Comparative Studies on the Inhibitory Effect of Morphine, Pentobarbital and Ethanol on the Electrically Evoked Contractions of Isolated Mouse Vas Deferrers

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To demonstrate the difference in the action mechanisms of morphine (Mor), pentobarbital (Pent) and ethanol (EtOH), comparative studies on the inhibitory effect on the electrical stimulation (ES)-evoked contractions of isolated mouse vas deferens (MVD) were undertaken. All the drugs inhibited the ES-evoked contractions of MVD preparations in a concentration-dependent manner, however, the effects were completely different among 3 drugs in the preparations after incubation in the medium containing each drug and also after acute or chronic treatment with the drugs. The differences among 3 drugs are as follows:

1) The in vitro incubation with Mor, but not with Pent and EtOH developed tolerance.
2) MVD from Mor and EtOH tolerant animals were also tolerant to the inhibitory effect of Mor and EtOH in vitro, respectively.
3) K⁺ and Ca²⁺ stimulated the contractions of MVD and the effect was enhanced further after incubation in Mor.
4) The administration of Mor and Pent dose-dependently enhanced the effect of K⁺ and Ca²⁺, but this effect of Mor was lost following repeated injections.
5) No cross tolerance was developed among 3 drugs in the inhibitory effect following in vitro incubation or in vivo treatment.

Thus, the effects of in vitro incubation with Mor, Pent and EtOH, and acute or chronic administration of the drugs on the response of isolated MVD preparations are different from each other suggesting the difference in the underlying mechanisms of the drugs.

Keywords — morphine; pentobarbital; ethanol; mouse vas deferens; tolerance; cross tolerance; potassium ion; calcium ion

Introduction

Isolated smooth muscle preparations, such as guinea pig ileum (GPI)¹,² and mouse vas deferens (MVD)² have been used widely for the study of the action of opiates. Namely, the electrically evoked contractions of these preparations were dose dependently inhibited by opiates and the effects were reversed by naloxone (Nx). Furthermore, the preparations isolated from morphine (Mor) tolerant guinea pig³,⁴ and mouse⁵ are tolerant to the in vitro inhibitory effect, and in vitro incubation of these preparations in the medium containing opiates develop tolerance and dependence,⁶-⁸ thus, they are available for the study of mechanisms of the phenomena. Another advantage of these preparations is that the mechanisms of the contractions induced by electrical stimulation (ES) have been well recognized, GPI contracts by the release of acetylcholine (Ach)¹,⁹ and MVD by noradrenaline (NE).¹⁰,¹¹

On the other hand, pentobarbital (Pent) and ethanol (EtOH) as well as Mor possess tolerance and dependence liability but they are classified into different type of dependent drugs from Mor according to the pharmacological characteristics and the cross dependency with Mor. The difference in the action sites among Mor, Pent and EtOH have also been demonstrated in the inhibitory effects on the ES-evoked contractions of the isolated guinea pig longitudinal muscle/myenteric plexus preparation.¹² However, they also reported the cross tolerance between Mor and EtOH in this preparation and stated the commonality between 2 drugs.¹³

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In order to demonstrate the difference in the mechanisms of Mor, Pent and EtOH, in the present experiment, comparative studies were made on the effects of these drugs on the ES-evoked contractions of MVD preparations from naive and drug treated animals. Experiments were further extended to study the effects of these drugs on the K\(^+\)- and Ca\(^{2+}\)-induced contractions of naive and drug treated MVD preparations since the release of NE is regulated by K\(^+\) and Ca\(^{2+}\).

**Materials and Methods**

**Preparation and Procedure** — Male mice of ddY strain, weighing 30—35 g, were sacrificed by decapitation and their vas deferens were isolated. Each preparation was mounted in a 10 ml organ bath filled with modified Krebs–Henseleit solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl\(_2\), 1.1 mM KH\(_2\)PO\(_4\), 25 mM NaHCO\(_3\), 11 mM glucose) at 37 \(^\circ\)C and aerated. The muscle preparations were stimulated transmurally with square-wave electrical pulses of 100 V, 10 ms duration at a frequency of 0.1 Hz through a platinum ring electrode. The contractions were recorded through an isotonic transducer.

