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Calcium channel inhibitors suppress the morphine-withdrawal syndrome in rats

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1 The effects of the Ca^{2+}-channel blockers verapamil and nimodipine, on the behavioural signs of naloxone (1 mg kg^{-1})-induced abstinence syndrome in morphine-dependent rats, were evaluated. The content of noradrenaline (NA) and of its metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) was measured, using high performance liquid chromatography and electrochemical detection or gas chromatography-mass spectrometry, in various brain regions of these animals. Possible interactions of nimodipine and verapamil with opioid receptors were evaluated by examining their ability to displace [^{3}H]-naloxone binding to brain membranes.

2 Verapamil (5, 10 and 50 mg kg^{-1}) and nimodipine (1, 5 and 10 mg kg^{-1}) dose-dependently reduced most of the signs of morphine abstinence.

3 Naloxone-precipitated abstinence decreased the NA content in the cortex, hippocampus, brainstem and cerebellum. In the same brain regions the content of MHPG increased, suggesting an increased release of the amine during morphine abstinence.

4 Nimodipine (10 mg kg^{-1} i.v.) did not change the content of NA or MHPG in the cortex, hippocampus and brainstem. However, nimodipine pre-treatment markedly reduced the changes in NA and MHPG content induced by the abstinence syndrome.

5 Neither verapamil nor nimodipine displaced [^{3}H]-naloxone from its binding sites.

6 These results suggest that Ca^{2+}-channel blockers suppress the behavioural and neurochemical expressions of morphine abstinence by a mechanism that differs from those of opioids or α_2-adrenoceptor agonists.

Introduction

The administration of opiates or opioid peptides modifies the content and movement of Ca^{2+} in the brain of several animal species (for reviews see: Sanghvi & Gershon, 1977; Ross & Cardenas, 1979; Chapman & Way, 1980). This biochemical action of opioids has been correlated both with their anti-nociceptive properties and with their dependence liabilities. Molecules able to chelate Ca^{2+} (e.g. (EGTA) or to antagonize Ca^{2+}-fluxes (e.g. lanthanum, D-600, verapamil) potentiate the analgesic action of morphine (Harris *et al.*, 1975), opioid peptides (Chapman & Way, 1982) and of several benzomorphans classified as κ-receptor agonists (Ben-Sreti *et al.*, 1983). Physical dependence on morphine is associated with a large increase in the content of Ca^{2+} in the brain of mice and rats. Naloxone prevents this effect and during naloxone-precipitated withdrawal syndrome these elevated Ca^{2+} levels quickly return toward control values (Yamamoto *et al.*, 1978).

Thus, modifications of Ca^{2+} disposition seem to play a basic role not only in the acute effects of opioids but also in the manifestations of the morphine abstinence syndrome in rodents (Harris *et al.*, 1975; 1976). Recently, it has been shown that the number of [^{3}H]-nitrendipine binding sites is greatly increased in the brain of morphine-tolerant mice (Ramkumar & El-Fakahany, 1984). Since [^{3}H]-nitrendipine binding sites in brain synaptosomes are strictly related to voltage-operated Ca^{2+}-channels (Turner & Goldin, 1985) it is likely that during morphine dependence important changes in voltage operated Ca^{2+}-channels occur.

To our knowledge, however, no information is available on the effects of organic Ca^{2+}-channel blockers in the morphine abstinence syndrome in rodents. We have shown here that verapamil and nimodipine, two chemically unrelated Ca^{2+}-channel

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blockers, suppress the behavioural expression of the abstinence syndrome. Furthermore, nimodipine also reduces the modifications of NA metabolism which are associated with the abstinence syndrome (Gunne *et al.*, 1969; Crawley *et al.*, 1979).

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Methods

Induction of morphine dependence, morphine abstinence syndrome and quantitation of the withdrawal signs

Under light ether anaesthesia one morphine-containing pellet was implanted subcutaneously on the first day and two further pellets implanted on the third day in male rats (Nossan strain) weighing 160–200 g. Each pellet contained 75 mg of morphine base and was prepared according to Gibson & Tingstad (1970). The abstinence syndrome was evoked by challenge on the fifth day with naloxone (1 mg kg^{-1}, i.p.).

