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PHARMACOLOGICAL CHARACTERIZATION OF OPIATE PHYSICAL DEPENDENCE IN THE ISOLATED ILEUM OF THE GUINEA-PIG

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1 Physical dependence was produced in ilea from naive guinea-pigs by exposure of the tissues to different opiates for logarithmically-spaced periods of time (20–320 min). The responsiveness of the tissue to naloxone, as indicated by a strong contracture of the ileum, was enhanced in contrast to that found in intestines not exposed to opiates.

2 The dose-response curves to naloxone obtained in tissues individually exposed to different opiates showed that their relative potency in increasing sensitivity to naloxone was as follows: levorphan > morphine > Met-enkephalin > nalorphine > pentazocine.

3 The naloxone-induced response was dose-dependent and was directly related to the opiate concentration and length of exposure.

4 Dextrorphan, the inactive isomer of levorphan, did not increase the responsiveness of the tissues to the narcotic antagonist, indicating that the phenomenon is stereospecific.

5 The naloxone-induced contraction in ilea exposed for 320 min to morphine (1×10^{-6} M) was not prevented or suppressed by the administration of a large dose of morphine (1×10^{-5} M) before or immediately after the naloxone challenge.

6 The evidence presented here shows that a phenomenon resembling *in vivo* opiate physical dependence can be acutely produced *in vitro* with pharmacological characteristics similar to other naloxone-induced abstinence effects.

Introduction

The main problem associated with the chronic use of opiates is the development of tolerance and dependence. A great deal of effort has been expended to find animal models in which the addictive properties of narcotic drugs can be reproduced, predicted and studied (Seevers, 1936; Halbach & Eddy, 1963; Martin, 1967; Villarreal, 1973). However, the complexity of the existent models has led to the search for less involved systems.

One of the models widely used to study the acute effects of narcotics is the isolated ileum of the guinea-pig (Paton, 1957; Cox & Weinstock, 1966; Kosterlitz & Waterfield, 1975). The narcotic 'receptors' of the myenteric plexus show characteristics similar to those in the central nervous system (Cox & Weinstock, 1966; Harris, Dewey, Howes, Kennedy & Pars, 1969; Kosterlitz & Waterfield, 1975). There is strong evidence that the binding 'sites' for narcotics in the guinea-pig ileum are pharmacologically relevant (Creese & Snyder, 1975), suggesting that this tissue might be suitable for the study of the chronic effects of narcotic drugs. Recently, it has been shown that the prolonged exposure of ilea to narcotics *in vitro* and *in vivo* produces tolerance, defined as a shift to the right in the dose-response curve for morphine (Goldstein & Schulz, 1973; Shoham & Weinstock, 1974; Opmeer & van Ree, 1978). Furthermore, manifestations of physical dependence to morphine have been observed in this preparation, as indicated by a strong contracture upon the addition of naloxone to intestines obtained from animals chronically exposed to morphine (Ehrenpreis, Light & Schonbuch, 1972; Frederickson, Hewes & Aiken, 1976; Schulz & Herz, 1976a; Rodríguez, Luján, Campos & Chorné, 1978) or to intestines obtained from naive animals exposed to opiates *in vitro* (Villarreal, Martínez & Castro, 1977, Luján & Rodríguez, 1978).

The purpose of the present investigation was to determine the pharmacological characteristics of opiate physical dependence *in vitro* by the use of a new approach that would allow the rapid production and manipulation of physical dependence and to study whether such characteristics are analogous to those found in other tests for narcotic addiction. A preliminary account of this work has been given at the annual meeting of the American Society of Pharmacology and Experimental Therapeutics (Luján & Rodríguez, 1978).

