MECHANISM OF BUFALIN-INDUCED BLOCKADE OF NEUROMUSCULAR TRANSMISSION IN ISOLATED RAT DIAPHRAGM

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Abstract The mechanism of blockade by bufalin at the neuromuscular junction was examined by using the isolated diaphragm of the rat. Bufalin appears to reduce acetylcholine sensitivity at the motor end-plate by causing prolonged depolarization. According to the results of the studies of the post-tetanic potentiation, bufalin appears to have both stimulant and depressant phases of action on the motor nerve terminals, i.e., in smaller doses, it acts as a stimulant and in larger doses (corresponding to the doses of blockade), as a depressant. The amount of acetylcholine release was considerably increased immediately after washing out of bufalin treatment (incubated for 30 min with \(10^{-4}\) g/ml), whereas in the presence of the same concentration of bufalin, obviously decreased. With regard to blocking effect, bufalin added to the bathing fluid was more potent than infused i.v. which indicates that the pre-junctional effect of this agent is essential for its blockade at the neuromuscular junction.

Bufalin is not only a cardiotonic steroid but also a powerful topical anesthesia, and is one of the various active constituents of the Chinese drug ch'ian su (senso in Japan), which is prepared from the skin of Bufo bufo gargarizans.

Effects of cardiotonic steroids, such as ouabain or digoxin, on the neuromuscular junction have been studied by many investigators, however considerable data is yet to be elucidated.

In a previous paper (1), experiments were done with several cardiotonic steroids including this agent, which showed a blockade of neuromuscular transmission in isolated rat diaphragm, gastrocnemius muscle in situ or chick biventer cervicis preparations.

Reported herein is a study of the mechanism of the blockade by bufalin on the neuromuscular junction of rat diaphragm.

MATERIALS AND METHODS

All experiments were performed on isolated diaphragm nerve-muscle preparations from male Wistar rats weighing 250-300 g. The preparation was placed in Tyrode solution having the following composition (expressed in mM/litre): NaCl, 137; KCl, 2.6; CaCl₂, 1.8; MgCl₂, 1.05; NaHCO₃, 11.9; NaH₂PO₄, 0.41; glucose, 5.5 and gassing with a mixture of 5% CO₂ in O₂. The temp. of the bathing fluid (40 ml) was maintained at 28-30°C and the phrenic nerve was stimulated by Nihon Kohden electronic stimulator Model MSE-3R at a frequency of 0.1 Hz with supramaximal rectangular pulses of 0.2 msec duration.
1. **Muscle twitch response**

The preparation was essentially the same as that described by Bülbbring (2) except for the electrode for stimulating the nerve as described by Mogey, Trevan and Young (3).

2. **Retrograde injections of acetylcholine and other drugs**

The method of making the preparation was similar to that as described by Paterson (5). The right hemidiaphragm, with the corresponding phrenic nerve, and the thoracic and abdominal inferior vena cava, was put into oxygenated bathing fluid. After ligature of the abdominal inferior vena cava, a polyethylene cannula was inserted and made stationary in the thoracic vena cava.

When a minute amount of acetylcholine was rapidly injected into the cannula, a tetanic twitch response was observed. The magnitude of the tetanic twitch height was to some extent dependent on the injection speed. Although Harris and Leach (6) have devised a technique for constant injection, the authors found, after several attempts that the constant injection can be manually controlled.

A further experiment was performed by using the “retrograde injection” technique for quite a different purpose, that is the test solution was infused slowly and continuously through the cannula replacing the acetylcholine rapid injection. This technique was attempted in order to verify whether or not the effect of test solutions on neuromuscular junctions differ regarding the two routes of application.

3. **Post-tetanic potentiation of muscle twitch response**

As in the method described by Segawa et al. (7), when studying the effects of drugs on the post-tetanic potentiation (PTP) of muscle twitch response, the tetanic stimulation, 30 Hz for 10 sec, was used to induce the PTP.

4. **Chronic denervation of the right hemidiaphragm**

Rats were anesthetized with ether and the right phrenic nerve was cut at the level of the right atrium through an intercostal incision. The rats were then allowed to recover and were sacrificed 6 days after the denervation. The denervated diaphragm was then set up in the same way as for the innervated preparation.

5. **Assay of acetylcholine release**

The whole diaphragm with intact phrenic nerves and minimal costal attachments was immersed in 5 ml of Tyrode solution aerated with a mixture of 5% CO₂ in O₂, at a room temp. of 25-27°C. In order to reduce the pH and minimize the spontaneous hydrolysis of acetylcholine, the NaHCO₃ in Tyrode solution was replaced with an equimolar amount of NaCl. The preparation was incubated for 30 min with eserine sulfate (10⁻⁵ g.ml) before each experiment to inhibit acetylcholinesterase. Supramaximal indirect stimulation was given for 10 min using a 0.2 msec pulse duration and stimulus frequency of 50 Hz. Samples for assay of acetylcholine were collected only after 5 min to allow time for the released acetylcholine to diffuse out into the medium. Between successive periods of stimulation, preparations were left still for at least 10 min.

