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Dibutyryl-cyclic GMP induces peripheral antinociception *via* activation of ATP-sensitive K^+ channels in the rat PGE_2-induced hyperalgesic paw

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1 Using the rat paw pressure test, in which increased sensitivity is induced by intraplantar injection of prostaglandin E_2, we studied the action of several K^+ channel blockers in order to determine what types of K^+ channels could be involved in the peripheral antinociception induced by dibutyrylguanosine 3 : 5′-cyclic monophosphate (DbcGMP), a membrane permeable analogue of cyclic GMP.

2 DbcGMP elicited a dose-dependent (50, 75, 100 and 200 μg paw^{-1}) peripheral antinociceptive effect. The effect of the 100 μg dose of DbcGMP was considered to be local since only a higher dose (300 μg paw^{-1}) produced antinociception in the contralateral paw.

3 The antinociceptive effect of DbcGMP (100 μg paw^{-1}) was dose-dependently antagonized by intraplantar administration of the sulphonylureas tolbutamide (20, 40 and 160 μg) and glibenclamide (40, 80 and 160 μg), selective blockers of ATP-sensitive K^+ channels.

4 Charybdotoxin (2 μg paw^{-1}), a selective blocker of high conductance Ca^{2+}-activated K^+ channels, and apamin (10 μg paw^{-1}), a selective blocker of low conductance Ca^{2+}-activated K^+ channels, did not modify the peripheral antinociception induced by DbcGMP.

5 Tetraethylammonium (2 mg paw^{-1}), 4-aminopyridine (200 μg paw^{-1}) and cesium (800 paw^{-1}), non-selective voltage-gated potassium channel blockers, also had no effect.

6 Based on this experimental evidence, we conclude that the activation of ATP-sensitive K^+ channels could be the mechanism by which DbcGMP induces peripheral antinociception, and that Ca^{2+}-activated K^+ channels and voltage-dependent K^+ channels appear not to be involved in the process.
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Abbreviations: 4-AP,4-aminopyridine; Ach, acetylcholine; ATP, adenosine 5′-triphosphate; ChTX, charybdotoxin; DbcGMP, N^2, 2′-O- dibutyrylguanosine 3:5′-cyclic monophosphate; K_{ATP}, ATP-sensitive K^+ channels; L paw, left paw; L-NMMA, L-N^G- monomethylarginine; NO, nitric oxide; NOS, nitric oxide synthase; PGE_2, prostaglandin E_2; R paw, right paw; TEA, tetraethylammonium

Introduction

In addition to the spinal and supraspinal antinociceptive sites of action of cholinergic drugs (Metys *et al*., 1969; Pedigo *et al*., 1975; Yaksh *et al*., 1985; Iwamoto & Marion, 1993; Brodie & Proudfit, 1984; Katayama *et al*., 1984), Ferreira & Nakamura (1979) described a peripheral analgesic effect of cholinergic agents. Because dibutyryl cyclic GMP (DbcGMP) mimicked ACh-induced analgesia, they suggested that cholinergic agents might cause analgesia by increasing cyclic GMP at the nociceptor level.

Duarte *et al*. (1990) demonstrated that intraplantar injection of ACh and sodium nitroprusside (SNP) induced antinociception in the rat paw rendered hyperalgesic with prostaglandin E_2 (PGE_2). The antinociceptive effect of ACh was blocked by L-N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of nitric oxide synthase (NOs). The antinociceptive action of both ACh and SNP was blocked by methylene blue (MB), an inhibitor of guanylate cyclase (GC), and was potentiated by MY5445, an inhibitor of cyclic GMP phosphodiesterase. In the studies by Duarte & Ferreira (1992), they described the involvement of the L-arginine/NO/cyclic GMP pathway in central morphine-induced antinociception.

Until now nothing is known about what happens after production of cyclic GMP at the nociceptor level that induces the state of antinociception. However, with respect to the participation of cyclic GMP in other biological effects such as vasodilatation, there is better evidence about the possible mechanism triggered by cyclic GMP. It is known that NO, by increasing cyclic GMP, can activate different types of potassium channels in different types of tissues (Thornbury *et al*., 1991; Kubo *et al*., 1994; Murphy & Brayden, 1995; Armstead, 1996; Zhuo *et al*., 1997; Carrier *et al*., 1997).

