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Morphine withdrawal in cortical slices: suppression by Ca^{2+}-channel inhibitors of abstinence-induced [^{3}H]-noradrenaline release

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1 The effects of morphine withdrawal were evaluated *in vitro* by monitoring the actions of naloxone on the depolarization-induced release of [^{3}H]-noradrenaline (NA) in cortical slices taken from naïve or dependent rats. The effects of dihydropyridine molecules acting on Ca^{2+}-channels (nimodipine and Bay K 8644) were also studied in this model.

2 Naloxone (10^{-8}–10^{-5} M) dose-dependently enhanced the K^+ induced release of [^{3}H]-NA in slices taken from dependent rats, but failed to modify the [^{3}H]-NA release from 'naïve' slices.

3 The naloxone-induced potentiation of release was significantly reversed by nimodipine (10^{-8}–10^{-6} M). These doses of nimodipine did not change [^{3}H]-NA release (both basal and K^+ induced) in preparations obtained from naïve rats.

4 Bay K 8644 potentiated the K^+-induced [^{3}H]-NA release from cortical slices taken from naïve rats to a similar extent as that of naloxone in dependent rats.

5 These results suggest that the naloxone potentiation of the depolarization-induced [^{3}H]-NA release in slices taken from dependent rats may be considered a model of morphine withdrawal *in vitro*. In this model dihydropyridine Ca^{2+}-channel antagonists suppress morphine-withdrawal effects in a similar manner to observations made *in vivo*.

Introduction

Opiates and opioid peptides reduce voltage-dependent calcium entry in neurones (North & Williams, 1983; Duggan & North, 1984). This results in an opiate-induced decrease of several Ca^{2+}-dependent processes amongst which is neurotransmitter release (Paton, 1957; Montel *et al.*, 1974; Kosterlitz & Waterfield, 1975; Moroni *et al.*, 1977; Schoffelmeer & Mulder, 1983; Jackisch *et al.*, 1986).

Chronic morphine administration results in tolerance to these effects (Casamenti *et al.*, 1980) and during morphine dependence an increase in the abundance of voltage operated Ca^{2+}-channels, as measured by [^{3}H]-nitrendipine binding techniques (Turner & Goldin, 1985), has been described in the brain of rodents (Ramkumar & El-Fakahany, 1984). This could indicate that chronic morphine exposure causes the appearance in neuronal membranes, of an increased number of Ca^{2+}-channels. An interaction between opiates and Ca^{2+}-channel inhibitors has been demonstrated in several laboratories: molecules able to antagonize Ca^{2+} fluxes potentiate the actions of morphine and of other opiate agonists (Harris *et al.*, 1975; Ben-Sreti *et al.*, 1983; Pillai & Ross, 1986) and facilitate behaviour possibly mediated by endogenous opioids (Kavaliers, 1987). Finally, we previously observed that several organic Ca^{2+}-channel blockers prevent most of the behavioural and biochemical manifestations of the morphine abstinence syndrome in dependent rats (Bongianni *et al.*, 1986). In order to investigate further the mechanism whereby dihydropyridine calcium channel inhibitors prevent the manifestations of morphine abstinence, we studied a simple *in vitro* model of morphine withdrawal.

In this paper we describe the model and the actions of nimodipine and of Bay K 8644 on stimulation-induced noradrenaline (NA) release from brain slices taken from the cortex of dependent rats.

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Methods

Induction of morphine-dependence

Male Wistar rats (140–160 g) were implanted subcutaneously, under light ether anaesthesia, with one morphine-containing pellet on the first day and two further pellets on the third day. Each pellet contained 75 mg of morphine base and was prepared according to Gibson & Tingstad (1970). The experiments were carried out on the fourth day. The induction of morphine tolerance and dependence at that stage has been previously assessed (Bongianni *et al.*, 1986).

Preparation and labelling of the slices

Control or dependent rats were decapitated and their brains rapidly removed and placed in cold oxygenated (95% O_2 plus 5% CO_2) Ringer solution (composition in mM: NaCl 118, KCl 6, KH_2PO_4 1.2, $CaCl_2$ 1.8, $NaHCO_3$ 25 and glucose 10).