**Drugs** — Morphine-HCl (Mor, Takeda), pentobarbital-Na (Pent, Tanabe). Ethanol (EtOH) and other chemicals were reagent grade obtained from commercial source (Nakarai). In *in vitro* assay, drugs were dissolved in the medium and added directly to the organ bath so as to the amount was contained in 0.1 ml, and the dose was expressed by final concentration added in the bath. For *in vivo* administration, Mor and Pent were dissolved in saline and were injected s.c. EtOH was administered s.c., or by inhalation according to the method of Goldstein.\(^{14}\)

**Estimation of Drug Effect** — The inhibitory effects were compared in IC\(_{50}\) values, concentration required for 50% inhibition of the ES-evoked contractions, estimated from the concentration–response curve for each drug of at least 3 separate experiments.

**Incubation in Drug Containing Medium** — Tissue preparations were incubated in the medium containing 10 \(\mu\)M Mor, 2 mM Pent or 350 mM EtOH at 37 \(^\circ\)C for 90 min, and after washing with normal buffer, the IC\(_{50}\) for the drugs were determined and compared with those incubated in the drug free medium.

**Effect of Acute and Chronic Treatment** — Animals were treated with a single s.c. dose of 50 mg/kg Mor, 50 mg/kg Pent or 3 g/kg of EtOH, 30 min prior to sacrifice. Control animals were treated with saline instead of drugs. In chronic treatment test, Mor and Pent, 50 mg/kg, was injected s.c. twice daily (9:00 a.m. and 6:00 p.m.) for 5 d. In case of EtOH, mice were exposed to alcohol vapor for 3 d according to the method of Goldstein *et al.*\(^{14}\) These treatments rendered the animals tolerant to the analgesic effect of Mor, to the hypnotic effect of Pent, and to the motor incoordinating effect of EtOH, respectively. Animals were sacrificed 30 min after the final administration of Mor and

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**Fig. 1.** Inhibitory Effect of Mor, Pent and EtOH on the ES-Evoked Contractions of Isolated MVD Preparations and Antagonism by Nx

The isolated MVD preparation was stimulated transmurally with square-wave electrical pulses of 100 V, 10 ms duration at a frequency of 0.1 Hz, and drugs were applied directly to the incubation bath. The dose of drug was expressed as the final concentration in the bath. ↓, Mor; ↓, Nx.
Table 1. The IC₅₀ Values for Mor, Pent and EtOH in the MVD Preparations after Incubation in the Medium Containing Each Drug

<table>
<thead>
<tr>
<th>Drug</th>
<th>Control</th>
<th>Incubation in</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Mor (10μM)</td>
<td>Pent (2 mM)</td>
<td>EtOH (350 mM)</td>
</tr>
<tr>
<td>Mor</td>
<td>0.53 ± 0.06 μM</td>
<td>2.4 ± 0.14 μM a)</td>
<td>0.48 ± 0.09 μM</td>
<td>0.61 ± 0.09 μM</td>
</tr>
<tr>
<td>Pent</td>
<td>0.34 ± 0.01 mM</td>
<td>0.35 ± 0.01 mM</td>
<td>0.36 ± 0.02 mM</td>
<td>0.33 ± 0.02 mM</td>
</tr>
<tr>
<td>EtOH</td>
<td>12.5 ± 1.8 mM</td>
<td>12.8 ± 1.3 mM</td>
<td>13.1 ± 2.0 mM</td>
<td>12.6 ± 2.1 mM</td>
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Isolated MVD preparations were incubated in the medium containing 10 μM Mor, 2 mM Pent or 350 mM EtOH for 90 min. Values are the means ± S.E. from 3 experiments. Significantly different from the IC₅₀ of respective control, a) p < 0.001.

Pent, or 30 min after exposure to EtOH vapor. The IC₅₀ for Mor, Pent and EtOH was estimated in each preparation.