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Methods

Preparation

Male guinea-pigs (300–500 g) that had been fasted overnight were killed by a blow on the head. The terminal portion of the ileum was removed and the 10 cm nearest to the caecum was discarded. The intestine was placed in a petri dish with Krebs solution (NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaH_2PO_4.H_2O$ 1.2, $NaHCO_3$ 25 with glucose 11, and choline chloride 0.929 mM) at room temperature (20–25°C) and was cut into small (2–3 cm long) pieces. The intestines were then gently but thoroughly washed free of faecal matter by flushing Krebs solution through the lumen. Each segment was set up in a 10 ml organ bath containing Krebs solution and bubbled with 95% O_2 and 5% CO_2. The temperature of the bath was maintained at 37°C by means of an external jacket through which warm water was circulate.

Before the administration of any drug, the tissues were fixed at a resting tension of 1 g and allowed to equilibrate for 40 min. They were constantly perfused (10 ml/min) with warm Krebs solution that was kept in a reservoir and continuously bubbled with 95% O_2 and 5% CO_2 to maintain the pH at 7.4. The spontaneous activity of the ileum was recorded isometrically with a FT 03 Grass force transducer connected to a 7D Grass polygraph, calibrated before every experiment so that a pen displacement of 1 cm was equal to 1 g.

Perfusion of narcotic drugs

Dependence was produced by the exposure of the tissue to one of the opiates which was added to the Krebs solution reservoir at various concentrations. The Krebs solution containing the narcotic was warmed (37°C) and perfused to the tissue at a flow rate of 10 ml/min during logarithmically-spaced periods of time (20–320 min). When the predetermined period had elapsed, the perfusion was stopped and naloxone was added directly to the organ bath in 100 μl of distilled water. The contractile response induced by naloxone was defined as the peak tension observed within 1 min after administration. Each segment was tested only once.

With the same procedure, it was possible to quantify the changes in tension produced by altering the concentration of the perfused opiates (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M) as well as the naloxone dose (3.84, 38.4, 384.0, 3840.0 $\times 10^{-9}$ M).

Drugs

All chemicals used to prepare the Krebs solution were of reagent grade. The following drugs were employed: morphine HCl (Merck), levorphan and dextrorphan tartrate (Hoffman-La Roche), naloxone HCl and naltrexone HCl (Endo), pentazocine (Winthrop), nalorphine HCl (Merck), and methionine-enkephalin (Met-enkephalin) (Sigma). All drugs were dissolved in distilled water with the exception of pentazocine which was dissolved in a minimal amount of 0.1 M HCl, partially neutralized with NaOH, and finally diluted to the desired volume with distilled water at pH 6.

Statistics

The parallel line assay of Finney was used to test the similarity of the curves and to determine the relative potency within 95% confidence limits. Analysis of variance was used when necessary.

Results

Naloxone-induced contraction in the guinea-pig isolated ileum

The addition of naloxone to intestines exposed to opiates for 320 min produced a dose-dependent response characterized by an immediate and strong contraction followed by a tonic contracture that lasted several minutes. In contrast, the administration of the antagonist to intestines not previously exposed to narcotics resulted in a small dose-dependent contraction of short duration (Figure 1). In both cases, the responses could be partially blocked by atropine (1×10^{-7} M).

Relative potency of the narcotic drugs in inducing dependence

The dose-response curves for naloxone in ilea exposed to opiates (1×10^{-6} M) for 320 min showed that the agonists levorphan, morphine and Met-enkephalin increased the responsiveness of the intestinal tissue to a higher degree than did nalorphine or pentazocine (Figure 2). The dose-response curves for naloxone in intestines exposed to morphine or levorphan (1×10^{-6} M) for 320 min was markedly shifted to the left of those obtained with ilea incubated with vehicle (1 ml saline/l Krebs solution) or dextrorphan (1×10^{-6} M). No quantification of the shift was made since the curves were not parallel: however, it could be estimated that intestines exposed to morphine or levorphan were about 10–100 times more sensitive to naloxone than those exposed to vehicle or dextrorphan. There was also a three to five fold increase in the maximal response to the antagonist in tissues treated with the two former drugs (Figure 2).

