

## Synergistic Interaction of Morphine and Halothane in the Guinea Pig Ileum: Effects of Pertussis Toxin

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The effects of pertussis toxin on the actions of morphine and halothane in the guinea pig ileum are described. Both morphine and halothane produce a dose-related inhibition of electrically induced muscle contraction. The  $IC_{50}$  of morphine was unchanged by the toxin ( $2.1$  and  $2.2 \times 10^{-7}$  M in control and toxin-pretreated animals). However, the  $IC_{50}$  of halothane was increased from  $2.1$  to an extrapolated value of  $9.1$  vol/vol% by pertussis toxin. At high levels of inhibition the interaction between morphine and halothane was synergistic and was converted to additive in the presence of the toxin. These results demonstrate that in the myenteric longitudinal muscle preparation the effects of halothane, but not those of morphine, are mediated by the substrate for pertussis toxin, possibly a  $G_i$  membrane protein. The present study provides significant evidence that the effects of halothane on neuronal tissue are dependent upon an interaction with a specific membrane protein. (Key words: Anesthetics, intravenous: morphine. Anesthetics, mechanism: G proteins; pertussis toxin. Anesthetics, volatile: halothane. Guinea pig ileum. Interactions, drug: halothane; morphine. Receptors: opioid.)

THE GUINEA PIG ILEUM has been widely used as an *in vitro* model to characterize the effects of opiates<sup>1</sup> and has also proven to be useful in the study of the effects of inhalation anesthetics.<sup>2,3</sup> Both types of agents are frequently used together in the practice of anesthesia. In a previous article we described an interaction between these two groups of compounds, which was synergistic and concentration-dependent.<sup>2</sup> The present study attempts to elucidate the mechanism of action of these agents alone and in combination in the guinea pig ileum. In this preparation the effects of morphine are mediated by binding to opioid receptors, mainly  $\mu$  and  $\delta$ .<sup>4</sup>

It has been suggested that the effects of many hormones and transmitters, including opioids, are mediated by a group of guanine nucleotide binding proteins (G proteins) located within cell membranes.<sup>5</sup> However, previous studies of the role of G proteins on opioid activity in the guinea pig ileum have been inconclusive.<sup>6,7</sup> A group of closely related G proteins are irreversibly ribosylated and thus inactivated by pertussis toxin.<sup>5,8</sup> This well-established ef-

fect has been used in the present study as a means of investigating the role of pertussis-sensitive G proteins in the effects of morphine and halothane.

### Materials and Methods

The experiments were carried out on the guinea pig ileum myenteric plexus-longitudinal muscle preparation (MPLM). The experimental protocol was approved by the Animal Studies Committee. Anesthesia was not provided prior to decapitation because of the possible impact of an anesthetic on tissue and on the interactions of morphine and halothane. Male albino guinea pigs (English short hair, weight 300–400 g) were decapitated and the terminal portion of the ileum dissected. The MPLM strip was prepared as described by Puig *et al.*<sup>9</sup> Each strip was suspended in a 10-ml organ bath containing Krebs bicarbonate solution at 37° C, and gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub>. When halothane was used, the O<sub>2</sub>/CO<sub>2</sub> gas mixture was passed through a Fluotec Mark II vaporizer and the delivered concentration of halothane verified by gas chromatography (Hewlett Packard 5830A gas chromatograph). After an equilibration period of 60 min (resting tension 0.3 g), the preparation was stimulated with platinum ring electrodes placed at the top and bottom of the preparation and separated by a distance of 6 cm. The parameters of stimulation were as follows: 0.1 Hz, 1 ms duration and supramaximal voltage (35 V). The stimuli were generated by a Grass stimulator (S-88), mixed with the aid of two stimulus isolation units (Grass, SIU 5), fed into an audio amplifier (Crown DC 300), and then applied to the electrodes. The voltage and duration of the stimuli were constantly monitored on a Tektronix R5103N oscilloscope. The isometric contractions of the muscle were then transduced (Grass force transducer FT03C) and recorded by a Grass polygraph.

