Characteristics of Post-tetanic Contraction Induced by Naloxone in Guinea Pig Ileum

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The characteristics of isolated guinea-pig ileal contractions of basal tension after tetanic stimulation in the presence of a high concentration of naloxone (NLX) [post-tetanic contraction] were investigated. The post-tetanic contraction did not occur in the absence of NLX, but did occur in a concentration-dependent manner in the presence of a high concentration of NLX (5×10⁻⁷, 10⁻⁶, 10⁻⁵ and 5×10⁻⁴ M), the concentration of which was higher than that required for antagonizing post-tetanic twitch inhibition. The contraction in the presence of 10⁻⁴ M NLX was diminished by washing NLX from the preparation with Krebs-bicarbonate solution. The contraction under 10⁻⁴ M NLX occurred in a frequency-dependent manner (5, 10 and 20 Hz), but not at 0.1 Hz. Tetanic stimulation (5, 10 and 20 Hz) without NLX did not induce this contraction. The post-tetanic contraction with 10⁻⁴ M NLX had a tendency to be antagonized in the presence of 5×10⁻⁵ M atropine. Methysergide (5×10⁻⁵ M) had no effect on this contraction. Spantide (10⁻⁵ M) largely inhibited the contraction, and indomethacin (5×10⁻⁶ M) and tetrodotoxin (5×10⁻⁷ M) completely inhibited this contraction. These results indicate that tetanic stimulation in the presence of a high concentration of NLX induces contraction of the ileal muscle due to the release of endogenous ileal contractile substances (substance P, prostaglandins and acetylcholine), and suggests that these contractions are closely linked to the endogenous opioid system induced by tetanic stimulation in the ileum.

Key words  naloxone; post-tetanic contraction; endogenous opioid; tetanic stimulation; ileum

Isolated guinea pig ileum is a useful model with which to investigate the dynamics of opioid action in vitro, since these actions can be detected through the inhibition of twitch contraction by field stimulation. In 1977 and 1978, Pug and co-workers reported the opiate-like inhibition, prevented by naloxone (NLX), of twitch contractions induced by low-frequency electrical field stimulation after high-frequency stimulation in isolated guinea pig ileum. This post-tetanic opiate-like inhibition of the twitch contraction (post-tetanic twitch inhibition) is a useful indicator with which to study the role of endogenous opioid in this tissue. However, this inhibition has not been well characterized. We previously reported some characteristics of the post-tetanic twitch inhibition in guinea pig ileum, i.e., the effects of peptidase inhibitors, the participation of opioid receptor types and the effect of dopamine antagonists.

In the present study, we report an increase in the basal tension [post-tetanic contraction] after tetanic stimulation in the presence of a higher concentration of NLX than that necessary to block the post-tetanic twitch inhibition, and attempt to further characterize the post-tetanic contraction. We also discuss the relationships between these contractile responses and the endogenous opioids induced by tetanic stimulation (post-tetanic twitch inhibition) which we previously reported.

MATERIALS AND METHODS

The experiments were performed essentially as we previously described.

Animals and Drugs All experiments were performed on isolated ileum obtained from randomly-bred male guinea pigs weighing between 350 and 450 g. The animals were stunned and decapitated, and the ileum was quickly isolated about 10 cm from the ileo-caecal junction. The myenteric plexus-longitudinal muscle (MPLM) was prepared by the method described by Rang. A glass rod was inserted into the lumen of an intestinal segment, and the MPLM was removed by rubbing the segment with a cotton swab soaked in Krebs’ solution. The preparations (2–2.5 cm in length) were suspended at a resting tension of 500 mg in a 5 ml organ bath between platinum ring electrodes (3.5 cm apart) placed at the top and bottom of the bath. The bath contained Krebs-bicarbonate solution (mm: NaCl 118; KCl 4.7; CaCl₂ 2.5; KH₂PO₄ 1.2; MgSO₄ 1.2; NaHCO₃ 25; Glucose 10) at 37 °C, and was bubbled with 95% O₂/5% CO₂.