Since the dose-response curve for naloxone of ilea perfused with levorphan was shifted to the left of and

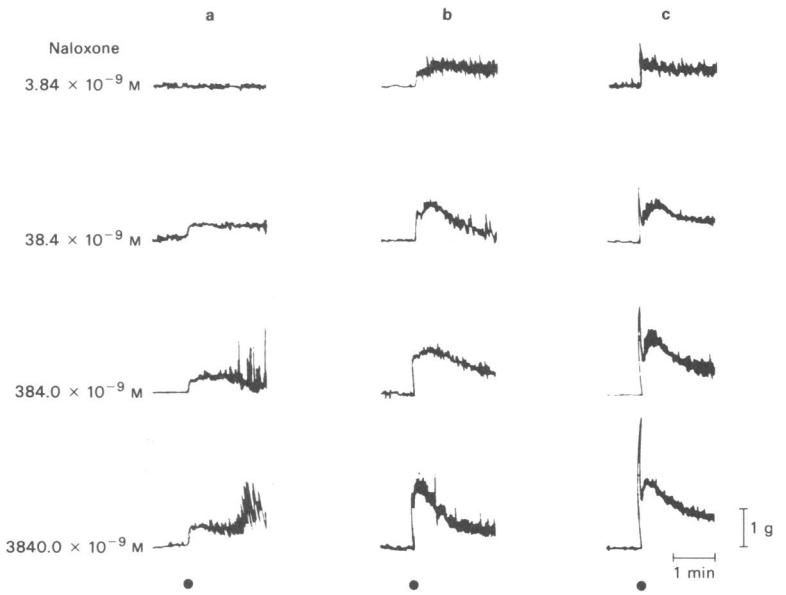

Figure 1 Nature of naloxone-induced contractions in the guinea-pig ileum. Traces show the contractions produced by naloxone (3.84–3840.0 × 10^{-9} M) in segments exposed to the vehicle alone (a), nalorphine (1 × 10^{-6} M, b), or morphine (1 × 10^{-6} M, c) for 320 min. The responses to naloxone in segments incubated with the opiate agonist morphine (c) are characterized by an immediate and intense contraction of the tissue, followed by a tonic contracture lasting for several minutes. The first component of this type of response is lacking in segments incubated with the agonist-antagonist nalorphine (b). Ilea not exposed to narcotics (a) also respond to naloxone but with a small contracture. In all cases, the response is clearly related to the antagonist dose. The dots represent the addition of naloxone to the bath at the final concentration indicated. Each segment was used for only one determination.

was parallel to the curve for morphine, a formal comparison of potency was carried out using factorial coefficients (Emmens, 1948). This procedure showed that levorphan was 3.4 (1.8–7.7 C.L.) times more potent than morphine. Met-enkephalin also sensitized the ileum to naloxone, but was 2.2 (0.95–5.2 C.L.) times less effective than morphine. The agonist-antagonist drugs sensitized the ileum to naloxone as well, although the contracture was of less intensity and the maximal response was small (ceiling effect) compared with the 'pure' agonist drugs. The pattern of contraction induced by naloxone in intestines incubated with nalorphine or pentazocine was somewhat different from that in ilea incubation with 'pure' agonist (Figure 1). It is important to point out that it was not possible to make a comparison of the potency of the 'pure' agonists and the mixed agonist-antagonist drugs because the condition for a valid bioassay (parallel dose-response curves) was not met; however, quantification of the potency of nalorphine and pentazocine revealed that the former was 4.0 (2.1–10.3 C.L.) times more potent than the latter.

The addition of naloxone to intestines not exposed to opiates produced a small but consistent dose-dependent contraction. Using a one-way analysis of variance, it was found that the responses induced by increasing doses of naloxone in untreated ilea were significantly different ($P < 0.01$). Additionally, Scheffé's test (Scheffé, 1953) revealed that the response of naive ilea to a dose of 3840 × 10^{-9} M was different from those obtained at 3.84 and 38.4 × 10^{-9} M ($P < 0.01$).