The MPLM from two groups of animals were used: 1) controls that received an intraperitoneal (ip) injection of 0.3 ml of 0.9% NaCl, and 2) animals that received 60  $\mu$ g/kg of pertussis toxin ip in 0.3 ml of saline.<sup>6,7</sup> Each animal served as the source of two muscle strips, each of which was used for generating one set of dose–response data. All animals were killed 6 days after injection, and identical series of experiments were performed in both groups. Pertussis toxin (Islet-Activating Protein, List Biological Labs, Campbell, California) was stored as lyophilized powder at 4° C and dissolved in saline immediately prior to administration.

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When halothane was used, it was delivered to the organ bath at concentrations of 0.4 to 4 vol/vol%. The response of the tissue to electrical simulation was monitored as the halothane was administered. After the effect of halothane was stabilized, the preparation was equilibrated for an additional 15 min in the presence of the anesthetic. When morphine was used, the tissue was exposed to the opiate for 3 min, the effect was recorded, and then it was washed with drug-free Krebs solution. Morphine solutions were made daily with glass distilled water and added to the bath in a volume of 0.1 ml. When the maximal effect of the drug (morphine or halothane) was attained, the height of the contraction (in millimeters) was compared with its pretreatment values, and the percent inhibition was then plotted against the log of the dose. Log dose-response curves were obtained for morphine alone, halothane alone, and morphine in the presence of 1.6% halothane. This concentration of anesthetic was used because it approximates the  $IC_{50}$ .<sup>2</sup> In calculating the results of these experiments, the data were normalized such that the magnitude of response of a drug was expressed as the percent of the size of the contraction immediately prior to its administration. As a result, in experiments in which morphine was added in the presence of halothane, the contribution of halothane to the total inhibitory effect was excluded. The curves were used to determine the  $IC_{50}$  of morphine and halothane. This value corresponds to the concentration that produces a 50% inhibition of the amplitude of the contractions. The experiments were carried out both in control and pertussis-treated animals.

The nature of the interaction between halothane and morphine was investigated by multiple-drug effect analysis with the aid of a microcomputer.<sup>10</sup> For this procedure, three dose-response curves were obtained: morphine alone, halothane alone, and a third curve in which morphine and halothane were combined in a fixed proportion. In the latter experiments, the lowest doses used were 4

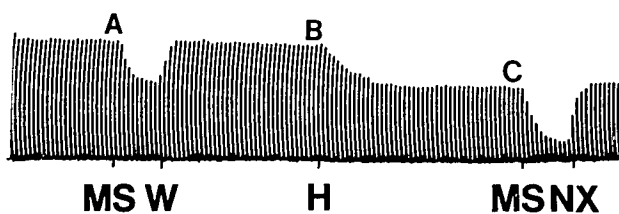


FIG. 1. Morphine (MS,  $1 \times 10^{-7}$  M) was added to the MPLM preparation at point A. After washing (W), halothane (H, 1.6%) was started at point B. When the MPLM was stabilized, the same concentration of MS was added (C). Subsequently, naloxone (NX,  $2 \times 10^{-7}$  M) was added to the bath. The effect of morphine was calculated by measuring the height of contraction after MS and determining the percent inhibition relative to point A. The effects of halothane alone and morphine in the presence of halothane were calculated in a similar manner using points B and C, respectively.

TABLE 1. Effect of Pertussis Toxin on Morphine-Halothane Interaction

	Saline Pretreatment		Pertussis Pretreatment	
	No Halothane	1.6% Halothane	No Halothane	1.6% Halothane
Morphine $IC_{50} \times 10^{-7}$ M	2.1	0.67	2.2	1.8*
95% CL	1.4-3.3	0.5-0.8	1.7-2.7	0.9-3.4
No. of experiments	5	6	5	6

The potency of morphine is expressed as the  $IC_{50}$  obtained from log dose-response curves under the various experimental conditions. CL = confidence limits.

\* Significant ( $P < 0.05$ ) difference versus saline-pretreated group.

