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Influence of pertussis toxin on the calcitonin-opioid interaction in isolated tissues

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1 In order to clarify one of the mechanisms involved in the analgesic effect of calcitonin, we have tested the *in vitro* modifications induced by calcitonin on the effect of opioids.

2 The inhibition of the contractions induced by opioids or clonidine, in guinea-pig ileum or in mouse vas deferens, were significantly reduced in tissues incubated with pertussis toxin (PTX). When tissues were incubated with PTX and calcitonin, the inhibitory effect was restored.

3 These results suggest that calcitonin is able to potentiate a non-PTX-sensitive mechanism of transduction and support the possibility of involvement of similar G-proteins in the effects of opioid and α_2-adrenoceptor agonists.

Keywords: calcitonin analgesia; opioid; clonidine; G-proteins; pertussis toxin

Introduction

Calcitonin (CT) is a polypeptide hormone concerned with calcium homeostasis that possesses anti-inflammatory and analgesic effects in a variety of osteoarticular disorders and on cancer pain.

The relationship between CT-analgesia and the opioid system has been demonstrated previously by the enhancement of endogenous opioid release found after CT administration (Collin *et al.*, 1989) and by the modifications induced by salmon-calcitonin (SCT) *in vivo* and *in vitro* on opioid effects (Martín *et al.*, 1992; 1993). Nevertheless, the mechanisms underlying the antinociceptive effect of CT are not fully understood.

It is well known that most of the *in vivo* and *in vitro* effects of opioids imply the activation of pertussis toxin-sensitive G proteins: pretreatment with pertussis toxin (PTX) significantly reduced some actions of the opioids such as antinociception (Wong *et al.*, 1992), or inhibition of cholinergic transmission in the ileal myenteric plexus (Kojima *et al.*, 1994).

In an attempt to clarify one of the potential mechanisms having a role in the analgesic effect of CT, we have tested the *in vitro* modifications induced by SCT on the effect of opioids, using tissues pretreated with PTX.

Taking into account our previous results, and the preferential presence of different kinds of opioid-receptors in each tissue, the research was carried out using: guinea-pig ileum to test the effect of μ and κ agonists, and mouse vas deferens to test the effect of a δ agonist. The presence in the mouse vas deferens of μ and κ receptors is well known; however, considering the low κ-receptor density and that the results obtained with the μ-agonist are less consistent in this tissue, it was used only to study the effect of the δ-agonist.

Furthermore, in the guinea-pig myenteric plexus, multiple inhibitory receptor types, such as opioids or α_2 make use of a common pool of G proteins (Ammer *et al.*, 1991). In order to exclude the possibility of interactions due to modifications on the opioid-receptor, the interactions of SCT with the α_2-adrenoceptor agonist, clonidine, were studied.

Methods

Male guinea-pigs weighing 300–450 g, and male CD-1 mice weighing 25–30 g were used.

Myenteric plexus-longitudinal muscle strips (MP-LM) were isolated from guinea-pig ileum, and vasa deferentia were carefully dissected from mice. Tissues were suspended in a 10 ml organ bath containing gassed Krebs solution, kept under 0.5 g of resting tension at 32°C and stimulated through platinum electrodes. MP-LM strips were stimulated with pulses of 70 V, 0.1 ms duration and 0.3 Hz, and mouse vas deferens were stimulated with trains of 15 pulses of 70 V, 15 Hz and 2 ms duration every minute.

Four types of experiments were carried out, as follows:

First Cumulative concentration-response curves were constructed for: [D-Ala^2, N-Me-Phe^4, Gly^5-ol] enkephalin (DAMGO) (5 to 80 nM), *trans*-(±)-3,4-dichloro-N-methyl-N-[2-1(-pyrrolidinyl)cyclohexyl]-benzene-acetamide methane sulphonate (U-50, 488H) (3 to 24 nM) and clonidine (25 to 200 nM) in MP-LM strips, and for [D–Pen^2,D-Pen^5]enkephalin (DPEN) (1 to 8 nM) in mouse vas deferens. The effect was evaluated as % inhibition. The agonists were added 3 h after beginning stimulation.

Second The concentration-response inhibition induced by opioids and clonidine was analyzed after 3 h of incubation of the tissues with PTX (300 ng ml^{-1}).

Third SCT (0.2 iu ml^{-1}) was added to the organ bath 2.5 h after checking the presence of a proper contractile response, and 30 min later the concentration-response curves were obtained.