Stereospecificity of the phenomenon

Dextrorphan, the inactive isomer of levorphan, did not sensitized the ileum to naloxone; in fact, the responses to naloxone in intestines incubated with the former did not vary from those obtained in intestines not exposed to opiates (Figure 2).

In all cases, doses of naloxone higher than 3840 × 10^{-9} M did not produce a further increase in the magnitude of the contraction, and doses above 7680 × 10^{-9} M actually decreased the contraction. It is well known that suppression of the precipitated withdrawal of narcotic drugs is not easily accomplished. With this in mind, we decided to test whether high doses of morphine (1 × 10^{-5} M) could inhibit or suppress the contraction produced by naloxone (3840 × 10^{-9} M) in intestines previously perfused for 320 min with morphine (1 × 10^{-6} M). We found that under these conditions, the administration of morphine

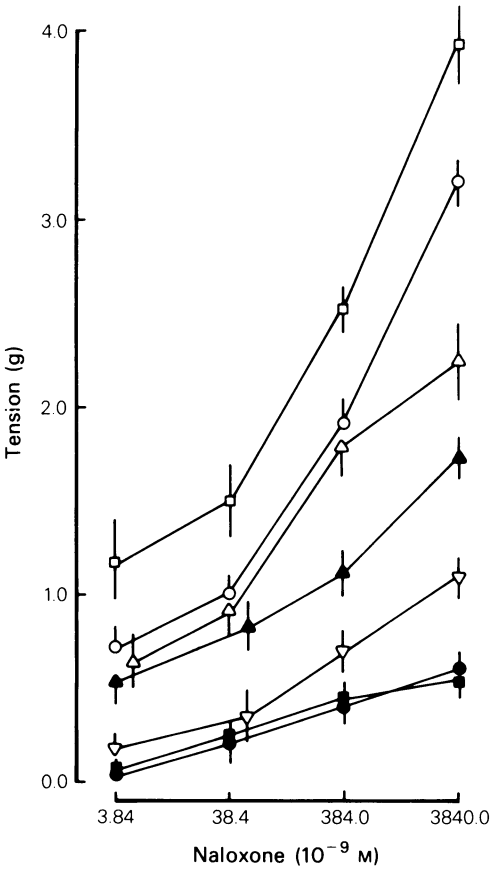

Figure 2 Narcotic dependence in the guinea-pig isolated ileum. Dose-response curves for naloxone-induced abstinence contractions are shown in ilea not exposed (●) or exposed for 320 min to different narcotics at a concentration of 1×10^{-6} M: (□) levorphan; (○) morphine; (△) Met-enkephalin; (▲) nalorphine; (▽) pentazocine; (■) dextorphan. Abscissa scale, concentration of naloxone; ordinary scale, response in g of tension. Each point is the mean of 10 tests carried out in segments obtained from different animals and used for only one determination; vertical lines show s.e. mean.