Results

Inhibitory Effect in Naive Preparations and Their Nx Sensitivity

Electrically induced contractions of naive MVD preparations were inhibited in a concentration dependent manner by the addition of Mor, Pent and EtOH. The IC₅₀ for Mor, Pent and EtOH were 0.53 μM, 0.34 and 12.5 mM, respectively. The inhibitory effect of Mor was reversed by Nx, while the effect of Pent and EtOH were insensitive to Nx (Fig. 1, Table I).

Tolerance Development by in Vitro Incubation

In the preparations incubated in the medium containing 10 μM Mor, the contractility to the ES were reduced by about 70% of that of naive preparations, and the concentration–response curve for Mor shifted to the right indicating the development of tolerance to its suppressive effect. However, incubation in 2 mM Pent or 350 mM EtOH containing medium did not affect the contractility of the preparations to ES and the concentration–response curve for each drug remained unchanged (Fig. 2). Exposure of the tissue preparations in the medium containing higher concentration of Pent and EtOH, more

Fig. 2. Concentration–Response Curve for Mor, Pent and EtOH of the Inhibitory Effect on the ES-Evoked Contractions of Isolated MVD Preparations after Incubation in Normal or Drug Containing Medium

The tissue preparations were incubated in normal medium (open symbols) or in the medium containing 10 μM Mor, 2 mM Pent or 350 mM EtOH (closed symbols). Each point is the mean ± S.E. from at least 3 experiments. For other details, refer to the footnote of Fig. 1.
Fig. 3. Concentration-Response Curve for Mor, Pent and EtOH of the Inhibitory Effect on the ES-Evoked Contractions of Isolated MVD Preparations Obtained from Acutely or Chronically treated with Respective Drugs

Acute treatment (half closed symbols); animals were treated with a single dose of 50 mg/kg of Mor or Pent, and 3 g/kg of EtOH. Chronic treatment (closed symbols); animals were made tolerant by the twice daily injections of 50 mg/kg of Mor or Pent for 5 d, and by inhalation of alcohol vapor for 3 d. Control animals (open symbols) were treated with saline instead of the drugs. MVD preparations were isolated 30 min after the final injection of Mor and Pent, or 30 min after the inhalation of alcohol vapor. For other details, refer to the footnote of Figs. 1 and 2.

Fig. 4. No Existence of Cross Tolerance between Mor, Pent and EtOH in the Inhibitory Effect on the ES-Evoked Contractions of MVD Preparations Isolated from Tolerant Animals

Mice were made tolerant by 5 daily injections of Mor and Pent or 3 d inhalation of alcohol vapor. MVD preparations isolated from naive animals (open symbols: Mor, ○; Pent □; EtOH, △) and from tolerant animals (closed symbols). For other details, refer to the footnote of Figs. 2 and 3.
Fig. 5. Effect of K⁺ and Ca²⁺ on the ES-Evoked Contractions of MVD Preparations Incubated in the Medium Containing Mor, Pent or EtOH

MVD preparations were incubated in normal (open symbols) or drug containing (closed symbols; 10 μM Mor, 2 mM Pent or 350 mM EtOH) medium for 90 min. KCl (○) and CaCl₂ (□) were added, the additional amount was indicated as the final concentration, directly to the organ bath. The height of the ES-evoked contractions of each preparation before addition of the ions was taken as standard (=1) and the effect was expressed in the increment against standard. For other details, refer to the footnote of Fig. 2.

taining Mor, Pent and EtOH are summerized as the change in IC₅₀ values for each drug in Table I. Change was only observed in IC₅₀ for Mor but not for Pent and EtOH in Mor tolerant preparations. Thus, cross tolerance was not developed between Mor and other two drugs. No appreciable changes were obtained in the IC₅₀ values for each drug in Pent and EtOH treated preparations (Table I).