Fourth Tissues were incubated with PTX; 2.5 h later SCT was added to the organ bath and 30 min later the concentration-response curves were constructed.

Selective antagonists were added to the organ bath to antagonize the effect of the opioids. Naloxone, 1 nM, was used to antagonize the effect of DAMGO, nor-binaltorphimine, 5 nM, for U-50,488H and naltrindol, 10 nM, for DPEN.

Each tissue was employed to construct only one concentration-response curve. Tissues showing contractile responses to the electrical stimulation smaller than 0.5 g were discarded.

Drugs

The following were used: clonidine (a gift of Boehringer Ingelheimen S.A., Spain), [D-Ala^2,N-Me-Phe^{4}Gly^5-ol]enkephalin, [D-Pen^2,D-Pen^5]enkephalin and naloxone-HCl (Sigma

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Spain); naltrindol-HCl (Research Biochemical Inc. USA); norbinaltorphimine-HCl (Research Biochemical Inc. U.S.A.); salmon-calcitonin (a gift of Rhône-Poulenc Rorer Spain, S.A.); pertussis toxin (Research Biochemical Inc. U.S.A.); U-50, 488H (The Upjohn Co. U.S.A.).

Comparisons between groups were made by the two-way ANOVA and LSD (Low Statistical Difference) tests.

Results

Incubation of the tissues with PTX, SCT or both substances simultaneously did not modify the contractile response.

When cumulative doses of DAMGO, U-50, 488H or clonidine were administered to MP-LM strips of guinea-pig ileum or DPEN to mouse vas deferens, the amplitude of the contractions induced by electrical stimulation decreased in a concentration-dependent manner. The percentages (±s.e.mean) of inhibition of the contractile response in control tissues were: for DAMGO (5, 10, 20, 40 and 80 nM): 24±13.1, 42.9±7.9, 64.8±9.1, 83.6±6.8 and 96.6±3.2; for U-50, 488H (3, 6, 12 and 24 nM): 24±5.5, 47.9±6.8, 72.4±6 and 86.3±4.7; for DPEN (1, 2, 4 and 8 nM): 21.3±5.4, 39.1±7.6, 59.1±6.8 and 74.3±7.4, and for clonidine (25, 50, 100 and 200 nM): 36.1±3.8, 45.1±6, 50.2±6.9 and 52±7.8.

The dose-dependent inhibition induced by opioids or clonidine was significantly ($P<0.01$) reduced in all the tissues incubated with PTX when compared with the respective control values. The percentages (±s.e.mean) of inhibition recorded were: for DAMGO (5, 10, 20, 40 and 80 nM): 5.5±1.3, 12.1±3.8, 24±6.9, 37.3±9.2, 50.5±12.8; for U-50, 488H (3, 6, 12 and 24 nM): 24.9±5.6, 24.3±5.6, 37.5±6.5 and 49.3±6.5; for DPEN (1, 2, 4 and 8 nM): 9.7±2.7, 20.9±5.9, 29.2±4.7 and 36.9±4.6, and for clonidine (25, 50, 100 and 200 nM): 22.9±4.6, 30.2±5, 34±5.9 and 35.6±6.2.

The incubation of the tissues with SCT significantly increased the inhibitory effect of U-50,488H ($P<0.05$) and DPEN ($P<0.05$) versus control values but did not modify the effect of DAMGO or clonidine. The percentages (±s.e.mean) of inhibition were: for DAMGO (5, 10, 20, 40 and 80 nM): 20±3.9, 39.1±6.3, 67.5±8.8, 84±6.7, 94.4±3.7; for U-50, 488H (3, 6, 12 and 24 nM): 55.5±3.8, 65.4±6.6, 84.3±5.6 and 98.4±2.4; for DPEN (1, 2, 4 and 8 nM): 23.6±3.1, 52.1±6.1, 71.4±6.2 and 87±4.7, and for clonidine (25, 50, 100 and 200 nM): 27.9±3.7, 38.6±3.9, 44±5.4 and 48.9±6.2.