Each rat was placed in a perspex cage (42 × 26 × 16 cm) 10 min before naloxone administration and was observed for 60 min after the injection of the drug. The following signs were monitored: wet dog shakes, grooming, agitation, teeth chattering, lacrimation and penile discharge. These signs were rated every 10 min using a scale from 0 to 3: 0 indicates sign absent during the 10 min of observation, 1 present but of low intensity, 2 average intensity, 3 maximal intensity. Diarrhoea was scored every 20 min. With this rating system, it was thus possible to evaluate each sign for each rat by obtaining a score from 0 to 18. For diarrhoea the scores ranged from 0 to 9. Each animal was weighed immediately before the naloxone injection and at the end of the observation period to monitor the loss of body weight during the abstinence syndrome.

In order to study the actions of Ca^{2+}-channel inhibitors on the abstinence syndrome, nimodipine was administered intravenously and verapamil subcutaneously 10 min before naloxone.

Determination of noradrenaline (NA) and 3-methoxy-4-hydroxyphenylglycol (MHPG)

Brain regions were homogenized in 10 volumes of ice cold 0.1 M formic acid containing 0.5% EDTA, 0.5 μM dihydroxybenzylamine hydrobromide (DHBA), and 0.2 nmol deuterated MHPG as internal standards. The homogenization was performed in polycarbonate tubes using a Polytron homogenizer (Brinkan Co.). The tubes were centrifuged (10 min at 10,000 *g*) and the supernatants divided into two aliquots. The first was used for high performance liquid chromatography (h.p.l.c.) determination of NA, the second for gas chromatographic-mass spectrometric (g.c.-m.s.) determination of MHPG. The first aliquot was mixed with 50 mg of acid washed alumina and an equal volume of 1 M Tris (pH 8.6). The alumina was retained on Whatman GF/B paper by centrifuging the mixture in a blue Eppendorf tip according to Van Valkenburg *et al.* (1982) and using the same procedure, it was washed 3 times with 0.5 ml of 0.1 M Tris (pH 8.6).

Catechols were subsequently eluted by twice passing 0.3 ml of 0.1 M perchloric acid containing 0.1% EDTA through the alumina. An aliquot (50 μl) of the eluate was injected into a liquid chromatography apparatus. This consisted of a Perkin-Elmer LC 600 autoinjector connected to a Waters M 45 pump and a Perkin-Elmer reversed-phase C-18 column (25 cm × 4.6 mm; 10 μm particle size). The flow rate was 1 ml min^{-1} and the column effluent was passed through an EDT Mod.LCA-15 cell where the catechols were detected using an EDT amperometric detector operated at + 0.70 V. The mobile phase was 70 mM NaH_2PO_4 (pH 3.8) with 8% methanol and 1 mM heptane sulphonic acid.

Since the h.p.l.c.-ED methodology we have was not sensitive enough to measure the brain regional contents of MHPG, we always employed a g.c.-m.s. method (Wood *et al.*, 1982). Briefly, the second aliquot of the supernatant was incubated for 24 h at 37°C with a mixture of sulphatase (1.5 mg) and glucuronidase (1 mg) dissolved in 2 ml of 2 M sodium acetate buffer (pH 6.2); 5 ml of ethyl acetate was then added. The samples were vortexed for 5 min and then left to stand for 10 min before being centrifuged at 600 *g* for 5 min. The organic extract was then evaporated under vacuum and the residue transported to a 1 ml reactivial with 200 μl of methanol. This extract was dried under nitrogen and reacted with 100 μl pentafluoropropionic anhydride and 50 μl hexafluoroisopropanol (Moroni *et al.*, 1979). The samples were then dried under nitrogen and dissolved in 10–12 μl of ethyl acetate. Aliquots of 1–2 μl were injected into an LKB 2091 g.c.-m.s. equipped with a multiple ion detector. The chromatrographic conditions were: silanized glass column (2 m × 1.5 mm) packed with 5% OV-17 on Gas Chrom Q (Chromopack). The carrier gas was helium at a flow of 15 ml min^{-1}; the oven temperature was 155°C and the flash heater set at 190°C. The mass spectrometric conditions used were: separator temperature 240°C; ion source temperature 240°C; electron energy 20 eV; accelerating voltage 3.5 kV; instrument resolving power 500. Only the peaks having an m/z of 458/460 and corresponding to MHPG and to its deuterated internal standard were recorded (Wood *et al.*, 1982).