Effect of Acute and Chronic Injection

The preparations obtained from the animals given Mor and Pent were less sensitive to the ES and the contractions were reduced dose dependently. A single large dose of Mor and Pent, 50 mg/kg, produced about 50 and 80% reduction in the contractility, respectively. However, even with such a marked reduction in the contractility, the concentration-response curves in these preparations were not altered from those in control preparations. Acute administration of EtOH, at the dose up to 3 g/kg did not affect the contractility of the preparations and the concentration-response curve (Fig. 3).

On the other hand, the preparations isolated from Mor and EtOH tolerant animals were tolerant to the suppressive effect of respective drugs and the concentration-response curve shifted to the right, while in the preparations from Pent tolerant animals the concentration-response curve remained unchanged (Fig. 3).

Cross Tolerance between Mor, Pent and EtOH

The concentration-response curve for Pent and EtOH did not change in Mor tolerant preparations, and vice versa (Fig. 4).

Effect on KCl and CaCl₂ Induced Contractions

The contractions induced by ES of naive MVD preparations were enhanced by the addition of KCl and CaCl₂ in a concentration dependent manner. The enhancing effects of K⁺ and Ca²⁺ were potentiated further significantly in the preparations which have been incubated in Mor containing buffer and were tolerant to Mor. However, in the tissues incubated in Pent or EtOH containing buffer, the effect of K⁺ and Ca²⁺ remained unaltered (Fig. 5).

Enhancing effects on the ES-evoked contractions by the addition of K⁺ and Ca²⁺ were facilitated further in the preparations obtained from the animals pretreated with a single s.c. dose of Mor and Pent 30 min before sacrifice. The effect
Fig. 6. Changes in the K⁺-Induced Enhancement of the ES-Evoked Contractions of MVD Preparations Isolated from the Animals Treated Acutely or Chronically with Mor, Pent or EtOH.

Acute treatment (Δ, □); mice were treated with a single dose of 10 and 50 mg/kg of Mor or Pent, or with 1 and 3 g/kg of EtOH, respectively. Chronic treatment (closed symbols); mice were made tolerant by the twice daily injections of 50 mg/kg of Mor or Pent for 5 d, and by inhalation of alcohol vapor for 3 d. Control animals were treated with saline instead of drugs (○). MVD preparations were isolated 30 min after the final injection of Mor and Pent, or 30 min after the inhalation of alcohol vapor. For other details, refer to the footnote of Figs. 2 and 5.

was proportional to the dose of Mor and Pent, while EtOH was ineffective at any dose employed. The potentiating effect of Mor, however, was lost in the preparations obtained from Mor tolerant animals, but the effect of Pent was substantially the same even after chronic treatment (Figs. 6, 7).

Fig. 7. Changes in the Ca²⁺-Induced Enhancement of the ES-Evoked Contractions of MVD Preparations Isolated from the Animals Treated Acutely or Chronically with Mor, Pent or EtOH.

Refer to the footnote of Figs. 5 and 6.
Discussion

Pent and EtOH as well as Mor inhibited ES-evoked contractions of isolated MVD preparations. Although the effect of Mor was antagonized by Nx, as expected, the effect of Pent and EtOH were not reversed by naloxone suggesting that the inhibitory effects of these drugs are not mediated through opioid receptors. The differences in the inhibitory effect between Mor and the other two drugs were also evident since tolerance to the inhibitory effect was only developed in the preparations incubated in the medium containing Mor but not Pent or EtOH. Further, in the preparations made tolerant to Mor by in vitro incubation the inhibitory effects of Pent and EtOH did not change from those in naive control preparations. These results suggest that the mechanism for the development of tolerance is different between Mor and the other two drugs.

