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Cortical acetylcholine release is increased and γ-aminobutyric acid outflow is reduced during morphine withdrawal

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1 The effects of naloxone on acetylcholine (ACh) and γ-aminobutyric acid (GABA) outflow from the cerebral cortex of freely moving, morphine-dependent guinea-pigs was studied.

2 The cortical efflux of ACh in chronically-treated guinea-pigs was about half of that of normal animals. GABA efflux was unaffected.

3 During opioid withdrawal precipitated by naloxone (0.1–10 mg kg^{-1}, i.p.) the guinea-pigs showed jumping, hyperactivity and wet dog shakes, the intensity of which was directly related to the dose of naloxone. The withdrawal syndrome was accompanied by a dose-dependent increase in ACh release and reduction in GABA outflow; ACh release was increased by naloxone at doses lower (0.1–3 mg kg^{-1}) than those acting on GABA efflux (1–10 mg kg^{-1}). Atropine (10 mg kg^{-1}) and diazepam (5 mg kg^{-1}) did not prevent GABA and ACh changes.

Introduction

Intracerebroventricular injection of noradrenaline and electrical stimulation of locus coeruleus are known to cause sedation, decrease the release of acetylcholine (ACh) and increase the outflow of γ-aminobutyric acid (GABA) from the parietal cortex of normal, freely moving guinea-pigs (Beani *et al.*, 1978; Bianchi *et al.*, 1979; Moroni *et al.*, 1982).

During morphine withdrawal, the firing rate of locus coeruleus neurones and the turnover rate of noradrenaline are consistently increased (Redmond & Krystal, 1984). It has been suggested that these changes play an important role in the development of opioid withdrawal, thus supporting the use of clonidine, an α_2-adrenoceptor agonist which inhibits locus coeruleus cells (Aghajanian, 1978; Cedarbaum & Aghajanian, 1978), in the treatment of heroin addicts (Gold *et al.*, 1978).

Since the cortical release of ACh and GABA are controlled in part by the noradrenergic system, they would be expected to change during opioid withdrawal in a manner similar to that observed during locus coeruleus stimulation. However, some reports argue against this view. First, cortical ACh release has repeatedly been found to increase, rather than decrease, during naloxone-precipitated morphine abstinence in the rat (Jhamandas & Sutak, 1974; Casamenti *et al.*, 1980; Crossland & Ahmed, 1984). Second, activation of locus coeruleus neurones seems not to be important for the expression of opioid withdrawal, because the behavioural signs of abstinence are the same in normal rats and in those having lesions of the dorsal noradrenergic ascending bundle (Britton *et al.*, 1984).

No investigation has been reported hitherto concerning the changes in cortical GABA outflow during morphine tolerance and withdrawal. Thus, we examined in parallel the pattern of ACh and GABA release from the cortex of freely moving guinea-pigs, made tolerant to morphine and treated with naloxone. This approach may clarify the effects exerted by opioid abstinence on GABA, and may help to differentiate better between the neurochemical signs of morphine withdrawal and those of locus coeruleus stimulation. A preliminary report of this investigation has recently been presented (Antonelli *et al.*, 1985).

Methods

Guinea-pigs of 400–500 g of either sex were used. Morphine pellets (75 mg each; Gibson & Tingstad, 1970) were implanted subcutaneously according to the following schedule: 1 pellet on the first day and two pellets on the fourth day.

[1] Correspondence.

Behavioural studies

One group of animals was used to quantify the behavioural signs of naloxone-precipitated withdrawal. The seventh day after the first pellet implantation, the guinea-pigs were placed in individual rectangular plastic cages without water and food and their behaviour was examined from 20 min before to 80 min after treatment with naloxone (0.1, 0.3, 1, 3, 10 mg kg^{-1} i.p.). The evaluation of withdrawal was performed according to Bläsig *et al.* (1973) by scoring more than one objective sign, that is: locomotion, jumping and wet dog shakes. Other somatic and autonomic signs were also present, but they could not be easily quantified in this animal species.

Release experiments

On the fifth day other animals were implanted with an epidural cup on the left or right parietal bone (Beani *et al.*, 1978). On the seventh day, half of the guinea-pigs were used to study the efflux of ACh. Physostigmine (0.3 mM) was added to the saline solution (0.25 ml) placed in the cup and renewed every 30 min. Its ACh content was determined by bioassay on tetrodotoxin-pretreated guinea-pig ileum (Beani *et al.*, 1978). The other half of the animals was used to study the efflux of GABA. In order to inhibit GABA metabolism, ethanolamine-*O*-sulphate 2 mM was added to the saline solution in the cup (Moroni *et al.*, 1982) and renewed every 30 min, as it was for the ACh determination.