This solution always contained morphine (3×10^{-6} M) when the brain preparations of dependent rats were treated. The brains were divided into two halves and four slices of the frontoparietal cortex were prepared from each half. Preliminary experiments showed that [^{3}H]-noradrenaline (NA) release did not differ between the slices prepared from the right or from the left portion of the brain.

The slices were prepared by keeping the brains under oxygenated Ringer solution using a home-made vibratome (Beani *et al.*, 1978) and they had a thickness of 400 μm. The slices were placed for 2 h at room temperature in oxygenated Ringer solution and then for 20 min, at 37°C, in a solution containing [^{3}H]-NA (50 pM; 370–740 GBq mmol^{-1}). After a further 25 min of washing, the preparations were placed in a thermostated superfusion apparatus and superfused with oxygenated solution at a rate of 2 ml min^{-1}. The superfusion fluid was collected every 5 min and a portion of it was counted in a Packard (Tri Carb 460 C) liquid scintillation spectrometer using Instagel as a scintillation fluid. The slices were stimulated by passing over them an isotonic Ringer solution containing KCl 30 mM.

In the experiments aimed to study the withdrawal phenomena, one group of slices of the same animal was used as a control and the second group had naloxone (at different concentrations) in the perfusion medium instead of morphine. In several experiments nimodipine was added to the naloxone solution, in other experiments nimodipine was added to the control (morphine containing) superfusion fluid. Particular care was taken to avoid light-induced nimodipine inactivation.

At the end of the experiments the slices were solubilized in 1 ml of Protosol and the tritium content of the tissue monitored.

Calculations

The fractional release of tritium and the stimulation-evoked overflow were calculated according to Taube *et al.* (1977), with minor modifications: the basal outflow was expressed as (c.p.m. tritium outflow per 5 min)/(c.p.m. of tritium in the slice at the beginning of the respective 5 min period), while the stimulation-evoked overflow was calculated by integrating the area under the stimulation curve and by subtracting the basal outflow from it.

Drug effects were evaluated by comparing the overflow evoked by K^+ in the slices taken from the half of the brain used as control with the slices taken from the other half perfused with drugs (naloxone, nimodipine, Bay K 8644).

Statistical analysis was performed by use of Dunnet's *t* test.

Identification of the tritium released by the slices

In several experiments a portion of superfusion fluid and an equal volume of Ringer solution containing [^{3}H]-NA were immediately acidified by adding HCl 1 M (200 μl) containing a detectable amount of unlabelled NA (5 nmol). These samples were then stored at − 50°C and processed within 3 days by mixing them 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 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 passing 0.5 ml of 0.1 M perchloric acid. An aliquot (175 μl) of the eluate was injected into an h.p.l.c. apparatus. This consisted of a Perkin-Elmer series 10 Liquid Chromatograph 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 a Bio-Analytical System LC-4B amperometric detector operated at + 0.70 V. The mobile phase was 70 mM Na H_2PO_4 (pH 3.8) with 5% methanol and 1 mM heptane sulphonic acid.

Materials

Morphine hydrochloride was supplied by the Hospital pharmacy, and morphine base pellets were prepared by Dr Giannini (Malesci S.p.A. Florence) according to Gibson & Tingstad (1970).

Nimodipine and Bay K 8644 were kindly supplied by Dr Hoffmeister (Bayer-AG, Wuppertal). Naloxone

was obtained from Crinos (Como, Italy).

[^{3}H]-NA was purchased from Amersham. Scintillation fluids were from Packard.

Results

The K^+ evoked tritium release

During K^+-stimulation the fractional release of ^{3}H from the slices increased approximately 15 times. H.p.l.c. purification showed that 80 ± 5% of the stimulation-induced tritium overflow and 62 ± 3% of the basal tritium outflow had a retention time identical to that of authentic [^{3}H]-NA. Part of the remaining 20% from the stimulated overflow and of the remaining 38% from the basal outflow were eluted with the solvent and some was lost in the alumina purification pathway. No differences were observed between the two halves of the frontal cortex.