More recently, our group demonstrated the participation of ATP-sensitive K^+ channels in the peripheral antinociception induced by morphine (Rodrigues & Duarte, 2000) and the NO donor sodium nitroprusside (Soares *et al*., 2000), which led us to assume that nociceptor desensitization may occur through the activation of K^+ channels leading to the alteration of threshold neuronal sensitivity to pain.

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The aim of the present study was to verify the possible relation between increased intracellular levels of cyclic GMP induced by DbcGMP and activation of K^+ channels causing neuronal desensitization, and to determine what types of K^+ channels may be involved in this effect.

Methods

Animals

The experiments were performed on 180–250 g male Wistar rats from CEBIO-UFMG (Animal House of Universidade Federal de Minas Gerais). The animals were housed in a temperature-controlled room ($23 \pm 1^{\circ}C$) on an automatic 12-h light/dark cycle (0600 to 1800 h of light phase). All testing was concluded during the light phase (1200 to 1700 h). Food and water were freely available until the beginning of the experiments. Naïve animals were used throughout.

Measurement of hyperalgesia

Hyperalgesia was induced by a subcutaneous injection of prostaglandin E_2 (PGE_2, 2 μg) into the plantar surface of the rat's hindpaw and measured by the paw pressure test described by Randall & Selitto (1957). An analgesimeter (Ugo-Basile, Italy) with a cone-shaped paw-presser with a rounded tip was used to apply a linearly increasing force to the rat's right hindpaw. The weight in grams required to elicit nociceptive responses such as paw flexion or struggle was determined as the nociceptive threshold. A cut-off value of 300 g was used to prevent damage to the paws. The nociceptive threshold was measured in the right paw and is reported as the average of three consecutive trials recorded before (zero time) and 3 h after PGE_2 injection (peak of effect). The results were calculated by the difference between these two averages (Δ of nociceptive threshold) and expressed as grams.

Experimental protocol

DbcGMP was injected subcutaneously into the right hindpaw 2 h after the local injection of PGE_2. In the protocol used to determine whether DbcGMP acts at sites outside the injected paw, PGE_2 was injected into both hindpaws while DbcGMP was injected 2 h later into the left paw (Ferreira & Nakamura, 1979). The nociceptive threshold was always measured in the right hindpaw. All the K^+ channel blockers were injected subcutaneously into the right hindpaw. The sulphonylureas (glibenclamide and tolbutamide) were administered 5 min before DbcGMP while all the other K^+ channel blockers were injected 45 min after DbcGMP (Wild *et al*., 1991; Ocaña & Baeyens, 1993; Yonehara & Takiuchi, 1997).

Chemicals

Prostaglandin E_2 (Sigma, U.S.A.) was used as the hyperalgesic agent and DbcGMP (Sigma, U.S.A.) as the antinociceptive drug. The K^+ channel blockers were glibenclamide (Sigma, U.S.A.), tolbutamide (ICN Biomedicals, U.S.A.), charybdotoxin (ChTx, Sigma, U.S.A.), apamin (Apa, Sigma, U.S.A.), tetraethylammonium chloride (TEA, Sigma, U.S.A.), 4-aminopyridine (4-AP, Sigma, U.S.A.), and cesium (Cs, Mitsuwa's Pure Chemicals, Japan). All drugs were dissolved in isotonic saline, except the sulphonylureas, which were dissolved in Tween 80 (2% in saline), and injected in a volume of 100 μl per paw.

Statistical analysis

Data were analysed statistically by one-way analysis of variance (ANOVA) with *post-hoc* Bonferroni's test for multiple comparisons. Probabilities less than 5% ($P<0.05$) were considered statistically significant.