The sources of the drugs were: naloxone hydrochloride (Endo Laboratories Inc., Garden City, NY, U.S.A.), atropine sulfate (Merck, Darmstadt, Germany), methysergide maleate (Sandz Inc., Tokyo, Japan), spantide (Peptide Institute, Minoh, Japan), indomethacin (Ciba-Geigy, Inc., Takarazuka, Japan), and tetrodotoxin (Sankyo, Inc., Tokyo). All other drugs were obtained commercially. Spantide was dissolved in 0.01 M acetic acid. Indomethacin was dissolved in 0.047 M sodium carbonate. Tetrodotoxin was dissolved in 0.01 M hydrochloric acid. Methysergide was dissolved in methanol. All other drugs were dissolved in distilled water and applied singly to the bath in a volume of 5 μl.

Electrical Field Stimulation and Tetanic Stimulation Rectangular electrical pulse field stimulations were applied at 0.1 Hz, 0.5 ms pulse width and maximum intensity using a DPS-160 B stimulator (NEC-Sanei Co., Tokyo, Japan) with a DPS-122 isolator (NEC-Sanei Co., Tokyo, Japan), and the responses were recorded isometrically on an SP-H5P recorder (Riken Denshi Co., Tokyo, Japan). Tetanic stimulations of 10 Hz (0.5 ms pulse width, maximum intensity, for 1 min) were repeated every 30 min (indicated by the stimulation number described below). A diagram of the experimental protocol was given previously.

The first tetanic stimulation was applied 40 to 50 min after the preparation was set up. The preparation was washed 6 times with 5 ml of Krebs-bicarbonate solution 13 to 15 min
after tetanic stimulation.

**Post-tetanic Contraction Induced by Naloxone**  Tetanic stimulation (10 Hz, 0.5 ms pulse width, maximum intensity, for 1 min) was applied in the presence of various concentrations of NLX (5×10^{-2}, 10^{-6}, 10^{-5} and 5×10^{-5} M) applied 5 min before the tetanic stimulation. Various frequencies (5, 10 and 20 Hz) of tetanic stimulation (0.5 ms pulse width, maximum intensity, for 1 min) were also applied in the presence of naloxone 10^{-6} M which was added 5 min before stimulation number 6 according to the experimental protocol. To examine the disappearance of post-tetanic contraction caused by the removal of naloxone, naloxone 10^{-6} M was applied 5 min before tetanic stimulation number 6, and we compared the contraction before (stimulation number 5) and after (stimulation number 7) the application of NLX.

**Effects of Various Contractile Antagonists on Post-tetanic Contraction**  Effects of atropine (5×10^{-5} M), sphincter (10^{-5} M), methysergide (5×10^{-5} M), indomethacin (5×10^{-5} M) and tetrodotoxin (5×10^{-5} M) 10 min before tetanic stimulation number 6 on the post-tetanic contraction induced by tetanic stimulation (10 Hz, 0.5 ms pulse width, maximum intensity, for 1 min) in the presence of NLX (10^{-6} M) were investigated. The preparation was washed to remove NLX and these antagonists from the medium 6 times with 5 ml of Krebs-bicarbonate solution 13 to 15 min after the tetanic stimulation.

**Quantification of Post-tetanic Contraction Caused by Naloxone**  The contraction was defined as the maximum basal tension height (mm) after the cessation of tetanic stimulation, and is expressed as the contraction percentage of the twitch contraction height (mm) before the tetanic stimulation.

**Statistics**  Values are expressed as means and standard error of the mean (S.E.M.) with the number of preparations in parentheses. The significance of differences in the presence or absence of antagonists was evaluated by one-way analysis of variance with Dunnett’s test. Differences were considered significant at p<0.05.

**RESULTS**

**The Post-tetanic Contraction Induced by Naloxone**  A typical tracing of the effects of NLX on the contraction of basal tension induced by tetanic stimulation (10 Hz, 0.5 ms pulse width, maximum intensity, for 1 min) [post-tetanic contraction] is shown in Fig. 1. Naloxone at 10^{-6} M did not produce the post-tetanic contraction, as previously described, but the contraction was increased in a manner that depended on the concentration of NLX (5×10^{-2}, 10^{-5}, 10^{-4} and 5×10^{-5} M) (Fig. 2). These contractions gradually faded after cessation of the tetanic stimulation and a return of the frequency to 0.1 Hz. Post-tetanic twitch inhibition was also antagonized by these concentrations of NLX. The effects of various frequencies (5, 10 and 20 Hz) of tetanic stimulation on the development of post-tetanic contraction in the presence of 10^{-6} M NLX were also investigated. Typical tracings of these effects are shown in Fig. 3. The post-tetanic contractions were induced by various frequencies of tetanic stimulation in a frequency-dependent manner (Fig. 4). The post-tetanic contraction occurred in the presence of 10^{-6} M NLX (stimulation number 6), unlike before NLX (stimulation number 5), and disappeared following the removal of NLX (stimulation number 7) (Fig. 5).