Opioid receptor binding

Rat brain membranes were prepared according to the procedure described by Zukin (1984). Displacement curves were performed using [^{3}H]-naloxone (1 nM) and increasing concentrations of various opioids, verapamil and nimodipine in a medium containing 100 mM sodium (Pert & Snyder, 1974).

Statistical analysis

Statistically significant differences in the content of NA, MHPG and behavioural signs were determined by the Dunnet test (Winer, 1971).

Materials

Sulphatase type H-1, β-glucuronidase, DHBA and MHPG were purchased from Sigma Chemical Co. (St. Louis, MO); MHPG d_3 from Merck Frosst (Montreal); [^{3}H]-naloxone from New England Nuclear (Boston, MS). Naloxone was supplied from Crinos (Como). Morphine pellets were prepared by Dr Giannini (Malesci, Firenze). Verapamil was supplied by Knoll-AG (Liestal) and nimodipine by Bayer-AG (Wuppertal).

Results

Morphine withdrawal signs

Table 1 and Table 2 present the withdrawal signs we monitored in two different groups of morphine-dependent rats injected with naloxone intraperitoneally. Under our experimental conditions the animals constantly lost approximately 8% of their body weight in 1 h. Furthermore, they displayed diarrhoea, wet dog shakes, agitation, grooming, teeth chattering, lacrimation and emission of aqueous-bloody material from the penis. The administration of morphine 20 mg kg^{-1} s.c., completely prevented the behavioural expression of the syndrome (data not shown).

Changes in the brain content of NA and of its metabolite MHPG during the abstinence syndrome

The content of NA in the cortex, hippocampus, brainstem and cerebellum did not change after acute morphine (5 mg kg^{-1} s.c., 1 h), chronic morphine (3 pellets in 5 days) or naloxone (1 mg kg^{-1}) administration to naive rats (data not presented). However, when naloxone (1 mg kg^{-1} i.p., 1 h) was injected to morphine-dependent animals the content of NA decreased significantly (Figure 1). Furthermore, in the same rats the content of MHPG almost doubled in the frontoparietal cortex, hippocampus, brainstem and cerebellum (Figure 1).

Verapamil and nimodipine antagonism of the naloxone-precipitated morphine withdrawal signs

Verapamil (50 mg kg^{-1}) and nimodipine (10 mg kg^{-1}) administered to naive animals caused a decrease of the spontaneous motor activity. Lower doses did not affect the gross behaviour.

Verapamil, administered to morphine-dependent rats 20 min before naloxone challenge reduced the appearance of most of the behavioural signs of the abstinence syndrome (Table 1). The diarrhoea and the body weight loss were greatly reduced by 5 mg kg^{-1} of verapamil. Higher doses were necessary to reduce the appearance of most of the other signs. The emission of bloody fluid from the penis was not affected.

The administration of nimodipine (1, 5, 10 mg kg^{-1}) to dependent animals prevented the appearance of most of the naloxone precipitated withdrawal signs in a dose-dependent manner. Similar to verapamil, the diarrhoea and the body weight loss were reduced at the lowest dose of nimodipine used (1 mg kg^{-1} i.v., 10 min before naloxone). Most of the other signs were greatly reduced by higher doses of the drug (Table 2). The