Results

Antinociceptive action of DbcGMP

Figure 1 shows that intraplantar (right paw) administration of DbcGMP (50, 75, 100 and 200 μg) antagonized the hyperalgesic effect of PGE_2 (2 μg paw^{-1}), in a dose-dependent manner. Maximal antinociceptive effect of DbcGMP is at 1 h after administration and last for plus 2 h (data not shown). DbcGMP at the dose of 100 μg paw^{-1} when injected into the left hindpaw (contralateral) did not produce antinociception in the right hindpaw, whereas DbcGMP at doses of 300 μg, when injected in to the left hindpaw induced a potent antinociceptive effect in the contralateral paw (Figure 2).

Antagonism of DbcGMP-induced antinociception by tolbutamide and glibenclamide

The intraplantar injection of tolbutamide (20, 40 and 160 μg) reduced the peripheral antinociception induced by DbcGMP (100 μg) in a dose-dependent manner (Figure 3). The other K^+

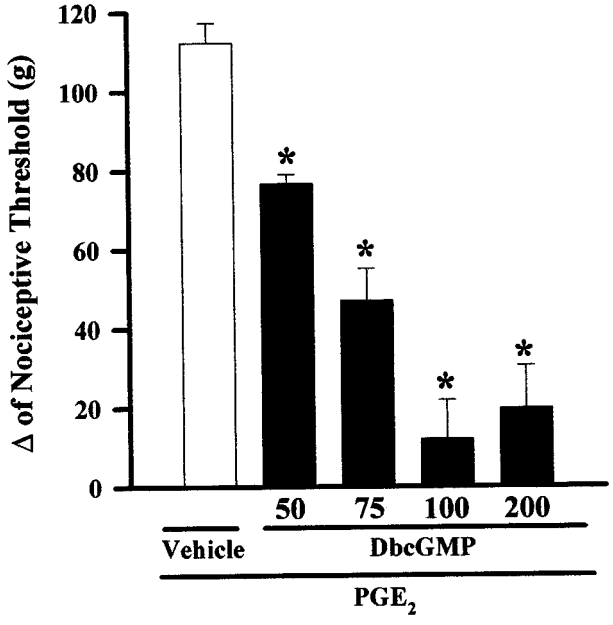

Figure 1 Effect of DbcGMP on the nociceptive threshold in rats with PGE_2-induced hyperalgesia. DbcGMP (μg paw^{-1}) was administered 2 h after local administration of 100 μl of PGE_2 (2 μg). The antinociceptive response was measured in the paw pressure test as described in Methods. Each column represents the mean + s.e.mean ($n=10$). *Indicates a significant difference from the PGE_2 + vehicle injected control ($P<0.05$, ANOVA + Bonferroni's test).

ATP channel blocker tested, glibenclamide (40, 80 and 160 μg paw^{-1}), also significantly inhibited the DbcGMP-induced peripheral antinociceptive effect (Figure 4). Maximal dose of sulphonylureas, when injected in contralateral paw, did not antagonize the antinociception (not shown). Neither sulphonylurea tested significantly modified the nociceptive threshold in control animals (data not shown), or induced any overt behavioural effect at the doses used. Furthermore, glibenclamide had no significant effect on plasma glucose level (results not shown).

Effect of ChTX, apamin, TEA, 4-AP and Cesium on DbcGMP-induced antinociception

Figure 5 shows that Charybdotoxin (2 μg), apamin (10 μg), TEA (2 mg), 4-AP (200 μg) and cesium (800 μg), injected into the paw, did not modify significantly the antinociception induced by DbcGMP (100 μg paw^{-1}).

Discussion

In the present study, DbcGMP, a permeable cyclic GMP analogue, had a dose-dependent peripheral antinociceptive effect on the hyperalgesia induced by PGE_2. Several studies have also demonstrated an antinociceptive effect of this compound through the activation of the L-arginine/NO/cyclic GMP pathway. Ferreira *et al.* (1991) reported that the antinociceptive effect of low doses of morphine administered into paws treated with PGE_2 was inhibited by two blockers of the enzymatic synthesis of NO from L-arginine, L-NIO and L-NMMA and also by methylene blue, a guanilate cyclase