Naloxone-induced [^{3}H]-NA release from brain slices of dependent animals

The release of [^{3}H]-NA significantly increased in slices taken from dependent animals and in which morphine was replaced by naloxone. Figure 1 shows that the effects of naloxone reached a maximum at 10^{-7}–10^{-6} M. These doses of naloxone did not affect [^{3}H]-NA overflow in non-dependent animals (data not presented).

A typical experiment showing the actions of naloxone (10^{-6} M) is presented in Figure 2. The figure shows that a small increase of ^{3}H-outflow also occurred

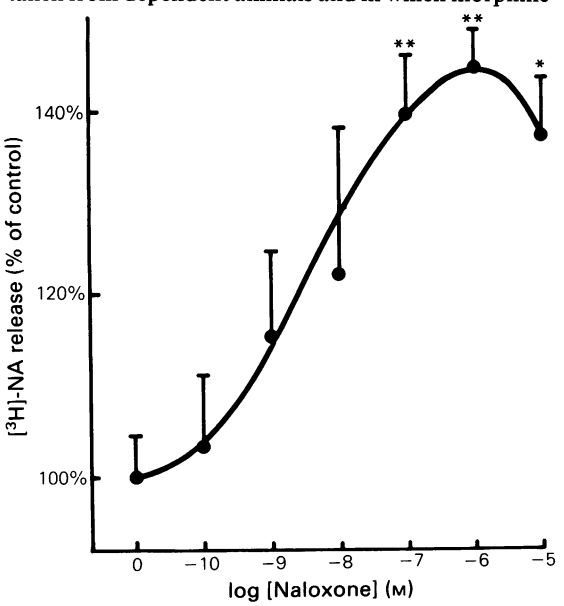

Figure 1 Dose-response curve for the effect of naloxone on [^{3}H]-noradrenaline (NA) release induced by K^+ (30 mM) from cortical slices of dependent rats. Four cortical slices originating from the left and right hemispheres were used and the effect of naloxone was evaluated by comparing the ^{3}H overflow in its presence with that of the corresponding half of the brain in which no withdrawal was challenged. Each point is the mean of at least 8 experiments. Vertical lines indicate s.e. $*P<0.01$, $**P<0.001$.

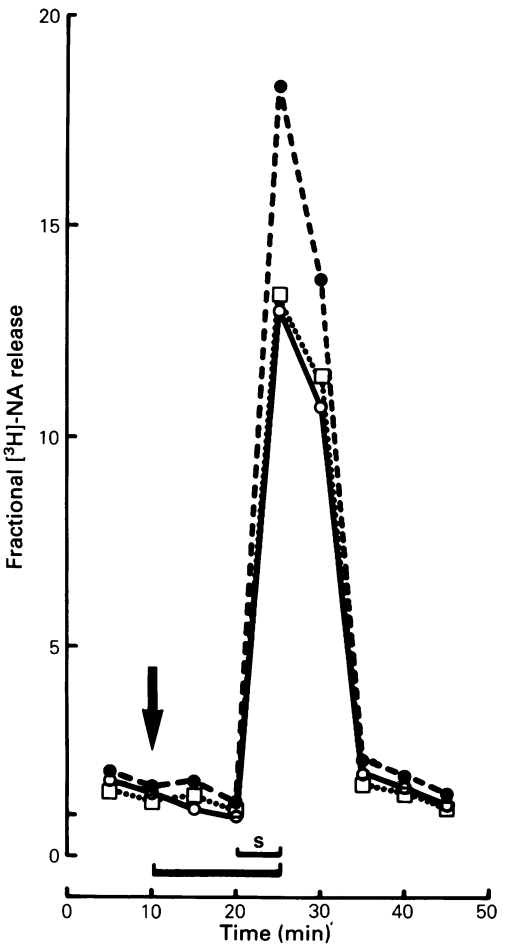

Figure 2 A typical experiment showing the effect of nimodipine on naloxone-evoked [^{3}H]-noradrenaline (NA) release. Three sets of four cortical slices were used. They were prepared from dependent animals. In one set of slices the withdrawal was challenged by naloxone (10^{-6} M) (●——●) and in another set it was challenged by naloxone (10^{-6} M) and nimodipine (10^{-6} M) (□ ···· □). In the control (○——○) set of slices no withdrawal was challenged. Naloxone or naloxone plus nimodipine was applied 10 min after the beginning of the collection of the samples (see arrow) and maintained for 15 min. Ten min after the beginning of the superfusion with naloxone an isotonic Ringer solution containing K^+ (30 mM) was applied (S).

immediately after the slices were superfused with naloxone containing Ringer solution, before the K^+-stimulation. This effect was constant but in terms of c.p.m. is relatively small and has not been studied further.