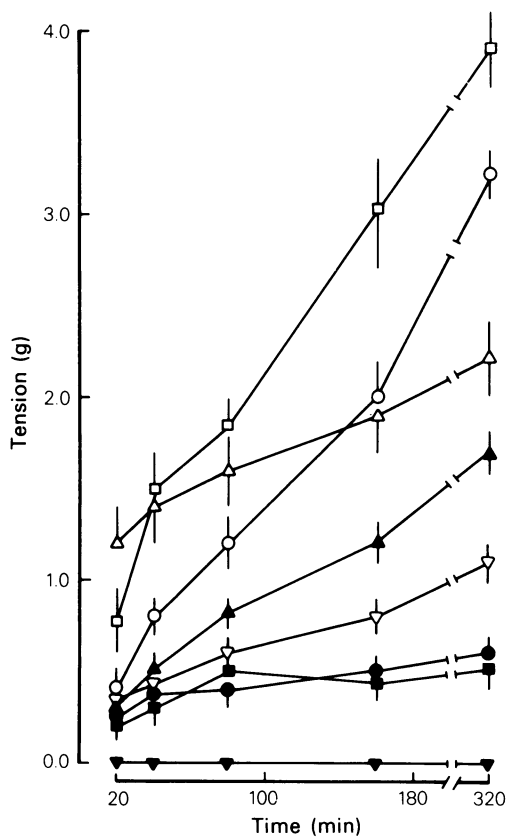

Figure 3 Influence of the length of exposure to narcotics (1×10^{-6} M) on the responsiveness of the guinea-pig isolated ileum to naloxone: (□) levorphan; (○) morphine; (△) Met-enkephalin; (▲) nalorphine; (▽) pentazocine; (●) vehicle; (■) dextrorphan; (▼) naltrexone. Challenge to naloxone (3840×10^{-9} M) was conducted 20–320 min after perfusion was begun. The mean values (n = 10 for each point) for the naloxone-induced contractions in g of tension are shown; vertical lines indicate s.e. mean.

before or immediately after naloxone challenge failed to block or suppress the contraction.

Effect of opiate dose and length of exposure on the responsiveness of naloxone in the guinea-pig ileum

By use of a dose of naloxone that produced a maximal response, it was possible to investigate the relationship between the length of exposure to an opiate and the magnitude of the contraction. In all cases, the response to naloxone was dependent on the length of opiate perfusion, reaching a peak at 320 min (Figure 3). Perfusion of the tissue with opiates for 640 min or more led to a diminution of the naloxone-induced contraction and of the response to acetylcholine, a finding which probably reflects tissue deterioration.

The naloxone-induced contraction was also dependent on the concentration of the narcotic that was perfused to the intestine (Figure 4). In general, the response to naloxone was proportional to the opiate dose, but concentrations higher than 1×10^{-6} M resulted in a reduction of the antagonist-induced contraction in all cases.

Discussion

For many years, it has been believed that the abstinence symptoms upon which the assessment of physical dependence is based were only present at a

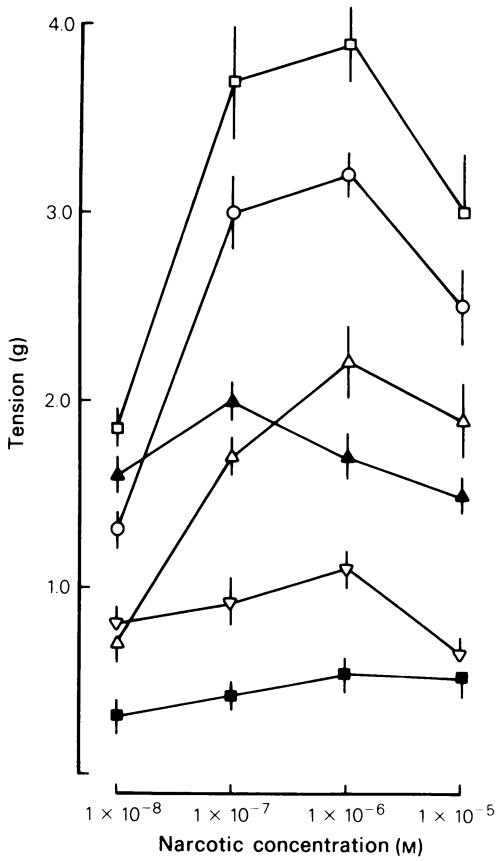

Figure 4 Influence of narcotic concentration on the responsiveness of the guinea-pig ileum to naloxone (3840.0×10^{-9} M). Mean values ± s.e. for the naloxone-induced contractions in g of tension obtained in ilea incubated for 320 min with opiates at concentrations ranging from 1×10^{-8} to 1×10^{-5} M are shown: (□) levorphan; (○) morphine; (△) Met-enkephalin; (▲) nalorphine; (▽) pentazocine; (■) dextrorphan.

high level of physiological organization (Halbach & Eddy, 1963). Therefore, the intact animal was used as a rule. However, the recent view that the neural elements of the myenteric plexus anatomically and neurochemically closely resemble those of the central nervous system (Gershon & Bursztajn, 1978), together with the discovery of opiate receptors in this tissue has supported the use of the guinea-pig isolated ileum as a model to test opiate physiological dependence.