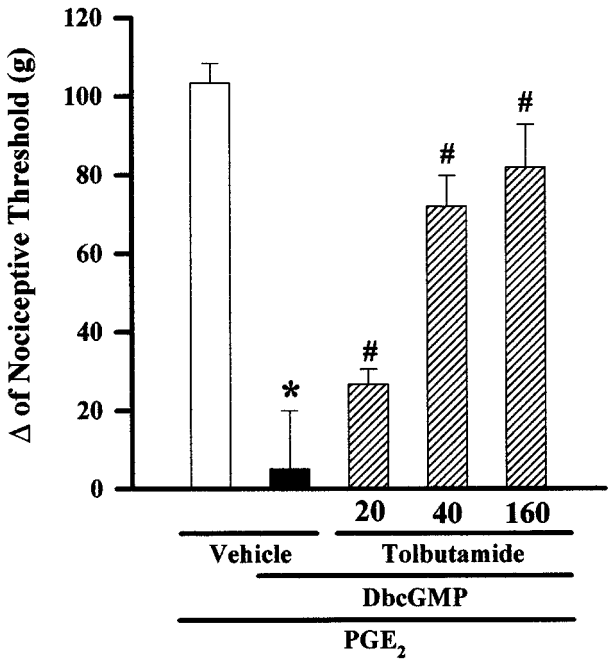

Figure 3 Tolbutamide induces a dose-dependent antagonism of the DbcGMP-induced antinociception of hyperalgesic paws (PGE_2, 2 μg). Antagonism induced by intraplantar administration of tolbutamide of the peripheral antinociception produced by DbcGMP in hyperalgesic paws (PGE_2, 2 μg). Tolbutamide (μg paw^{-1}) was administered 5 min before DbcGMP (100 μg paw^{-1}). Each column represents the mean ± s.e.mean ($n=5$). *, # Indicate significant differences compared to PGE_2 + vehicle- and PGE_2 + DbcGMP + vehicle-injected controls, respectively ($P<0.05$, ANOVA + Bonferroni's test).

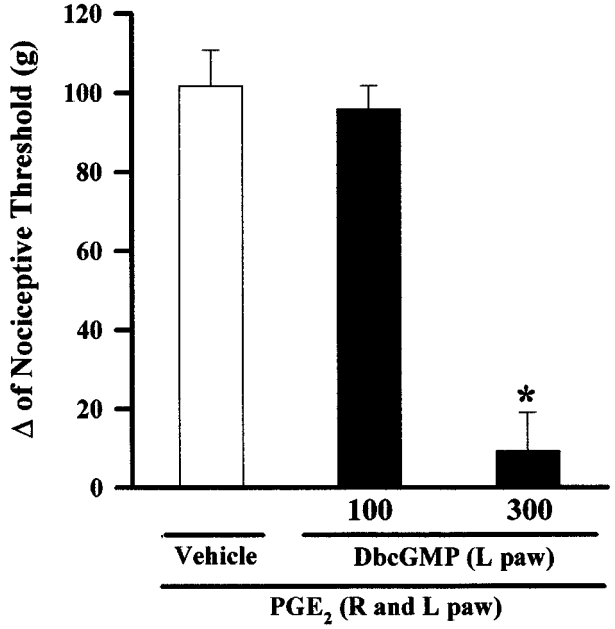

Figure 2 Exclusion of an outside paw antinociceptive effect of DbcGMP. DbcGMP (μg) was administered into the left (L) paw 2 h after PGE_2 (2 μg) administration into both hindpaws, right (R) and left. The analgesic response of the right hindpaw was measured by the paw pressure test as described in Methods. Each column represents the mean ± s.e.mean ($n=10$). *Indicates a significant difference from the PGE_2 + vehicle injected control ($P<0.05$, ANOVA + Bonferroni's test).