$\times 10^{-8}$  M morphine and 0.4% halothane (the approximate threshold concentration of each agent). For the production of dose-response curves, the doses were increased, but the ratio was maintained by using multiples of the above concentrations. Hill coefficients and  $IC_{50}$  for each curve were determined. With those obtained from the dose-response curves of individual agents, a calculation was made of the concentrations (in the previously mentioned proportion) that would be necessary to produce a particular degree of inhibition under the assumption that the drugs were merely additive. This pair of concentrations was then compared with the actual concentrations that were needed to produce the same degree of inhibition in experiments utilizing both drugs combined. The ratio of the actual over expected concentrations is the combination index. A combination index of 1 indicates that the effects of both drugs were additive when combined. A combination index  $< 1$  indicates that lower drug combinations were needed to produce the stated level of response than would be expected if the drugs were additive, thus demonstrating synergism. Conversely, a combination index  $> 1$  indicates a less than additive interaction (antagonism). Combination indexes were calculated for each of a number of degrees of inhibition. The resulting graph shows the nature of the interaction at different levels of inhibitory response. Combination indexes were calculated in control and pertussis-treated animals.

The IC values were calculated using by the methods of Tallarida and Murray.<sup>11</sup> Values were considered to be significantly different if their 95% confidence limits (CL) did not overlap.

## Results

Morphine and halothane individually produced a dose-related inhibition of the electrically induced muscle contractions of the MPLM (fig. 1). The  $IC_{50}$  of morphine was  $2.1 \times 10^{-7}$  M and the  $IC_{50}$  of halothane was 2.1%. Pretreatment of animals with pertussis toxin did not sig-

nificantly change the  $IC_{50}$  of morphine (table 1). In contrast, the potency of halothane was significantly decreased following pretreatment with pertussis toxin (fig. 2; table 2). When the  $IC_{50}$  from the group pretreated with pertussis toxin was divided by the corresponding control (saline group), the resulting ratio showed a 4.3-fold reduction in the potency of halothane.

The interaction between morphine and halothane was studied in a series of experiments in which morphine dose-response curves were performed in the presence of 1.6% halothane (fig. 1). These experiments were carried out on MPLM from both saline and pertussis toxin-treated animals. As previously reported,<sup>2</sup> in the saline group, this concentration of halothane significantly increased the potency of morphine at degrees of inhibition above 50%, and this interaction was synergistic in nature. When similar experiments were repeated in animals pretreated with pertussis toxin, the  $IC_{50}$  of morphine was increased so that it was no longer significantly different from the value for morphine alone (without pertussis toxin; table 1), an indication that the toxin abolished the interaction of morphine and halothane. The effect of pertussis toxin on the morphine-halothane interaction is shown in table 1. In the saline group, 1.6% halothane increased the potency of morphine on the MPLM by approximately a factor of 3. In the pertussis group the  $IC_{50}$  of morphine in the presence of halothane did not significantly differ from the  $IC_{50}$  of morphine in the saline group without halothane (table 1), indicating that pertussis had no effect on

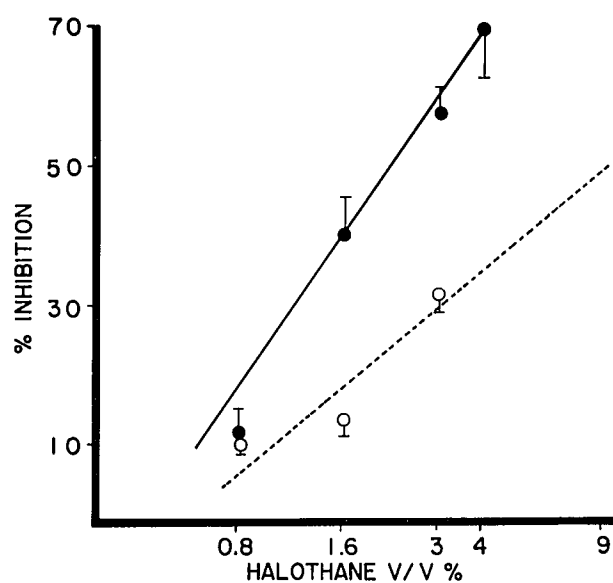


FIG. 2. Log dose-response curves for halothane in saline (closed circles) and pertussis-pretreated (open circles) animals. All data points represent the mean values obtained from five animals. The vertical bars indicate the standard errors. The line represents the computer generated best fit of the combined data.