The GABA content of the samples was measured by mass-fragmentographic analysis as previously described (Moroni *et al.*, 1983). The withdrawal was induced by injecting naloxone at different doses i.p. and the changes in release were measured over the following 2 h, at 30 min intervals.

Drugs

Freshly prepared solutions of the following compounds were used: noradrenaline hydrochloride, acetylcholine chloride and physostigmine sulphate (Sigma Chemicals Co.); atropine sulphate (Merck); diazepam (Roche); GABA (Calbiochem); GABAd (Thor Isotopes); naloxone hydrochloride and morphine hydrochloride (Salars, Italy); tetrodotoxin (Sankyo, Japan).

Statistical analysis

The differences in ACh and GABA release between groups or between pre- and post-treatment values were determined by analysis of variance and Dunnet's *t* test (Winer, 1971).

Results

Behavioural signs of chronic treatment with morphine and withdrawal

Morphine-tolerant guinea-pigs not receiving cup implantation appeared sedated and hyporeactive, and lost about 10% of their initial weight in 7 days. Naloxone-precipitated abstinence began 5–10 min after the antagonist injection and lasted for more than 1 h. The guinea-pigs exhibited hyperactivity, jumping and wet dog shakes in direct relation to naloxone dose, from 0.3 to 10 mg kg^{-1}. Doses of 0.1 mg kg^{-1} were ineffective.

Abstinence was similar in the animals with cups containing saline solution plus ethanolamine-*O*-sulphate. In contrast, immediately after naloxone, those guinea-pigs with cups containing the physostigmine solution frequently showed clonic movements of the neck and of the forelimb contralateral to the cup. In some cases, generalized convulsions developed.

Acetylcholine release

The ACh release measured in dependent guinea-pigs was approximately half that recorded in normal guinea-pigs run in parallel (normal guinea-pigs: 104 ± 9.9 pmol cm^{-2} 30 min^{-1}; tolerant guinea-pigs 47.9 ± 3.04 pmol cm^{-2} 30 min^{-1}, mean ± s.e., $n = 10$).

The decrease in release was similar to that produced by 5 mg kg^{-1} (i.p.) of morphine in naive guinea-pigs (Figure 1). Naloxone, by itself ineffective in normal animals up to 10 mg kg^{-1}, increased ACh release in a log dose-dependent manner (0.1–3 mg kg^{-1}) (Table 1). A naloxone dose of 3 mg kg^{-1} was maximal, since it caused the ACh release to increase to the same extent as did 10 mg kg^{-1}. The maximum increase in release after 3 mg kg^{-1} was +167% of the value before naloxone and only +4% of the control values. The antagonist effect disappeared within 2 h. The animals showing localized, moderate clonic movements after naloxone (30% of the cases) displayed the same increase in ACh release as those without any motor signs; therefore, their release values were included in the calculated means in Table 1.

The data from guinea-pigs showing generalized seizures (20% of the animals) were discarded, because the increase in ACh efflux was up to 3–4 times the control values.

GABA release

Morphine-tolerance and abstinence affected GABA release differently from ACh. First, GABA outflow was similar in normal and morphine-treated animals (normal guinea-pigs 1228 ± 164 pmol cm^{-2} 30 min^{-1}; morphine-treated guinea-pigs 1251 ± 112, mean ± s.e.