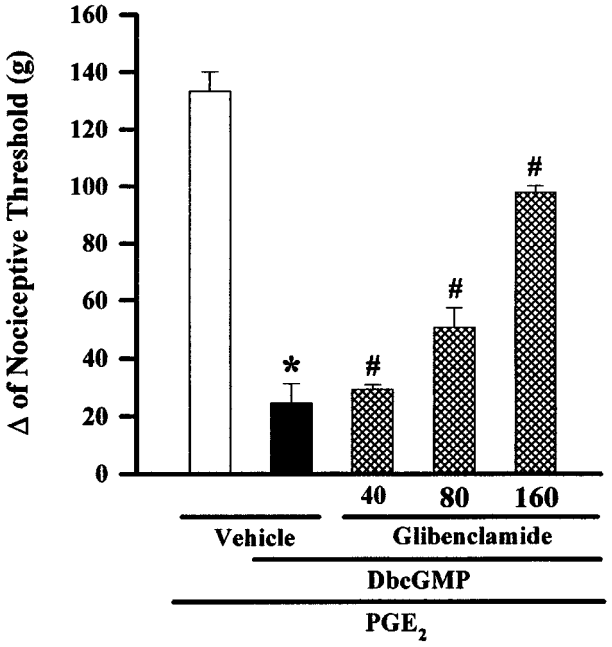

Figure 4 Glibenclamide induces a dose-dependent antagonism of the DbcGMP-induced antinociception of hyperalgesic paws (PGE_2, 2 μg). Glibenclamide (μg paw^{-1}) was administered 5 min before DbcGMP (100 μg paw^{-1}). Each column represents the mean ± s.e.mean ($n=5$). *, # Indicate significant differences compared to PGE_2 + vehicle- and PGE_2 + DbcGMP + vehicle-injected controls, respectively ($P<0.05$, ANOVA + Bonferroni's test).

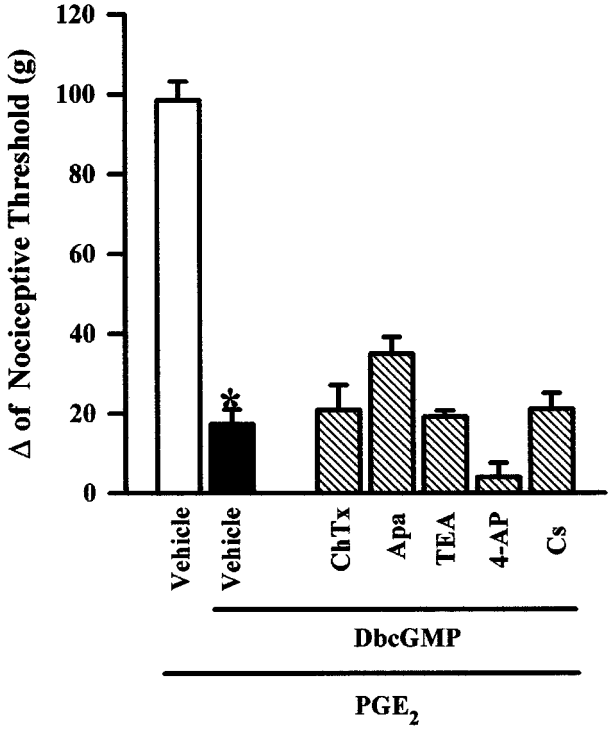

Figure 5 Effect of intraplantar administration of apamin (10 μg), charybdotoxin (2 μg), 4-AP (200 μg), TEA (2 mg) and cesium (800 μg) on the peripheral antinociception induced by DbcGMP in hyperalgesic paws (PGE_2, 2 μg). Drugs were administered 45 min after DbcGMP (100 μg paw^{-1}). Each column represents the mean ± s.e.mean ($n=5$). No statistically significant difference was found between the groups treated with PGE_2 + DbcGMP + vehicle and PGE_2 + DbcGMP + apamin, charybdotoxin, 4-AP, TEA or Cs. *Indicates a significant difference from the PGE_2 + vehicle injected control ($P<0.05$, ANOVA + Bonferroni's test).

inhibitor. Also, the antinociceptive effect was potentiated by MY5445, an inhibitor of the cyclic GMP-degrading enzyme. The activation of the NO/cyclic GMP pathway in primary sensory neurons seems to contribute to the antinociceptive action of dipyrone at the spinal and peripheral level since it was observed that the analgesia induced by intraplantar (Duarte *et al*., 1992), intraperitoneal and intrathecal administration was abolished by pretreatment with L-NMMA or methylene blue.