TABLE 2. Effect of Pertussis Toxin on the Potency of Halothane

	Pretreatment	
	Saline	Pertussis Toxin
$IC_{50}$ of halothane	2.1	9.1*
95% CL	1.7-2.5	5.2-15.7
No. of animals	5	5

Each value was obtained from the log dose-response curves of halothane in saline- or pertussis-treated animals. Halothane was used in concentrations of 0.4-3%. Any concentrations above 3% were obtained by extrapolation from the respective dose-response curves.

\* Significant difference ( $P < 0.05$ ) between pretreatment groups.

the potency of morphine itself but prevented the interaction of morphine and halothane.

The interaction of morphine and halothane in control and pertussis toxin groups was further investigated by multiple-drug effect analysis. The MPLM preparations were exposed to increasing concentrations of morphine and halothane in a fixed ratio and combination indexes were calculated. Figure 3 shows that the interaction be-

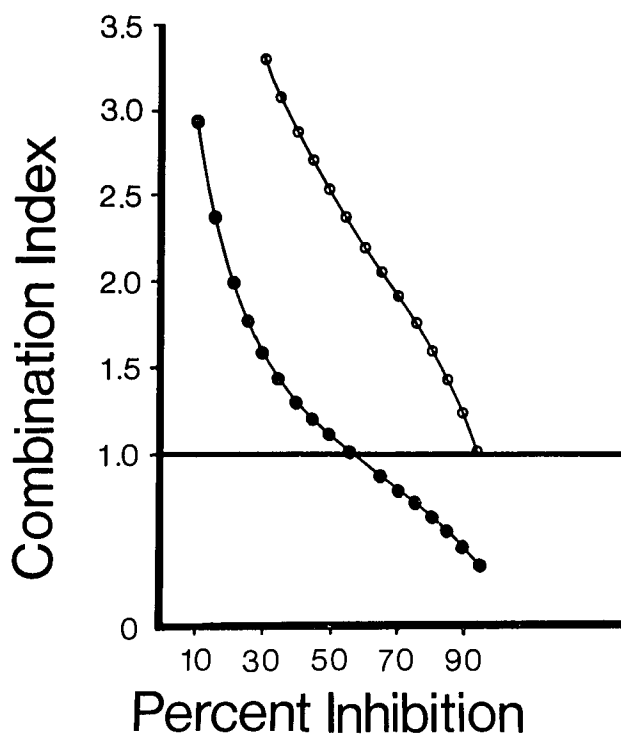


FIG. 3. The results of multiple-drug effect analysis. Values were obtained from three groups of dose-response curves: morphine, halothane, and both agents combined in fixed proportion. The ordinate indicates the combination index at each level of response (percent inhibition, abscissa). The line drawn with the closed circles represents the interaction in saline-pretreated animals, and the line drawn with the open circles represents the interaction in pertussis-pretreated animals.

tween morphine and halothane was complex. The line to the left (drawn with closed circles) was obtained from the saline group; it shows that the interaction was synergistic when the total level of inhibition was greater than 50% (combination index  $< 1$ ), whereas it was less than additive (antagonistic) when the total level of inhibition was  $< 50\%$  (combination index  $> 1$ ). The line to the right (open circles) demonstrates that pertussis toxin changed the nature of the interaction only at high levels of inhibition. Thus, for example, at 80% inhibition the interaction in the saline group was synergistic and changed to less than additive in the pertussis group. Similarly, at 50% inhibition the interaction was changed from additive (saline group) to less than additive (pertussis group).