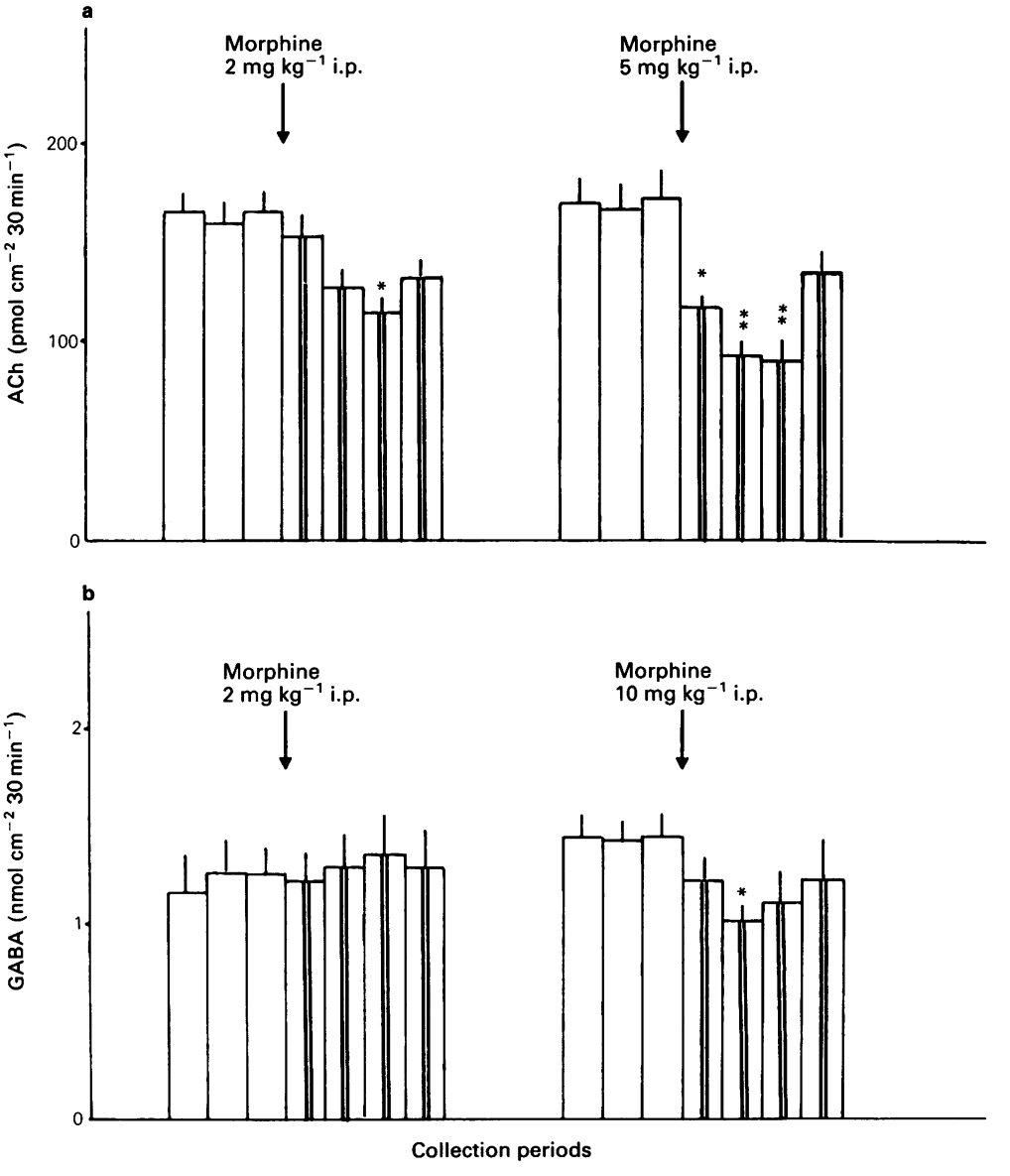

Figure 1 Effect of morphine on (a) acetylcholine (ACh, pmol cm^{-2} 30 min^{-1}) and (b) γ-aminobutyric acid (GABA, nmol cm^{-2} 30 min^{-1}) release from cerebral cortex of normal freely moving guinea-pigs. The drug was given at the arrow. The values are the mean of 5 experiments with s.e. shown by vertical lines. Significantly different from control: $^{*}P < 0.05$; $^{**}P < 0.01$.

Table 1 Effect of naloxone (injected i.p.) on ACh release (pmol cm^{-2} $30\,min^{-1}$ ± s.e.mean) from cerebral cortex of normal and tolerant guinea-pigs. The percentage with respect to the first collection period is shown in parentheses

