg) was administered 5 min before morphine (100 μg). Each column represents the mean $\pm$ s.e.mean ($n=5$). * and # $P<0.01$ as compared to (Carrageenan + vehicles) and (Carrageenan + morphine + vehicle)-injected controls, respectively (Bonferroni's test).

Figure 5 Effect of intraplantar administration of apamin and ChTX on the peripheral antinociception induced by morphine in hyperalgesic paws. Apamin and charybdotoxin (μg) were administered 45 min after morphine (100 μg). Each column represents the mean $\pm$ s.e.mean ($n=5$). No statistically significant differences were detected between the groups treated with morphine + vehicle and morphine + apamin or ChTX in any case. * $P<0.01$ vs carrageenan + vehicle-injected control (Bonferroni's test).

Discussion

The present study demonstrated that the sulphonylureas glibenclamide and tolbutamide could reverse the peripheral antinociceptive effect induced by intraplantar administration

Figure 6 Effect of intraplantar administration of 4-AP, TEA and Cesium on the peripheral antinociception induced by morphine in hyperalgesic paws. 4-AP, TEA and Cesium ($\mu\text{g paw}^{-1}$) were administered 45 min after morphine ($100 \mu\text{g paw}^{-1}$). Each column represents the mean $\pm$ s.e. mean ($n=5$). No statistically significant differences between the groups treated with carrageenan + morphine + vehicle and carrageenan + morphine + 4-AP, TEA or Cesium were found in any case. * $P < 0.01$ vs carrageenan + vehicle-injected control (Bonferroni's test).

of morphine in rats. Other K⁺ channel blockers such as apamin, ChTX, 4-AP, TEA, and Cesium did not exhibit any inhibitory effect. The doses of the ineffective blockers are compatible with those used to examine the involvement of potassium channels in the inhibitory prejunctional effect of morphine on peripheral sensory nerves *in vivo* (Yonehara & Takiuchi, 1997).

A growing number of both experimental and clinical studies demonstrated that locally administered opioids can produce pronounced analgesic effects by interacting with peripheral opioid receptors (Ferreira & Nakamura, 1979; Bentley, 1981; Smith, 1982; Stein *et al.*, 1990). According to Stein (1993), μ opioid agonists are more potent than δ or κ agonists in inducing peripheral antinociceptive effects. Thus, we used morphine because it has been described as an agonist of μ opioid receptors (Zimmerman *et al.*, 1987; Satoh & Minami, 1995). To exclude central effects of opioids many strategies can be used (Stein, 1993). In the present study, we used the strategy of evaluating the efficacy of ipsi- versus contralateral paw administration because the route and site of administration would be the same. Morphine at a dose of $100 \mu\text{g}$ was ineffective when administered into the contralateral paw, suggesting that at this dose morphine has a peripheral site of action in inflamed tissue. This effect seems to be specific and receptor mediated, since $50 \mu\text{g}$ of naloxone (when injected into the right paw, but not into the left), totally blocked the antinociceptive effect of morphine (result not shown).

Patch-clamp studies have shown that the sulphonylureas are selective inhibitors of ATP-sensitive K⁺ channels in pancreatic β -cells, cardiac myocytes and skeletal muscle cells (Edward & Weston, 1993). Indeed, the sensitivity to sulphonylureas, especially the potent glibenclamide, is commonly used to characterize the K_{ATP} channel (Babenko *et al.*, 1998). However, glibenclamide also blocks an ATP-independent K⁺

current in a human neuroblastoma cell line (Reeve *et al.*, 1992) and a delayed rectifier K⁺ current in neural and cardiac cells (Rosati *et al.*, 1998). Blockade of these currents might mimic the effects expected from K_{ATP} blockade, thus potentially confusing the interpretation of the results. Delayed rectifying K⁺ channels are blocked by TEA, 4-AP and cesium (Hille, 1992) and if morphine was acting through the activation of these channels both sulphonylureas and these other blockers would revert this effect.

Raffa & Codd (1994) demonstrated that glibenclamide cannot bind directly to μ , δ or κ opioid receptors because this drug cannot alter the binding of specific agonists of these receptors. The effect of sulphonylureas against morphine-induced antinociception should not be interpreted as a counteraction by a possible increased excitability induced by the blockers, since these drugs do not cause any hyperalgesic effect when alone. Our results agree with those obtained by Nichols & Lederer (1991) who described glibenclamide as more potent in blocking ATP-sensitive K⁺ channels than tolbutamide in pancreatic β -cells and in smooth and cardiac muscle. In the present study, the maximum dose of glibenclamide ($80 \mu\text{g}$), by the same route, did not alter significantly the plasma glucose level (results not shown). Furthermore, all sulphonylureas tested to date, when administered by the intracerebroventricular or intrathecal route, dose-dependently antagonized the antinociception induced by systemic administration of morphine (Ocaña *et al.*, 1990; 1995; Wild *et al.*, 1991), suggesting that opening of ATP-sensitive K⁺ channels in neurones of the central nervous system underlies the antinociceptive effect of morphine.

In the present study apamin, a protein extracted from bee venom and a selective blocker of small conductance Ca²⁺-activated K⁺ channels (Romey *et al.*, 1984), and ChTX, a toxin that blocks large conductance calcium-activated K⁺ channels (Miller *et al.*, 1985), failed to antagonize the peripheral antinociceptive effect induced by morphine. Stretton *et al.* (1992) demonstrated that activation of charybdotoxin-sensitive, but not apamin-sensitive K⁺ channels may be the mechanism for the prejunctional modulation of sensory nerves in guinea-pig airways by μ -opioid agonists and suggest that the same type of K⁺ channel activation could be involved in the modulation of pain sensation by opiates. Our results disagree with this hypothesis and exclude the involvement of both types of Ca²⁺-activated K⁺ channels in the peripheral antinociception induced by morphine. Also, according to Garcia *et al.* (1997), ChTX is not specific for the large conductance Ca²⁺-activated K⁺ channels, blocking a number of other K⁺ channels.

Our results show that 4-AP, TEA, and Cesium administered intraplantarly had no significant effect on the peripheral antinociception induced by morphine.

These drugs block different types of K⁺ channels, including calcium-activated and voltage-dependent K⁺ channels, although they are not specific for any of them in particular (Cook & Quast, 1990). Ocaña *et al.* (1995) showed that 4-AP and TEA have no effect on the central antinociception induced by μ -opioid receptor agonists. Finally, North & Williams (1985) demonstrated that the K⁺ channels activated by μ -opioid agonists are not sensitive to 4-AP or TEA.

In conclusion, we have found that two different sulphonylureas, glibenclamide and tolbutamide, antagonized the peripheral antinociceptive effect induced by morphine in rats, suggesting that ATP-sensitive K⁺ channels play an important role in this effect. It is important to consider that other potassium channels might be involved, such as G-protein coupled channels. Since other K⁺ channel blockers failed to

reverse this effect it may be inferred that other types of K⁺ channels such as large conductance Ca²⁺-activated, small conductance Ca²⁺-activated and voltage-dependent K⁺ channels appear not to be involved in the peripheral antinociceptive effect induced by morphine.

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(Received June 21, 1999)

Revised September 22, 1999

Accepted October 15, 1999