**Effects of Various Ileal Contractile Antagonists and

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Naloxone \(10^{-6}\) M was added to the bath 5 min (indicated by closed circles) before stimulation with each frequency (5, 10 and 20 Hz, 0.5 ms pulse width, maximum intensity, for 1 min), indicated by the solid line. The post-tetanic contraction is indicated by an arrow.

**Fig. 3. Representative Dynograph Tracing of the Effect of Different Frequencies on the Post-tetanic Contraction in the Presence of Naloxone (NLX)**

NLX \(10^{-6}\) M was added to the bath 5 min before stimulation with each frequency (5, 10 and 20 Hz at 0.5 ms pulse width, maximum intensity, for 1 min) of tetanic stimulation. The post-tetanic contractions were calculated as the maximum basal tension height (mm) after the cessation of tetanic stimulation and are expressed as the contraction percentage of the twitch contraction height (mm) before the tetanic stimulation, as indicated in the "Materials and Methods" section. The values and vertical bars represent the means and S.E.M. for the numbers of preparations shown in parentheses.

**Fig. 4. Effect of Different Frequencies on the Post-tetanic Contraction in the Presence of Naloxone**

Naloxone \(10^{-6}\) M was added to the bath 5 min before stimulation with each frequency (5, 10 and 20 Hz at 0.5 ms pulse width, maximum intensity, for 1 min) of tetanic stimulation. The post-tetanic contractions were calculated as the maximum basal tension height (mm) after the cessation of tetanic stimulation and are expressed as the contraction percentage of the twitch contraction height (mm) before the tetanic stimulation, as indicated in the "Materials and Methods" section. The values and vertical bars represent the means and S.E.M. for the numbers of preparations shown in parentheses.

**Tetrodotoxin on Post-tetanic Contraction** Figure 6 shows a typical tracing of the effects of various ileal contractile antagonists, atropine \((5 \times 10^{-6} \text{ M})\) for acetylcholine (ACh), spantide \((10^{-5} \text{ M})\) for substance P, methysergide \((5 \times 10^{-5} \text{ M})\) for serotonin, indomethacin \((5 \times 10^{-6} \text{ M})\) for prostaglandins and tetrodotoxin \((5 \times 10^{-7} \text{ M})\) for nervous conductance block. The respective antagonists did not inhibit the twitch response at 0.1 Hz (except for atropine and tetrodotoxin), applied 10 min before tetanic stimulation. In the presence of atropine and tetrodotoxin, a gradual and small increase in basal tension was observed after twitch inhibition occurred. Additionally, in the presence of atropine \((5 \times 10^{-6} \text{ M})\), the increase in basal tension during the tetanic stimulation developed somewhat slowly compared with the other contractile antagonists. The addition of methysergide \((5 \times 10^{-5} \text{ M})\) did not inhibit the twitch response at 0.1 Hz, but first increased the tone of the preparation, then it returned to the original level. No ileal contractile antagonist induced the post-tetanic contraction without NLX by tetanic stimulation (figure not shown). In these experiments, post-tetanic twitch inhibition was maximally antagonized, except by tetrodotoxin, for which the level antagonized was the same as that of our results (previously shown). As shown in Figs. 6 and 7, the post-tetanic contraction in the non-treatment condition (in the presence of naloxone \(10^{-6} \text{ M}\) alone) was 39.7 ± 5.3% \((n=19)\). In the presence of atropine \((5 \times 10^{-6} \text{ M})\), the post-tetanic contraction had a tendency to be antagonized, but not significantly \((30.2 ± 1.6\%, n=7)\). Methysergide \((5 \times 10^{-5} \text{ M})\) did not affect the contraction \((37.3 ± 5.8\%, n=3)\). The post-tetanic contraction in the presence of spantide \((10^{-5} \text{ M})\) was significantly inhibited \((10.9 ± 1.3\%, n=4, p<0.01)\), and also that in the presence of indomethacin \((5 \times 10^{-6} \text{ M})\) \((3.3 ± 2.0\%, n=4, p<0.01)\) or tetrodotoxin \((5 \times 10^{-7} \text{ M})\) \((0 ± 0\%, n=3, p<0.01)\) (Fig. 7).