		Collection periods					
		Before treatment		*After treatment*			
Treatment	n	1	2	3	4	5	6
Normal guinea-pigs							
Naloxone 10 mg kg^{-1}	5	103 ± 10	102 ± 9.2	111.6 ± 10.5 (107%)	106 ± 8.5 (102%)	108 ± 9.5 (104%)	96.6 ± 7.8 (94%)
Dependent guinea-pigs							
Naloxone 0.1 mg kg^{-1}	5	55.3 ± 5.03	56.3 ± 5.08	80.0 ± 4.3* (150%)	76.0 ± 3.2* (143%)	50.6 ± 12*	56.0 ± 15
0.3 mg kg^{-1}	5	52.4 ± 8.8	53.3 ± 8.0	97.2 ± 17* (185%)	70.9 ± 16 (135%)	55.2 ± 9.6	54.4 ± 7.1
1 mg kg^{-1}	5	53.1 ± 4.0	54.1 ± 3.2	144.0 ± 29** (260%)	92.2 ± 8.4** (174%)	58.5 ± 5.0	51.3 ± 1.9
3 mg kg^{-1}	5	41.0 ± 11	39.7 ± 9.9	109.3 ± 27** (267%)	100.5 ± 13.8** (245%)	82.8 ± 15.4** (202%)	64.0 ± 10.4* (172%)
10 mg kg^{-1}	5	44.2 ± 8.8	44.2 ± 7.7	102.7 ± 19** (232%)	120.5 ± 18.7** (283%)	102.7 ± 18.7** (232%)	84.5 ± 14.9* (191%)
Naloxone 10 mg kg^{-1} 1 h after diazepam 5 mg kg^{-1} i.p.	5	47.0 ± 4.5	47.0 ± 4.0	107.0 ± 13** (227%)	127.0 ± 12** (270%)	105.0 ± 18** (223%)	90.5 ± 12** (192%)

Naloxone was given between the second and third collection period.
Significantly different from 1st collection period: *$P < 0.05$: **$P < 0.01$.

Table 2 Effect of naloxone (injected i.p.) on GABA outflow (pmol cm^{-2} 30 min^{-1} ± s.e.mean) from cerebral cortex of normal and tolerant guinea-pigs. The percentage with respect to the first collection (control) is shown in parentheses

		Collection periods				
		Before treatment		*After treatment*		
Treatment	n	1	2	3	4	5
Normal guinea-pigs						
Naloxone 10 mg kg^{-1} i.p.	5	1238 ± 151	1164 ± 112 (94%)	1191 ± 87 (98%)	1158 ± 105 (94%)	1226 ± 95 (99%)
Dependent guinea-pigs						
Naloxone 1 mg kg^{-1} i.p.	5	1101 ± 57	1038 ± 42 (94%)	980 ± 124 (89%)	1027 ± 105	1050 ± 76
3 mg kg^{-1} i.p.	5	1078 ± 119	863 ± 104** (80%)	855 ± 78** (79%)	856 ± 68* (79%)	958 ± 105
10 mg kg^{-1} i.p.	5	1406 ± 103	1065 ± 117** (75%)	903 ± 103** (63%)	909 ± 139** (63%)	1022 ± 220* (71%)
Naloxone 10 mg kg^{-1} i.p. 1 h after atropine 10 mg kg^{-1} i.p.	5	1075 ± 127	859 ± 169* (79%)	709 ± 118** (66%)	727 ± 109** (67%)	980 ± 102

Naloxone was given between the first and second collection period.
Significantly different from the first collection period: *$P < 0.05$; **$P < 0.01$.

Table 3 Effect of naloxone (injected i.p.) on GABA outflow (pmol cm^{-2} 30 min^{-1} ± s.e.mean) from cerebral cortex of morphine-dependent guinea-pigs (ethanolamine-*O*-sulphate was omitted from the Ringer solution placed in the cup)

		Collection periods				
		Before treatment		*After treatment*		
Treatment	n	1	2	3	4	5
No treatment	5	654 ± 117	621 ± 48	559 ± 86	558 ± 55	556 ± 53
Naloxone 3 mg kg^{-1}	6	602 ± 121	550 ± 128	520 ± 102	529 ± 127	529 ± 106

Naloxone was given between the first and second collection period.

of 25 animals). This was in agreement with the finding that only at the high dose of 10 mg kg^{-1} was morphine able to reduce GABA outflow in naive guinea-pigs, whereas it reduced ACh release at 2 mg kg^{-1} (Figure 1). Naloxone, by itself ineffective in normal animals up to 10 mg kg^{-1}, hardly affected GABA outflow at 1 mg kg^{-1} in morphine-treated animals and exhibited maximal activity only at 10 mg kg^{-1} (Table 2). When ethanolamine-*O*-sulphate was omitted from the saline solution the amount of GABA recovered was about half of that found in the presence of ethanolamine-*O*-sulphate and was hardly affected during naloxone-precipitated withdrawal (Tables 2 and 3).