Classically, the measurement of physical dependence has been based on the evocation of abstinence signs, either by abrupt drug withdrawal or, more recently, by the administraiton of a narcotic antagonist (Maggiolo & Huidobro, 1961; Way, Loh & Shen, 1969; Rodríguez & Villarreal, 1974; Bläsig & Herz, 1977). This last approach was undertaken following the demonstration that pretreatment of animals with narcotic analgesics enhanced naloxone potency (Way *et al.*, 1969), which suggested that the increase in sensitivity could be a measure of opiate physical dependence. Villarreal & Castro (1979) extended this concept and proposed that the shift to the left and the increase in the maxima of the dose-response curves for naloxone were a sensitive measure of the magnitude of physical dependence. By analogy, the naloxone-induced contractions of the guinea-pig ileum after continuous perfusion with opiates was considered to be a withdrawal sign, implying that the exposure of the tissue to narcotic drugs led to the development of opiate physical dependence. Moreover, we have found that the replacement of the morphine-containing perfusion fluid with another without the opiate was sufficient to produce a contracture of the intestines, although of minor intensity and short duration (not shown). Similar findings have recently been reported in intestines from guinea-pigs implanted with morphine pellets (North & Zieglgänsberger, 1978). However, in our hands only precipitated withdrawal could be easily quantified.

Villarreal *et al.* (1977) have described a procedure in which they produced opiate physical dependence *in vitro* by the incubation of segments of guinea-pig ileum with narcotic drugs for 24 h at a low temperature (2–4°C). However, they failed to produce physical dependence in intestines maintained at 37°C for prolonged periods and pointed out that this was probably due to acute deterioration of the tissue. This observation, contradictory to ours, might be explained by methodological differences.

The dose-response curves for naloxone in intestines maintained at 37°C and in continuous contact with opiates reveal the existence of a close relationship between the capacity of narcotic drugs to increase the responsiveness of the ileum to naloxone and the induction of physical dependence in animals and man. Those drugs with low dependence liability in monkeys and man (Martin & Gorodetzky, 1965; Jasinski, Martin & Haertzen, 1967; Jasinsik, Martin & Hoeldtke, 1970; Villarreal, 1973) such as nalorphine and pentazocine also produced a low degree of physical dependence in the isolated ileum as measured by the contracture produced upon naloxone challenge. This is in contrast with the 'pure' agonists morphine and levorphan which greatly increased the responsiveness to naloxone, a finding to be expected since these compounds readily induce dependence in intact organisms (Deneau, McCarthy & Seevers, 1970; Villarreal 1973).

Dextrorphan, the isomer of levorphan that does not induce or maintain opiate physical dependence (Villarreal, 1970; Villarreal *et al.*, 1977), did not increase the sensitivity of the ileum to naloxone, providing evidence of the stereospecificity of the

phenomenon in this preparation and fulfilling one of the conditions for a model designed to study opiate physical dependence. Furthermore, the development of this phenomenon was inhibited when morphine or levorphan (1×10^{-6} M) were administered together with naloxone (1×10^{-7} M), suggesting that since the agonist could not occupy the 'receptors', the mechanisms involved in the development of physical dependence could not be initiated. Similar results have been reported in mice (Yano & Takemori, 1977) and monkeys (Seevers & Deneau, 1963), and more recent studies confirm this view (Bhargava, 1978).