Effects of nimodipine on the naloxone-induced release of [^{3}H]-NA from brain slices of dependent animals

Figure 2 shows that 10^{-6} M nimodipine, added to the slices together with naloxone, completely prevented the effects of the narcotic antagonist.

Nimodipine actions were dose-dependent and started to be significant at 10^{-7} M, reached a maximum at 10^{-6} M and were variable at 10^{-5} M (Figure 3).

The K^+-induced fractional release of [^{3}H]-NA in slices taken from non-dependent animals was not affected by nimodipine 10^{-6} M (data not presented).

Effects of Bay K 8644 on the release of [^{3}H]-NA

Bay K 8644 increased in a dose-dependent manner the K^+ (30 mM)-induced overflow of NA in slices prepared from non-dependent animals, thus showing an action similar to that of naloxone in dependent rats.

Figure 4 shows that the drug was active at very low concentrations (10^{-9} M) and reached its maximum at 10^{-7}–10^{-6} M. When naloxone (10^{-6} M) plus Bay K 8644 (10^{-7} M) were applied to slices of dependent animals no further potentiation of release was observed.

Discussion

Naloxone (10^{-7}–10^{-5} M) significantly increased the K^+ (30 mM)-evoked release of [^{3}H]-NA from cortical slices obtained from the brain of morphine-dependent rats. This increased release did not occur in slices prepared from naïve animals and it may therefore be considered a withdrawal correlated effect.

During morphine abstinence an increased release and turnover of transmitters has been previously shown both *in vivo* and *in vitro* (Jhamandas & Sutak, 1974; Collier, 1980; Casamenti *et al.*, 1980; Bongianni *et al.*, 1986). Also, it has been repeatedly demonstrated that the administration of receptor antagonists or of drugs able to reduce the release of noradrenaline, dopamine, acetylcholine and 5-hydroxytryptamine from neurones, prevents the behavioural expression of several signs and symptoms of the syndrome (Tseng *et al.*, 1975; Crawley *et al.*, 1979; Collier, 1980; Redmond & Krystal, 1984; Neal & Sparber, 1986). It therefore seems possible that an increase of neurotransmitter release could explain most of the biochemical and behavioural manifestations of morphine abstinence. The increased release of transmitter we observed may be explained by supposing that adaptive changes (namely an increase) of the neuronal permeability to

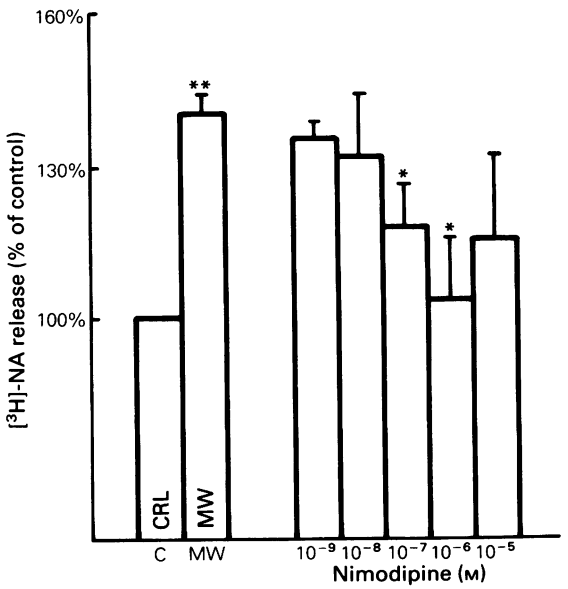

Figure 3 Effects of different concentrations of nimodipine on [^{3}H]-noradrenaline (NA) overflow evoked by naloxone in slices of dependent rats. The experimental procedure was the same as that of Figure 2. Each column is the mean of at least 7 experiments. Vertical lines indicate s.e. $*P<0.01$, compared to naloxone-induced release (MW). $**P<0.001$, compared to control release (C).