Table 1 Effects of verapamil on naloxone-precipitated morphine withdrawal signs

Withdrawal sign	*Control*	*Verapamil* (mg kg^{-1} s.c.) *5*	*20*	*50*
Body weight loss	8.1 ± 0.8	3.9 ± 0.1**	3.5 ± 0.6**	1.5 ± 0.4**
Diarrhoea	4.2 ± 0.3	2.9 ± 0.5**	2.2 ± 0.2**	1.4 ± 0.1**
Wet dog shakes	11.0 ± 1.2	9.0 ± 1.3	7.2 ± 1.1**	4.2 ± 0.6**
Grooming	9.0 ± 1.2	8.1 ± 1.1	3.1 ± 0.2**	2.1 ± 0.2**
Agitation	9.4 ± 1.1	5.3 ± 1.5**	5.5 ± 0.7**	3.0 ± 0.4**
Teeth chattering	7.2 ± 1.0	6.9 ± 1.2	3.0 ± 1.0**	2.6 ± 0.3**
Lacrimation	5.8 ± 0.7	4.3 ± 0.7	4.0 ± 0.3**	3.1 ± 0.6**

**$P < 0.01$. $n = 7$ each group.

Table 2 Effects of nimodipine on naloxone-precipitated morphine withdrawal signs

Withdrawal sign	*Control*	*Nimodipine* (mg kg^{-1} i.v.) *1*	*5*	*10*
Body weight loss	8.1 ± 0.8	3.6 ± 0.3**	2.1 ± 0.3**	1.4 ± 0.2**
Diarrhoea	4.2 ± 0.3	1.5 ± 0.1**	1.1 ± 0.1**	0.2 ± 0.01**
Wet dog shakes	11.0 ± 1.2	8.1 ± 0.9**	3.0 ± 0.2**	0.5 ± 0.01**
Grooming	9.0 ± 1.2	6.2 ± 0.7	3.1 ± 0.5**	0.3 ± 0.01**
Agitation	9.4 ± 1.1	6.2 ± 0.3**	4.8 ± 0.2**	2.1 ± 0.3**
Teeth chattering	7.2 ± 1.1	4.2 ± 0.2**	4.8 ± 0.3**	1.3 ± 0.4**
Lacrimation	5.8 ± 0.7	3.1 ± 0.1**	4.5 ± 0.6**	3.0 ± 0.2**

**$P < 0.01$. $n = 7$ each group.

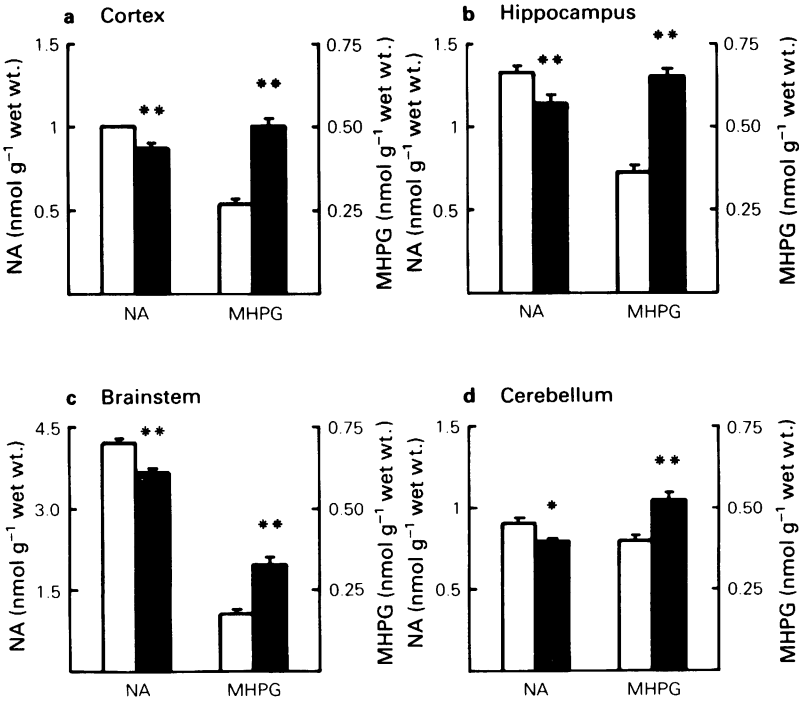

Figure 1 Changes in the content of noradrenaline (NA) and 3-methoxy-4-hydroxyphenylglycol (MHPG) in (a) cortex, (b) hippocampus, (c) brainstem and (d) cerebellum induced by naloxone-precipitated withdrawal from chronic morphine. Open columns represent content of NA and MHPG in controls and solid columns, content during the abstinence syndrome. Each column is the mean value of at least 7 rats. Vertical bars represent s.e.mean. **$P < 0.01$; *$P < 0.05$.

emission of bloody material from the penis was not affected by nimodipine (10 mg kg^{-1}).