**DISCUSSION**

We previously reported some characteristics of post-tetanic twitch inhibition in guinea pig ileum. The post-tetanic opiate-like inhibition of the twitch contraction (post-
Fig. 6. Representative Dynograph Tracings of the Effects of Some Antagonists on the Post-tetanic Contraction in the Presence of Naloxone

Each concentration of drugs (atropine $5 \times 10^{-6} \text{M}$ (●), methysergide $5 \times 10^{-7} \text{M}$ (○), spantide $10^{-5} \text{M}$ (▲), indomethacin $5 \times 10^{-7} \text{M}$ (■), and tetrodotoxin $5 \times 10^{-7} \text{M}$ (△)) was added to the bath 1 min before, and naloxone $10^{-6} \text{M}$ (●) was added 5 min before, tetanic stimulation (10 Hz, 0.5 ms pulse width, maximum intensity, for 1 min). The post-tetanic contraction is indicated by an arrow.

tetanic twitch inhibition) is a useful indicator with which to study the role of endogenous opioids in this tissue. In these studies, the concentration of NLX used to antagonize the post-tetanic twitch inhibition was lower than $5 \times 10^{-7} \text{M}$ and did not induce an increase in basal tension. However, concentration-dependent increases in basal tension after tetanic stimulation in the presence of higher concentrations of NLX ($5 \times 10^{-7}$, $10^{-6}$, $10^{-5}$ and $5 \times 10^{-5} \text{M}$) (post-tetanic contraction) than that used for the antagonism of post-tetanic twitch inhibition were observed (Figs. 1 and 2). In the present experiments, as the concentration of NLX used was higher than that of antagonizing actions via opioid receptors, it may be possible that these results may have been a non-specific effect occurring because of the high concentrations of NLX, so these effects had never been investigated. However, from a toxicological point of view, it is very important to clarify the characteristics of drug actions which seem to be non-specific. As shown in this report, even the high concentration of NLX induced different effects on some drugs. Therefore, it seems that the effects of these high concentrations of NLX cannot be excluded as non-specific effects; therefore, to standardize the conditions of our experiments, we studied the effects of the following parameters on the post-tetanic contraction.

It was observed that as the frequencies of the pulses of tetanic stimulation increased from 5 to 20 Hz in the presence of NLX ($10^{-6} \text{M}$), the post-tetanic contraction consequently increased (Figs. 3 and 4). The post-tetanic contraction diminished after washing NLX from the bath (Fig. 5), and these effects did not result from a higher concentration of NLX alone before tetanic stimulation or from tetanic stimulation alone. Moreover, post-tetanic twitch inhibition (the effect of endogenous opioids) was also antagonized by these concentrations of NLX (Figs. 1, 3, 5 and 6), which are the same inhibitions as described in our previous reports. These results indicate that both tetanic stimulation and the presence of a high concentration of NLX are essential for the development of post-tetanic contractions. And these mean that the endogenous opioid system and contraction-generating system are tightly linked.

The pharmacological characteristics of this contraction were investigated in the presence of various antagonists of ideal contractile substances and tetrodotoxin (Figs. 6 and 7), the concentration of which was high enough to block the maximum contractions caused by each contractile substance (ACH, serotonin, and tetrodotoxin), except for tetrodotoxin. The concentration of tetrodotoxin ($5 \times 10^{-5} \text{M}$), which inhibits 95% of twitch contraction, was used for detecting the action of endogenous opioid peptides (post-tetanic twitch inhibition), since higher concentrations of tetrodotoxin com-
pletely block the twitch contraction. This contractile response appeared to be derived from the enteric neurons, since tetrodotoxin completely blocked this contraction.

The contraction had a tendency to be decreased by atropine (5×10^{-6} M), but methysergide (5×10^{-5} M) did not affect the contraction. In the presence of atropine or tetrodotoxin, a gradual and small increase in basal tension was observed after twitch inhibition was produced by these drugs. These phenomena are sometimes observed after the cessation of an electrical field stimulation of low frequency, not tetanic stimulation, or under the decrease in the twitch potency by low frequency stimulation. But, it seems that these are obviously different from the post-tetanic contraction since these contractions continue longer than that in post-tetanic contraction. It is now impossible to explain this state of affairs. Additionally, in the presence of atropine (5×10^{-6} M), the increase in basal tension during tetanic stimulation developed somewhat slowly developed compared with that in the presence of other contractile antagonists. This indicates that ACh may be one of the components released during tetanic stimulation, which has not been clearly analyzed.