Sodium-dependence of acetylcholine and GABA release

From the above findings it is apparent that the GABA efflux was less sensitive than ACh release during naloxone-induced withdrawal. This difference could depend on the extraneuronal origin of GABA recovered in the cup. To check this point, ACh and GABA were measured before and after tetrodotoxin 0.5 μM had been added to the saline solution in the cup.

Ninety min after tetrodotoxin application, ACh release in normal, untreated animals was reduced to 15–25% of the initial level. On the other hand, GABA outflow was reduced to 50%, provided that ethan-

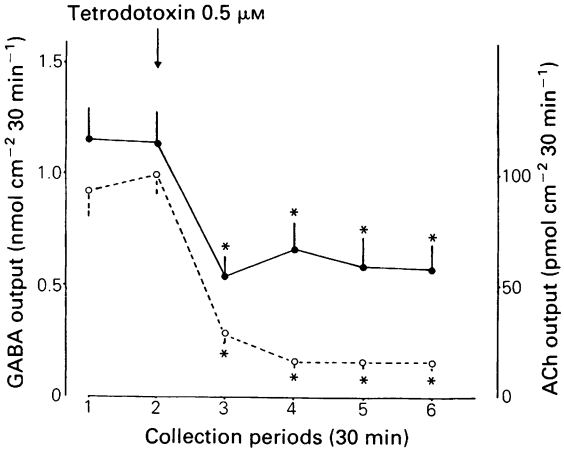

Figure 2 Effect of topical application of tetrodotoxin 0.5 μM (arrow) on γ-aminobutyric acid (GABA ●) and acetylcholine (ACh, ★) release from the cerebral cortex of freely moving guinea-pigs. The values are the mean of 5 experiments; with s.e. shown by vertical lines. Significantly different from control periods: $*P < 0.01$.

olamine-*O*-sulphate was also present (Figure 2). In the absence of ethanolamine-*O*-sulphate, tetrodotoxin (TTX) inhibited GABA outflow by only 25% (before TTX 630 ± 42, after TTX 432 ± 57 pmol cm^{-2} 30 min^{-1}, mean ± s.e. of 6 experiments).

Thus, it can be inferred that 80–85% of the cortical ACh release of normal, freely moving guinea-pigs can be ascribed to a sodium-dependent mechanism. In contrast, about 50% of the cortical GABA outflow appears to depend on the activity of the GABAergic neurones, the remainder probably being of glial origin.

Naloxone-induced acetylcholine and GABA changes are unaffected by diazepam and atropine pretreatment.

With the aim of ascertaining whether the increase of ACh release could in part depend on the reduction of GABA outflow, some morphine-treated animals were injected with diazepam (5 mg kg^{-1}, i.p.) 1 h before adminstration of naloxone 10 mg kg^{-1}. Diazepam reduced the cortical release of ACh to a smaller extent (from 58 ± 6 to 47 ± 4.5 pmol cm^{-2} 30 min^{-1} ± s.e., see Table 1) than in naive animals (Tanganelli *et al.*, 1985) but it did not prevent the increase of release caused by naloxone (Table 1). Diazepam reduced the withdrawal signs and in no instance did diazepam-treated animals show signs of localized or generalized seizures. In order to check whether the decrease in GABA efflux during withdrawal was a consequence of the increased cholinergic activity (Reiffenstein, 1979) the muscarinic antagonist atropine (10 mg kg^{-1}, i.p.) was administered 1 h before naloxone, 10 mg kg^{-1} i.p.

As shown in Table 2, GABA efflux was not affected by atropine, and it was decreased by naloxone as much as in the animals not pretreated with atropine. In addition, the withdrawal signs were unaffected by atropine.

Discussion

In morphine-dependent guinea-pigs, abstinence signs (hyperactivity, jumping, wet dog shakes) were well correlated with the naloxone doses in the range between 0.3 and 10 mg kg^{-1}. At the same time, the opioid antagonist increased ACh release and decreased GABA efflux (Tables 1 and 2). The enhancement of ACh release confirms previous reports in anaesthetized rats (Jhamandas & Sutak, 1974; Crossland & Ahmed, 1984) and conscious rats (Casamenti *et al.*, 1980). At variance with this last report, we did find the basal ACh efflux of dependent guinea-pigs significantly lower than in normal animals. Thus, the guinea-pig seems to differ from the rat in that the corticipetal cholinergic structures do not easily exhibit tolerance towards morphine. Differences in animal species and/or in treatment duration may account for this discrepancy. In any case, since the amount of ACh release in dependent animals was nearly halved, the actual effect elicited by naloxone was chiefly restricted to the reversal of opioid inhibition: naloxone 0.1–1 mg kg^{-1} tended to return the ACh release to normal levels and only at 3 mg kg^{-1} did naloxone slightly enhance the outflow above the control values (plus 4%). Diazepam did not change this response, indicating that the enhancement of GABA control of the cholinergic neurones did not prevent their response to naloxone.