Differences in the effects among Mor, Pent and EtOH on the MVD preparations were also observed after acute and chronic in vivo treatment with the drugs. No appreciable changes in the concentration–response curves were obtained in the preparations isolated from the animals treated with a single dose of the 3 drugs. However, the concentration–response curve shifted to the right in the preparations from the animals made tolerant to Mor and EtOH but not in the preparations from Pent tolerant animals. Cross tolerance has been reported between Mor and Pent in the analgesic effect,\(^{16}\) between Mor and EtOH in the inhibitory effect on the ES-evoked contraction of GPI\(^{13}\) and between Pent and EtOH in the hypnotic effect.\(^{16}\) Further, Pent and EtOH are classified into the same type of dependence liable drugs because of the cross dependency between them. However, in the present study, we could not demonstrate the cross tolerance between Mor and Pent, and also between Pent and EtOH suggesting that the mechanisms for the development of tolerance to the inhibitory effect of the 3 drugs on the ES-induced contractions of the isolated MVD preparations are different from each other.

The ES-evoked contraction of MVD preparations is mediated by the release of NE from the nerve terminals,\(^{10,11}\) and, as it is well recognized, the release of NE is regulated by K\(^+\) and Ca\(^{2+}\). Actually, the addition of these ions concentration-dependently accelerated the contractions of MVD preparations. The accelerating effect of K\(^+\) and Ca\(^{2+}\) was further enhanced in Mor tolerant preparations, rendered tolerant by in vitro incubation, but not in the preparations incubated in Pent and EtOH containing medium. Thus, only Mor tolerant preparations were more sensitive to K\(^+\) and Ca\(^{2+}\) and readily responded to NE.

On the other hand, in the preparations isolated from the animals treated with a single large s.c. dose of Mor, Pent and EtOH the concentration–response curve for the respective drug remained at the level in control preparations. However, in the preparations isolated from Mor and Pent injected animals, the contraction accelerating effects of K\(^+\) and Ca\(^{2+}\) were further enhanced in parallel with the dose of the drugs. On the contrary, EtOH injection did not affect the K\(^+\) and Ca\(^{2+}\) induced acceleration of the contractions of the preparations. Interestingly, the enhancement of the effect of K\(^+\) and Ca\(^{2+}\) by a single injection of Mor was lost in the preparations obtained from Mor tolerant animals. Differing from the effect of Mor, the enhancement by K\(^+\) and Ca\(^{2+}\) observed after a single dose of Pent was obtained in the preparations from Pent tolerant animals as well. Furthermore, the concentration–response curve for Mor and EtOH but not for Pent shifted to the right in the preparations isolated from tolerant animals. As is well known, the presence of Ca\(^{2+}\) is essential in the releasing mechanism of neurotransmitters and also in the mechanism of stimulation–contraction coupling of the muscle preparations. K\(^+\) depolarizes cell membrane and stimulates the release of neurotransmitters. Thus, Mor, Pent and EtOH affect the mechanism differentially in MVD for releasing NE, and the differences of the effects may reflect the differences in the sensitivity of the preparations to K\(^+\) and Ca\(^{2+}\).

The difference in the mechanisms for the development of tolerance is also evident since cross tolerance could not be obtained in the preparations isolated from tolerant animals to each drug. Mayer \textit{et al.}\(^{13}\) reported that chronic
morphinization of guinea pig by pellet implantation resulted in tolerance to the inhibitory effect of Mor in the isolated longitudinal muscle/myenteric plexus preparations, and in cross-tolerance to the inhibitory effect of EtOH. However, they also reported\(^{12}\) the differences in the action sites between Mor and EtOH and stated that Mor acts primarily pre-junctionally while EtOH acts pre- and post-junctionally for the inhibition of the contractions of the preparations. In the present experiment, we could not demonstrate cross tolerance between Mor and EtOH in isolated MVD preparations. The discrepancy between our data and that of Mayer \textit{et al.} may be due to the species difference of the animals used and to the difference of the neurotransmitters concerned in the stimulus-response process in the MVD preparations.

In conclusion \textit{in vitro} and \textit{in vivo} treatment of Mor, Pent and EtOH produce characteristic changes in the sensitivity of the isolated MVD preparations to K\(^+\) and Ca\(^{2+}\)-induced acceleration of the ES-evoked contractions. These changes may affect the responses of the preparations to each drug and also the development of tolerance to the effects and resulted in the differences of the effects among Mor, Pent and EtOH.

References