Nimodipine antagonism of the changes of NA metabolism induced by naloxone-precipitated abstinence syndrome

No changes in the content of NA and MHPG were observed in the cortex, hippocampus, cerebellum and brainstem of rats treated with nimodipine (5 and 10 mg kg^{-1} i.v., 1 h). When the drug (10 mg kg^{-1}) was administered to dependent animals 10 min before the naloxone challenge, the abstinence syndrome was almost completely prevented and the content of NA in the cortex, hippocampus, cerebellum and brainstem did not decrease. Similarly, the same dose of nimodipine completely prevented the withdrawal changes of the MHPG content in the brainstem. As shown in Figure 2, the withdrawal related increase in the concentration of MHPG in the cortex and in the hippocampus was significantly reduced.

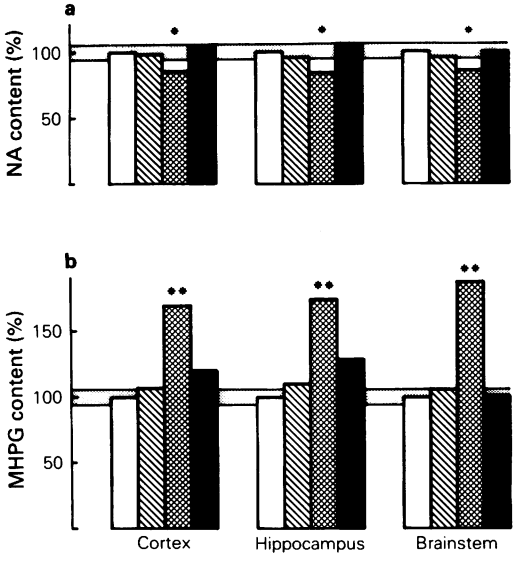

Figure 2 Effects of nimodipine on the changes in the content of (a) noradrenaline (NA) and (b) 3-methoxy-4-hydroxyphenylglycol (MHPG) in the cortex, hippocampus and brainstem induced by naloxone-precipitated withdrawal from chronic morphine. Open columns represent control levels of NA and MHPG; hatched columns, levels during chronic morphine treatment; cross-hatched columns, levels during the abstinence syndrome and solid columns, levels during the abstinence syndrome in nimodipine (10 mg kg^{-1})-treated rats. See Figure 1 for the values of NA and MHPG in controls and after withdrawal. Each column is the mean % value from at least 7 animals. $^{**}P<0.01$; $^{*}P<0.05$.

Effects of nimodipine and verapamil on [^{3}H]-naloxone binding

In order to clarify whether the actions of nimodipine and verapamil were related to a direct action on opioid receptors, their ability to displace the binding of [^{3}H]-naloxone to rat brain membranes was tested. The two Ca^{2+}-channel inhibitors did not affect [^{3}H]-naloxone binding up to a concentration of 10^{-4} M. Using the same incubation medium and membrane preparation, morphine (EC_{50} 10^{-7} M), etorphine (EC_{50} 6×10^{-9} M), methadone (EC_{50} 2×10^{-7} M) and naloxone (EC_{50} 2×10^{-9} M) displaced [^{3}H]-naloxone binding.

Discussion

The morphine abstinence syndrome can be effectively suppressed both in man and in animals by administering suitable doses of opioid agonists or of clonidine and related α_2-adrenoceptor agonists (Redmond & Krystal, 1984). The data presented here indicate that verapamil and nimodipine, two chemically unrelated molecules both exhibiting activity on voltage operated Ca^{2+}-channels (Spedding, 1985), also suppress most of the signs of the behavioural symptoms of the abstinence syndrome. Neither verapamil nor nimodipine displaced [^{3}H]-naloxone from its binding sites and therefore they are probably not active at the level of the opioid receptor. Furthermore, while verapamil seems to be able to increase NA release both in brain and peripheral tissues (Galzin & Langer, 1983; Zsotér *et al.*, 1984) and to interact with NA receptors (Nayler *et al.*, 1982; Galzin & Langer, 1983), nimodipine (10 mg kg^{-1} i.v.) does not alter the brain NA content or metabolism and at a concentration of 10^{-5} M does not interact *in vitro* with the actions of NA (Towart *et al.*, 1982). It is, therefore, extremely likely that the actions of verapamil and nimodipine in preventing the behavioural manifestations of the abstinence syndrome are related to changes in Ca^{2+} fluxes in the brain.