Methionine-enkephalin, an endogenous ligand for the opiate receptor, also sensitized the ileum to naloxone, a phenomenon which reflects its dependence liability properties. This finding confirms and extends the observation made by Schulz & Herz (1976b) that Met-enkephalin can replace morphine in intestinal 'strips'. Our study demonstrates that Met-enkephalin not only has the capacity to maintain opiate physical dependence, but also can induce it with a potency very similar to that of morphine, suggesting that this peptide can produce physical dependence in animals as has been shown by Bläsig & Herz (1976) and Wei & Loh (1976). Since Met-enkephalin, a normal constituent of the nervous system, is able to produce, maintain and reinduce physical dependence, it is posisble to postulate the existence of endogenous mechanisms that naturally generate and regulate this kind of phenomenon (Luján, Valencia-Flores & Rodíguez, 1980).

One interesting feature of the enkephalin-induced sensitization of the ileum to naloxone is its rapid onset as compared with the other opiates tested. Nevertheless, the maximal response to naloxone was reached at the same time by all the drugs, including Met-enkephalin. The significance of this phenomenon remains to be elucidated.

The finding that intestines not exposed to narcotics respond to naloxone with a small but consistent contracture confirms the results of Rodríguez *et al.* (1978) and contrasts markedly with those of other authors (Frederickson *et al.*, 1976; Schulz & Herz, 1976a; Villarreal *et al.*, 1977). This discrepancy might be due to methodological differences (Rodríguez *et al.*, 1978) or to a circannual rhythm in the response of the ileum to naloxone (Rodríguez, Luján & Vargas-Ortega, 1980). Naloxone-induced contractions in the naive guinea-pig ileum are not entirely unexpected since peptides with opioid-like action are physiological constituents of this tissue (Schulz, Wuster, Simantov, Snyder & Herz, 1977; Hughes, Kosterlitz & Sosa, 1978). Additionally, the incubation of intestines with naltrexone, considered to be a pure narcotic antagonist similar to naloxone (Blumberg & Dayton, 1973), blocks the subsequent response to naloxone in intestines not exposed to narcotic drugs. These findings suggest a possible role for the endorphins in the naloxone-induced contractions in naive intestines.

The administration of the same drug or another agonist to animals exhibiting abstinence signs after abrupt withdrawal from narcotics reduces the severity of, or entirely blocks the abstinence syndrome (Halbach & Eddy, 1963; Seevers & Deneau, 1963); however, in the precipitated abstinence, even large doses of narcotic agonists do not suppress this response (Cheney, Judson & Goldstein, 1972; Bläsig & Herz, 1977; Villarreal *et al.*, 1977). Using this approach, we found that the administration of high doses of opiate agonists (1×10^{-5} M) before or immediately after the contraction produced by naloxone in ilea exposed to narcotic drugs, did not suppress or prevent precipitated withdrawal. This confirms the results of Villarreal *et al.* (1977) and further supports the similarity of this test with others using intact organisms. The diminished degree of sensitization to naloxone resulting from very high doses of narcotic drugs is probably due to the self-antagonist properties of the opiates at high concentrations (Gyang & Kosterlitz, 1966; Dewey & Harris, 1971).

In the present experiments, the production of physical dependence *in vitro* follows a well-defined pattern: its development is related to the time that the tissues are in contact with the opiate, to the narcotic dose tested, and to the type of narcotic used. Furthermore, the phenomenon is prevented by opiate antagonists and is stereospecific. These characteristics are evidence of a clear quantitative and qualitative analogy between the phenomena observed in the guinea-pig isolated ileum and in intact animals. Also, they provide the first real support for the concept that shifts to the left with an increase in the maxima of the dose-response curves for naloxone constitute the most sensitive measure of the degree of physical dependence. The similarity of this *in vitro* model for the detection of opiate physical dependence with others in intact organisms supports its use in the study of: (a) the antagonist effects of narcotic drugs; (b) the production and maintenance of physical dependence by new narcotic drugs; and (c) the neurochemical changes that lead to the development and expression of opiate physical dependence.

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