When tissues were incubated with both PTX and SCT, the inhibitory effect of opioids and of clonidine was restored, the difference versus tissues incubated with PTX being statistically significant ($P<0.01$) for DAMGO and clonidine and ($P<0.05$) for U50,448H and DPEN. The percentages (±s.e.mean) of inhibition recorded were: for DAMGO (5, 10, 20, 40 and 80 nM): 20.6±4.2, 41.6±5.5, 67.8±9.8, 79.2±8.3, 93.4±4.3; for U-50, 488H (3, 6, 12 and 24 nM): 41.2±11.1, 50.1±5.8, 63.5±5.6 and 72.8±5.8; for DPEN (1, 2, 4 and 8 nM): 25.8±4.8, 33.7±6.6, 45±8.6 and 55.9±8.3, and for clonidine (25, 50, 100 and 200 nM): 33.9±3.1, 43.6±3.6, 49.5±4.6 and 51±4.5.

Figure 1 summarizes these results.

The inhibition induced by opioids was antagonized by administration of selective opioid antagonists.

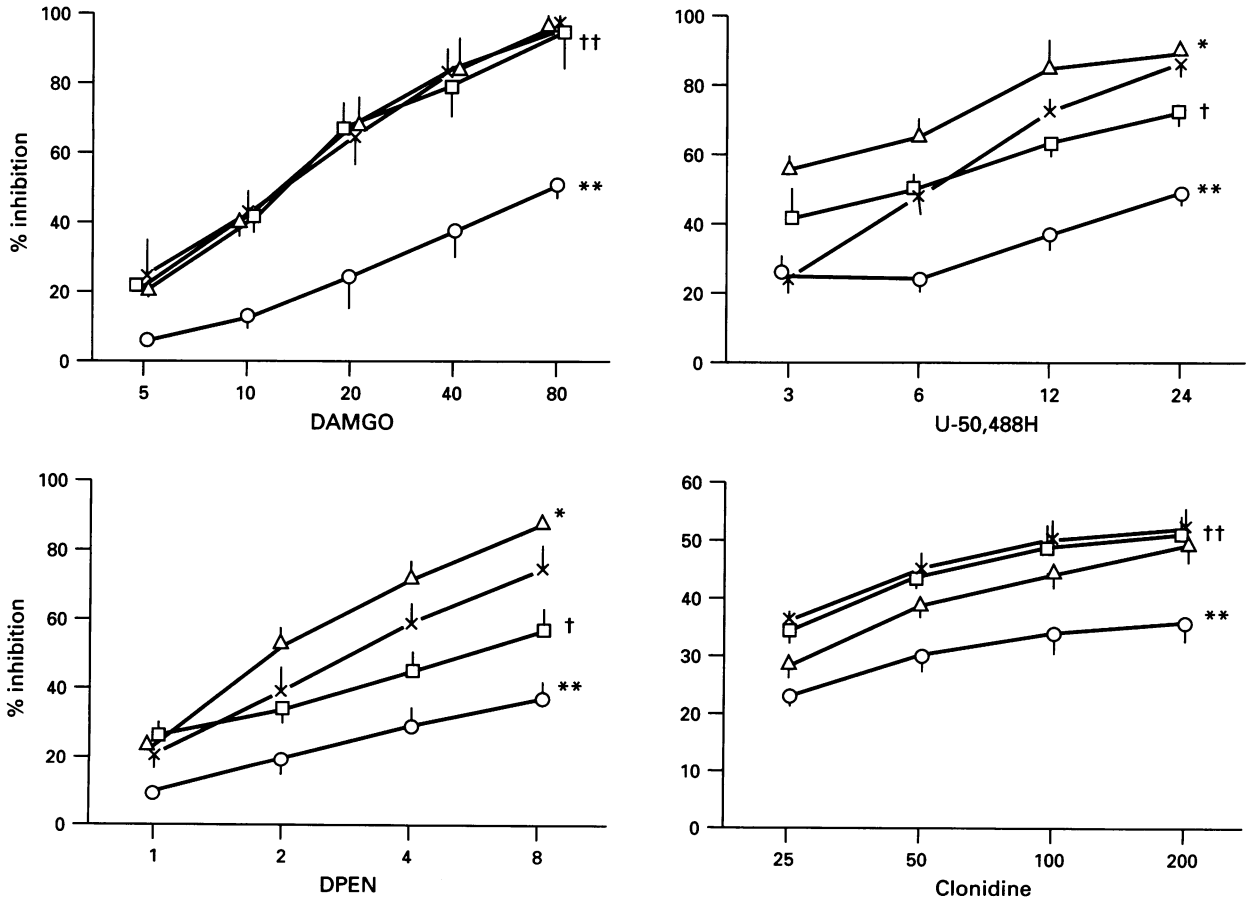

Figure 1 Inhibition of the contractile response after cummulative addition of nM concentrations of the drugs in: control tissues (X), and in tissues incubated with: salmon-calcitonin (SCT) (△), pertussis toxin (PTX) (○) or SCT+PTX (□). Curves for DAMGO, U-50,488H and clonidine were constructed in MP-LM strips and for DPEN in mouse vas deferens. Each point represents the mean±s.e.mean of at least 8 experiments. *$P<0.05$ and **$P<0.01$ significant differences versus control, and †$P<0.05$ and ††$P<0.01$ significant differences between SCT+PTX and PTX-incubated tissues.