### Discussion

In the present study we show that the effects of halothane on the MPLM preparation and the nature of its interaction with morphine are altered by pertussis toxin. The mechanism of action of inhalation anesthetics are not well established. Most theories propose that these agents interact with membrane structures, such as lipid bilayers or lipid interfaces.<sup>12,13</sup> More recent models suggest an interaction between general anesthetics and protein molecules.<sup>14-17</sup> The present study investigates the hypothesis that halothane interacts with a specific type of membrane protein in neuronal tissue, the guanine nucleotide-binding proteins (G proteins). More recently, evidence has been presented that halothane interacts with a pertussis-sensitive membrane protein in the mouse heart.<sup>18</sup> It is well established that opiates inhibit electrically induced contractions in the guinea pig ileum by binding to specific opioid receptors, mainly  $\mu$  and  $\delta$ .<sup>4</sup> In many tissues these receptors are coupled to adenylate cyclase by G proteins.<sup>19</sup> However, in the MPLM preparation the sequence of events following the opioid-receptor interaction is a subject of controversy. At present, at least four types of G proteins have been identified<sup>3</sup>; they appear to modulate the activities of adenylate cyclase, cyclic GMP phosphodiesterase, phospholipase C, and possibly regulate calcium and potassium ion channels. Several of these G proteins,  $G_i$  and  $G_o$ , are substrates for pertussis toxin. The former protein has been found in a variety of tissues and the latter protein has been isolated from brain. The ability of this toxin to alter a biologic effect is a currently accepted criterion for the involvement of G proteins in the system studied. Pertussis toxin irreversibly ribosylates  $G_i$  proteins. Thus, the ability of this toxin to alter the effects of morphine and halothane on the MPLM would be evidence that a G protein participates in the inhibitory responses. Sensitivity to pertussis implicates three possible G proteins:  $G_i$ , which mediates inhibition of adenylate cyclase,  $G_o$ ,

which has been found in the brain but has no known function, and  $G_i$ , which is found in the retina. The inhibitory nature of the response and the tissue used in the present study suggest that a  $G_i$  protein is involved in the responses of this tissue.

Both halothane and morphine produced a dose-related inhibition of the electrically induced contractions of the MPLM. Halothane at concentrations of 1.6% and 3% did not alter the response to acetylcholine ( $1 \times 10^{-6}$  to  $1 \times 10^{-3}$  M) in unstimulated MPLM preparations (unpublished observation). These findings demonstrate that the effects of halothane are not on cholinergic receptors in smooth muscle.

It has been previously established that pertussis toxin does not affect the response to electrical stimulation (twitch tension) or the neuronal mechanisms in the guinea pig ileum.<sup>7</sup> The effects of halothane were significantly reduced in pertussis-pretreated animals. In the present study, the potency of halothane was reduced by pertussis toxin from a value of 2.1 to one that extended beyond the limit of the dose-response curve but was extrapolated to a value of 9.1 (fig. 2). In contrast, the inhibitory effects of morphine were not affected by the toxin. The insensitivity of the morphine response further demonstrates that the effect of the toxin is not on the contractile process itself or the signal transduction mechanism distal to the G protein, whereas the ability of pertussis toxin to inhibit the response to halothane suggests that the toxin reached effective concentrations in the tissue. Morphine produces its effects in the MPLM preparation by binding to opioid receptors. Although the mechanism of action of halothane is not established, we have reported that it does not interact with opioid receptors.<sup>2</sup> The present study shows that these drugs also differ with respect to events that occur within the cell membrane. The inability of pertussis to alter the response to morphine suggests that  $G_i$ ,  $G_o$ , or  $G_i$  proteins are not involved in the transduction of the effects of opiates in this tissue. However, these findings do not exclude the possibility that other G proteins with different sensitivities to pertussis toxin may mediate these effects.<sup>20,21</sup> In contrast, the halothane response was significantly decreased by the toxin. This finding demonstrates that the effect of the anesthetic is dependent upon a pertussis-sensitive G protein in this preparation.

We have recently reported that the interaction between halothane and morphine is synergistic.<sup>2</sup> The present study demonstrates that this interaction is also affected by pertussis toxin. The potentiation of morphine by halothane was abolished by pretreatment with pertussis toxin (table 1). This was corroborated by separate experiments performed for the multiple-drug effect analysis (fig. 3), which demonstrates that those concentrations of morphine and halothane that produce a synergistic interaction in the presence of the toxin resulted in an additive interaction,

and the previously additive interaction became antagonistic.

The present study confirms an earlier report, which suggested that the effects of morphine in the guinea pig ileum were not dependent upon the function of a  $G_i$  protein.<sup>7</sup> More importantly, however, our results indicate that the mechanism of action of inhalational anesthetics on neuronal tissue may involve an interaction with a specific membrane protein.

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