Naloxone appreciably affected ACh release even at 0.1 mg kg^{-1}, whereas this drug induced detectable behavioural signs only at 0.3 mg kg^{-1}. In other words, the neurochemical variable was more sensitive to naloxone than the behavioural measures used.

The animals employed to study ACh release often showed localized clonic movements a few minutes after naloxone. In some instances the development of generalized seizures led us to exclude the animals from the study. The naloxone-precipitated abstinence presumably unmasked the latent hyperexcitability of the cortical areas in contact with the solution containing physostigmine in the cup (Bianchi & Beani, 1985).

The increase in ACh release following naloxone cannot be considered the only reason for the seizures. In fact, more consistent enhancement of corticipetal cholinergic transmission has been induced, for example by amphetamine or amantadine without leading to convulsions (Beani & Bianchi, 1973). Clearly, other

factors, intimately linked to the opioid withdrawal, must be involved.

Increased excitability of the central nervous system in the course of morphine withdrawal has been repeatedly reported (Freedman & Aghajanian, 1985). The neurochemical basis of this hyperexcitability can, at least partly, be related to the decreased availability of synaptic GABA. This change could enhance the facilitation of seizures determined by the local application of physostigmine. In support of this, guinea-pigs pretreated with diazepam did not exhibit any sign of convulsions after naloxone.

Since little is known about the influence of morphine on cortical GABA release, the following discussion is focused on this point. First, the local treatment with tetrodotoxin demonstrated that only about half of GABA diffusing into the cup came from the GABAergic neurones (providing that ethanolamine-*O*-sulphate was also present). Therefore, a decrease in total GABA release of 30% of the initial value after naloxone 10 mg kg^{-1} (see Table 2) corresponded to an actual decrease of the neuronal GABA of 60% with respect to pre-drug conditions. The tetrodotoxin- (and naloxone-) sensitive component of GABA efflux became evident only when the GABA-transaminases were inhibited by ethanolamine-*O*-sulphate. This suggests that the GABAergic synapses are surrounded by very efficient mechanisms of reuptake and metabolism (Iversen & Kelly, 1975; Bianchi *et al.*, 1982), which impede the free diffusion of the amino acid from the sites of its release.

The present data however do not allow us to specify the reason(s) for the decreased amino acid outflow during morphine withdrawal.

Clearly, the inhibition of GABA release cannot depend on the suppression of an opioid-mediated facilitation. In fact, chronic morphine-treatment does not change the basal GABA efflux and only at a relatively high dosage (10 mg kg^{-1}) does the opioid decrease GABA efflux in naive animals. Similarly, the observed reduction of GABA release cannot be related to the enhancement of cholinergic signals reaching the cerebral cortex. The possible control exerted by ACh through muscarinic receptors on the GABA structures has been stressed by some workers (Benjamin & Quastel, 1977; Reiffenstein, 1979; Coundray-Lucas *et al.*, 1984) and denied by others (Racagni *et al.*, 1978).

From our data the following may be inferred: (1) the cholinergic cells were more responsive to naloxone that the GABA neurones in morphine-dependent guinea-pigs. In fact, the threshold and the maximal doses of naloxone required to change ACh and GABA release were different (See Tables 1 and 2). Thus, the two changes do not run in parallel. (2) Atropine pretreatment did not appreciably affect basal GABA release and did not affect the reduction of GABA caused by naloxone. Therefore, a consistent muscarinic control of GABA cells in tolerant animals as well as a muscarinic step in decreasing the amino acid efflux during withdrawal can be ruled out.

The decrease in GABA efflux cannot even be attributed to the enhanced activity of the noradrenergic neurones since noradrenaline increases GABA release both *in vivo* and *in vitro*, at least in normal guinea-pigs (Moroni *et al.*, 1982; Beani *et al.*, 1986). Thus, further research is needed to explain the reduction of GABA outflow.

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