The addition of methysergide (5×10^{-5} M) first increased the tone of the preparation then returned it to the original level. This phenomena of methysergide was also reported by Kadlec et al., but was not discussed in terms of significance. Additionally, the contractile effect of LSD-25, which is also a serotonin antagonist, has also been observed by Drew. He mentioned that the contraction seemed to be mediated via the H_{3}-receptor since it was prevented by pretreatment with mepyramine. On the other hand, Gintzler et al., reported that LSD-25 could function as an agonist and/or antagonist of serotonin. Thus, the first increase in tone by methysergide may derive from the agonistic action of these antagonists such as LSD-25. Therefore, the participation of the histaminergic system and serotoninergic system in the post-tetanic contraction and the post-tetanic inhibition in our previous paper should be investigated further.

In the presence of spantide (10^{-7} M), the post-tetanic contraction was almost completely antagonized, and also by indomethacin (5×10^{-5} M) and by tetrodotoxin (5×10^{-7} M) (Figs. 6 and 7). Previous reports show that part of the contractile response to substance P is not affected by atropine, tetrodotoxin, morphine, enkephalins or naloxone, which indicates that the response is due to the direct action of substance P on the smooth muscle. The present study also shows that atropine did not completely block the post-tetanic contraction, though spantide almost completely blocked the post-tetanic contraction. These strongly indicate that substance P participates in the post-tetanic contraction. In the present experiment, this contractile response appears to be derived from the enteric neurons, since tetrodotoxin completely blocked this contraction.

Interestingly, indomethacin completely antagonized this contraction. It is known that prostaglandin E\textsubscript{2} and F\textsubscript{2\alpha} are candidates as prostaglandins which induce contractions of guinea pig ileum. As it is reported that prostaglandin E\textsubscript{2} potentiated both those contractions evoked directly by the stimulation of smooth muscle or evoked indirectly via the parasympathetic nerve supply, it is possible that the effect of indomethacin on the contraction in the present study may be due to the the inhibition of both components. Atropine inhibited the contraction of prostaglandins via a parasympathetic nerve supply, and the part of endogenous prostaglandins released by tetanic stimulation may also induce the contraction via direct stimulation. Therefore, the post-tetanic contraction may have only a tendency to be decreased by atropine. The characteristics of the post-tetanic contraction in the present study and the post-tetanic inhibition in our previous study as well as interactions between these contractile antagonists should be further investigated.

These results indicate that substance P, prostaglandins and acetylcholine from the enteric neurons induce post-tetanic contraction in the presence of NLX, which was essential for this response and also blocked the post-tetanic twitch inhibition (the endogenous opioid action) (Figs. 1, 3 and 5). Although these contractile substances are known to be co-localized in guinea-pig myenteric neurons depending on the frequency of electrical stimulation, they make differing contributions to the associated motor response. For example, low frequencies of electrical stimulation are entirely cholinergic, and substance P released by some conditions is involved in responses of longer duration. Therefore, the results of this study may indicate that contractile substances (substance P, prostaglandins, and ACh) and endogenous opioids, the response of which was blocked by NLX, are concomitantly released by tetanic stimulation, and that these contractile substance-induced contractions (post-tetanic contraction) may be maintained by involvement in contractile responses of longer duration, even after the cessation of tetanic stimulation.

Both tetanic stimulation and the presence of a high concentration of NLX were essential for the development of post-tetanic contractions. This may indicate two possible mechanisms. One is that the endogenous opioids inhibit the release of contractile substances from the enteric neurons, and the high concentration, not low concentration, of NLX removed the release-inhibition of these contractile substances which were blocked by endogenous opioids. Another possible mechanism is that a high concentration of NLX acts directly on the releasing system(s) of these contractile substances. Whichever occurred one, the other or both, these accumulated contractile substances were released by a high concentration of NLX with tetanic stimulation, so that a sustained response to the contractile substances might be detected.

In conclusion, the present results show that tetanic stimulation in the presence of a high concentration of NLX induces an increase in basal tension (post-tetanic contraction) in guinea-pig ileal muscle due to the release of endogenous ileal contractile substances (substance P, prostaglandins and ACh) from the enteric neurons. These results suggest that the endogenous opioid system induced by tetanic stimulation (post-tetanic twitch inhibition) participates in the response to these contractile substances in the ileum.

REFERENCES