Discussion

Opioid and α_2 receptors belong to the superfamily of receptors coupled to G-proteins. It is well documented that PTX produces a functional uncoupling of these receptors from certain G-proteins (G_i/G_0), and so PTX is able to prevent, at least partially, the analgesic effect of opioids and clonidine and their effects on the guinea-pig ileum (Lujan *et al.*, 1984; Kojima *et al.*, 1994).

When the MP-LM strips of guinea-pig ileum or the vasa deferentia of mice were incubated with PTX and SCT simultaneously, the inhibition of the effect of opioids or clonidine induced by PTX was significantly reduced. Different hypotheses may be postulated to explain these results. Considering that the action of the PTX is mediated by catalysation of the ADP-ribosylation of the α subunit of the G_i/G_0-proteins (Przewlocki *et al.*, 1987), it may be feasible that SCT reverses this ribosylation and, so, the responsiveness to opioids and clonidine could be restored.

An alternative explanation could be the induction by SCT of the synthesis of new α subunits: it was shown previously (Ammer *et al.*, 1991) that chronic exposure to opiate or clonidine increases the concentration of $G_0\alpha$ and $-\beta$ subunits; if SCT were able to increase the synthesis or the insertion in the membrane of new G-proteins not affected by PTX, the effect of opioids and clonidine would be re-established. However, it must be considered that the time of incubation with SCT is only 30 min which is perhaps too short a period to induce the synthesis of new G-proteins.

There is also evidence that opioid and α_2 receptors are not exclusively coupled to PTX-sensitive G-proteins. Results of various studies lead to the conclusion that these two receptors are susceptible to regulation by processes that might involve G_s-proteins (Sánchez-Blázquez & Garzón, 1991; Crain & Shen, 1992). Interestingly, the effects mediated through G_s-proteins became evident when small doses of opioids were used or in the presence of PTX when the effects mediated by G_i/G_0-proteins were blocked (Fan *et al.*, 1993). This could be the reason why the effect of SCT is not manifested for all the opioid agonists in naive tissues.

Our results show that incubation with PTX inhibits the *in vitro* effect of both opioid agonists and clonidine. These data agree with previous reports (Lujan *et al.*, 1984) and sustain the possibility of the existence of common mechanisms of transduction for α_2 and opioid receptors as has been proposed by Ammer *et al.* (1991). These authors suggest that, in the guinea-pig myenteric plexus, multiple inhibitory receptor types make use of a common pool of G-proteins.

Furthermore, it has been suggested that PTX-sensitive G-protein (G_i/G_0) and non-PTX-sensitive G-protein (G_s) are integral components of the mechanism that mediates opioid inhibition and opioid enhancement, respectively, of evoked enkephalin release in the guinea-pig ileum (Gintzler & Xu, 1991).

Tissues incubated with PTX and SCT show restoration of the inhibition of the contractile response induced by opioids or clonidine. From these results it may also be suggested that SCT is able to potentiate a non-PTX-sensitive mechanism of transduction. Perhaps, in these tissues the inhibition of the contractile response may be mediated by enkephalin release evoked through a G_s-protein activation. After the incubation of the tissues with PTX the inhibition of the effect of the opioids was statistically significant ($P<0.01$); however, the highest tested doses were able to induce a decrease in the force of the electrically induced contractions. From this remaining activity it can be suggested that not all the G_i/G_0 proteins have been inactivated and so, if a release of opioid material takes place, it may increase the inhibition.

The hypothesis of a potentiation of the effect of the opioids through a non-pertussis toxin-sensitive G-protein is also supported by the fact that the analgesia induced by CT is not reversed after pretreatment with PTX (Guidobono *et al.*, 1991).

In addition current results obtained after the administration of clonidine suggest and support the possibility of involvement of similar G-proteins on the effects of opioid and α_2 agonists.

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