An interaction between acute and chronic morphine treatment and brain calcium content or movement has been proposed previously (Sanghvi & Gershon, 1977; Ross & Cardenas, 1979; Chapman & Way, 1980). In particular, it has been shown that acute injections of large doses of morphine reduced the synaptosomal content of Ca^{2+} in rats and mice (Harris *et al.*, 1976; Yamamoto *et al.*, 1978), while chronic morphine treatment increased brain Ca^{2+} levels by almost 100% (Yamamoto *et al.*, 1978). This increased content of Ca^{2+} rapidly returned towards normal values during precipitated withdrawal (Yamamoto *et al.*, 1978), thus

indicating important changes in Ca^{2+} disposition during the abstinence syndrome. Such important redistribution of Ca^{2+} is probably a key event for the neurochemical and behavioural expressions of the syndrome. It is therefore not surprising that molecules able to modify Ca^{2+} fluxes, such as lanthanum and copper, when injected intracerebroventricularly reduce most of the signs of morphine abstinence (Harris *et al.*, 1975; Bhargava, 1978). In this context, the recent demonstration of an increased number of [^{3}H]-nitrendipine binding sites (Ramkumar & El-Fakahany, 1984), and therefore probably of voltage operated Ca^{2+} channels (Turner & Goldin, 1985) in the brain of dependent mice, further support the idea that changes in Ca^{2+} disposition are involved in morphine-dependence.

Our experiments agree with these observations and demonstrate that not only lanthanum (Harris *et al.*, 1976), but also organic molecules able to interact with Ca^{2+} channels, suppress the neurochemical and behavioural expressions of morphine abstinence. The various signs of the syndrome are differentially reduced by verapamil and nimodipine. Body weight loss and diarrhoea were the most sensitive to the action of these drugs. The emission of aqueous red material from the penis was not modified after the largest doses used. Ejaculation and penile erection during opioid-abstinence have been associated with the release of ACTH-MSH like peptides from the hypophysis. Ca^{2+}-channel blockers do not inhibit this release (Fratta, personal communication). The possibility that peripheral sites of action contribute to the differential effect of Ca^{2+}-channel inhibitors should be considered. This could be important especially for the suppression of the body weight loss and diarrhoea. However, the data showing that nimodipine reduces the changes in brain NA metabolism occuring during the abstinence syndrome, in a manner similar to that of clonidine (Crawley *et al.*, 1979), suggest that central mechanisms are also operative.

The role of the voltage operated Ca^{2+}-channels in the brain is still a controversial issue. In fact, in spite of the existence of recognition sites for these drugs (Bellemann *et al.*, 1983; Gould *et al.*, 1983; Cortès *et al.*, 1984), at pharmacologically relevant concentrations they seem not to affect neuronal function (Daniell *et al.*, 1983; Starke *et al.*, 1984). Recently, it has been shown that when neurones release pathologically large amounts of transmitter because of abnormal activation of their voltage operated Ca^{2+}-channels, verapamil, nimodipine and related drugs become quite active (Middlemiss & Spedding, 1985). It is likely that a pathological activation of already modified Ca^{2+}-channels (Ramkumar & El-Fakahany, 1984) takes place during morphine abstinence. This could explain most of the neurochemical and behavioural events of the syndrome and also the data described here.

If this is true, the Ca^{2+}-channel inhibitors could be considered drugs acting on the basic mechanisms of the expression of morphine abstinence. In our opinion it would be rational to extend our studies to other molecules and other animal species, taking into consideration their possible therapeutic role in this pathological condition.

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