Furthermore, Dray *et al*. (1992) reported that DbcGMP inhibited the depolarization response evoked by bradykinin in the ventral roots of the medulla. Vocci *et al*. (1978) observed that DbcGMP injected i.c.v. reduced the tail flick reflex response to thermal stimuli in mice. In rats, injection of DbcGMP into the ventricles (Cohn *et al*., 1978) or i.t. (Jurna 1984) also caused antinociception, suggesting that DbcGMP may be involved in analgesia also at the central level.

Recently Germany *et al*. (1996) demonstrated the participation of the NO/cyclic GMP pathway in the antinociception induced by intracerebroventricular administration of bradykinin, since this antinociception was antagonized by prior administration of N^G-nitro-L-arginine methylester (L-NAME) and methylene blue.

To exclude central effects of analgesics many strategies can be used (Stein, 1993). In the present study, we used the strategy of evaluating the efficacy of i.p.s.i.-versus contralateral paw administration because the route and site of administration would the same. DbcGMP, at dose of 100 μg, was ineffective when administered into the contralateral paw, suggesting that at this dose, DbcGMP has a peripheral site of action.

The sulphonylureas tolbutamide and glibenclamide reversed the peripheral antinociceptive effect of DbcGMP in a dose-dependent manner. These drugs specifically block ATP-sensitive K^+ channels, with no effect on Ca^{2+}-activated or voltage-dependent K^+ channels (Amoroso *et al*., 1990; Davies *et al*., 1991; Nichols & Lederer, 1991; Edwards & Weston, 1993).

The central antinociceptive action of various substances such as R-PIA, an A_1 adenosine receptor agonist (Ocaña & Baeyens, 1994), prolactin (Shewade & Ramaswany, 1995) and various 5-HT_{1A} serotonin receptor agonists (Robles *et al*., 1996) also appears to be related to the activation of ATP-sensitive K^+ channels. Intracerebroventricular administration of glibenclamide (Ocaña *et al*., 1995) antagonized the central antinociceptive effect of morphine in mice, as measured by the hot-plate algesimetric test (Ocaña *et al*., 1990). Some studies using the tail-flick test have reported similar results, i.e., reversal of the central antinociceptive effect of morphine by glibenclamide.

In a study carried out in our laboratory using the rat paw compression test, Rodrigues & Duarte (2000) and Soares *et al*. (2000) demonstrated that glibenclamide and tolbutamide respectively reversed the peripheral antinociceptive action of μ opioids receptor agonists and of the nitric oxide donor, sodium nitroprusside, in a dose-dependent manner.

The peripheral antinociceptive action of DbcGMP seems to occur only through specific activation of ATP-sensitive K^+ channels since blockers of other types of K^+ channels, such as Ca^{2+}-dependent (apamin and charybdotoxin) and voltage-dependent (4-AP, TEA and Cs) channels, did not reverse such action. The results were still negative when these blockers were applied according to the same protocol as for the sulphonylureas, 5 min before DbcGMP (results not shown).

Ocaña *et al*. (1995), in a study of the central antinociceptive effect of morphine and fentanyl, Rodrigues & Duarte (2000), in a study of the peripheral antinociceptive effect of the same drugs, and Soares *et al*. (2000), in a study of peripheral antinociception induced by sodium nitroprusside, also observed that 4-AP and TEA did not reverse the effect of these agonists. Similarly, the antinociceptive action of R-PIA (Ocaña & Baeyens, 1994) and of 5-HT_{1A} serotonin receptor agonists (Robles *et al*., 1996) was not reversed by either 4-AP or TEA.

It is important to point out that, as mentioned earlier, on the one hand, several studied demonstrated the participation of the NO-cyclic GMP pathway in the analgesia induced by certain drugs such as opioids, and on the other, various studies have associated analgesia with the activation of ATP-sensitive K^+ channels. Thus, our results establish a link between these data by indicating that ATP-sensitive K^+ channels are involved in the peripheral antinociceptive effect of DbcGMP. This suggestion is based on the fact that the peripheral antinociceptive effect of this drug was reversed by tolbutamide and glibenclamide.

Finally, we still do not know whether the activation of ATP-sensitive potassium channels in the nociceptive terminal occurs directly *via* cyclic GMP or through the activation of PKG, this being an area for future investigation.

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