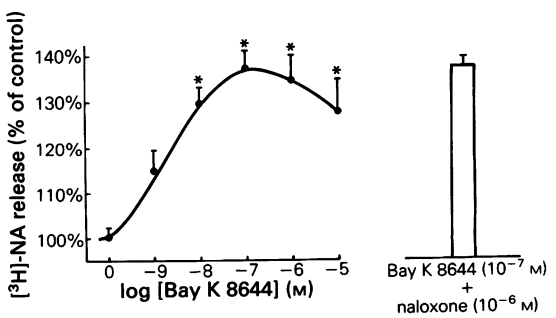

Figure 4 Effects of Bay K 8644 on the [^{3}H]-noradrenaline (NA) overflow. The actions of different doses of Bay K 8644 were evaluated in slices taken from non-dependent animals. Each point is the mean of at least 5 experiments; vertical lines indicate s.e. The column on the right was obtained using slices prepared from dependent rats ($n = 4$) (see Figure 2 for details). $*P<0.01$.

Ca^{2+} occur during the development of tolerance and dependence.

These changes seem to allow the neurones to deal with most of the Ca^{2+}-dependent process in spite of the presence of opioids. Such concepts are supported by the observations that the synaptosomal Ca^{2+}-content largely increases in the brain of dependent rats (Yamamoto *et al.*, 1978), that the number of voltage operated Ca^{2+}-channels, as measured by the [^{3}H]-nitrendipine binding technique (Turner & Goldin, 1985), is increased in the brain of dependent mice (Ramkumar & El-Fakahany, 1984) and that appropriate doses of Ca^{2+}-channel inhibitors suppress both the behavioural signs and the increased NA turnover associated with abstinence (Bongianni *et al.*, 1986). The data presented here further support this concept and extend it to *in vitro* models. In fact, nimodipine dose-dependently reversed the withdrawal-induced potentiation of [^{3}H]-NA release without affecting the K^+-evoked release from control slices. The last observation confirms previous results suggesting that dihydropyridine Ca^{2+}-channel blockers do not inhibit the stimulation-evoked transmitter release when tested at concentrations of 10^{-8}–10^{-6} M (Starke *et al.*, 1984; Godfraind *et al.*, 1986).

Thus it appears that dihydropyridine Ca^{2+}-entry inhibitors are not able to inhibit neurotransmitter release from neuronal tissue under basal situations, but become active when the transmitter release is triggered by an 'activated' state of the Ca^{2+}-channels. This particular state may be obtained during morphine or ethanol abstinence (data presented here; Little *et al.*, 1986) or in the presence of drugs such as Bay K 8644 (Middlemiss & Spedding, 1985). Interestingly enough, in our experimental conditions Bay K 8644 increased the [^{3}H]-NA release from control slices to an extent very similar to that of naloxone (10^{-7} M) from dependent slices. The actions of Bay K 8644 were not additive.

Our interpretation of the *in vivo* and *in vitro* data raises questions on the possible biochemical mechanisms occurring in neuronal membranes during chronic exposure to morphine, which lead to 'activation' of some kind of dihydropyridine sensitive Ca^{2+}-channels.

Recently, it has been shown that it is possible to 'recruit' new Ca^{2+}-channels by activating protein kinase C (Strong *et al.*, 1987). It has been suggested that these 'recruited' channels are dihydropyridine sensitive. An activation of protein kinase C results in an increased Ca^{2+}-entry and transmitter release in several neuronal preparations (Zurgil *et al.*, 1986; Nichols *et al.*, 1987). During the induction of morphine tolerance, changes of the function of protein-kinase C could occur which could result in changes of membrane permeability to calcium. Preliminary experiments from our group support these concepts (Moroni *et al.*, unpublished results).

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