Male guinea-pigs weighing 200-300 g were sacrificed by a blow on the head and the ileum was removed. The ileum was cooled for 5-8 hr at 2°C and afterward dissected.
into small segments (2-3 cm long) then suspended in 2 ml of oxygenated Tyrode solution containing CaCl₂ (10 times the normal concentration) and morphine hydrochloride (5 mg/l) at 22-25°C. Contractile response of the ileum was recorded on a smoked paper with a semi-isotonic lever. Before assay of acetylcholine, bufalin in samples was extracted by shaking three times with 5 ml of chloroform. The muscle preparation was exposed to the sample solution or the standard solution for 30 sec with considerable accuracy and contractile height was recorded. Between successive determinations of acetylcholine, muscle was left still for 45-90 sec. Assays were carried out by the 3-point method.

Alternatively, preliminary assays were performed on isolated heart of Meretrix lasoria (8) which was essentially the same as that of Venus mercenaria (9). As Meretrix heart is considered to be more specific for acetylcholine than guinea-pig ileum, accordingly the heart was tested to determine whether or not the ileum would be suitable for assay. An alkaline hydrolysis test was performed on the assayed sample to confirm that the assayed substance was acetylcholine.

6. Drugs used

Acetylcholine chloride (Ovisot, Daiichi), d-tubocurarine chloride (Amelisol, Yoshibari), succinylcholine chloride (Succin, Yamanouchi), ouabain (E. Merck), MgSO₄ (Kokusan Kagaku) and tetrodotoxin (Sankyo). Bufalin and resibufogenin, a cardiotonic steroid also derived from senso, were kindly provided by Dr. M. Komatsu and Dr. Y. Kamano, the Division of Organic Chemistry of Taisho Pharmaceutical Co., Japan. Bufalin and resibufogenin were prepared in a stock solution of 2 mg/ml in 50% ethanol in water.

RESULTS

1. Effects of bufalin and other chemicals on the twitch response induced by retrograde injection of acetylcholine

The mechanism of blockade by bufalin at the neuromuscular junction was examined using the isolated diaphragm of the rat. The initial consideration is whether or not the agent affects the post-synaptic membrane of the preparation, and the so-called retrograde injection technique, which was originally described by Burgen et al. (4) was utilized for determination.

Figs. 1 and 2 demonstrate the results of this experiment, in which the twitch response caused by retrograde injection of acetylcholine (1.25 mcg in 0.05 ml Tyrode) was depressed to the point of an artifact height, namely, 0.05 ml Tyrode-induced twitch height, with a dose of $5 \times 10^{-5}$ of bufalin. This dose of bufalin, as described (1), corresponds to that
Fig. 1. Effects of bufalin and other chemicals on the twitch response induced by the retrograde injection of acetylcholine (ACh). At dots, 1.25 mcg of ACh was injected every 3 min. At C, 0.05 ml of Tyrode solution was injected as an artifact height. At arrows, drugs were added to the bath. Frequency of the nerve stimulation was 0.1 Hz. SCH: succinylcholine chloride. EtOH: 50% ethanol solution, as a control for bufalin and resibufogenin.

Fig. 2. Effects of bufalin on the twitch response induced by the retrograde injection of ACh. (A) normal diaphragm. At dots, 2.0 mcg of ACh was injected every 3 min. At C, 0.05 ml of Tyrode solution was injected as an artifact height. At arrows, bufalin was added to the bath to give a final concentration of \(1.5 \times 10^{-5}\) g/ml. (B) diaphragm denervated for 6 days. At dots, 50 ng of ACh was injected every 3 min. Electrical stimulation was applied to the muscle (0.1 Hz).
producing the complete blockade of twitch response induced by nerve stimulation. This effect of bufalin on the twitch response induced by acetylcholine injection as well as the sensitivity to acetylcholine was obviously augmented in the preparations prepared from 6 days denervated animals (Fig. 2). These results indicate that bufalin reduces acetylcholine sensitivity at the motor end-plate by causing prolonged depolarization. After washing, the response to acetylcholine slowly returned to normal.

In addition to bufalin, effects of resibufogenin (like bufalin a cardiotonic steroid derived from senso) and ouabain were investigated. In a concentration of $5 \times 10^{-5}$, the twitch response was reduced in part in the former, while in the latter, no appreciable effect was observed with the same concentration.

Moreover, as a standard substance, succinylcholine chloride was approx. one hundred times more potent than bufalin.

2. Effects of bufalin on the PTP of muscle twitch response

In order to examine the pre-junctional component of action of bufalin, an experiment on the PTP was carried out. As described by Segawa et al. (7), a very simple method in which the modification of the increase in single twitch response after tetanic nerve stimulation was used as an index for the pre-junctional effect. Two values, PTP-magnitude and PTP-duration, have also been introduced by the same authors (7). The former indi-

![Graph showing effects of bufalin on PTP magnitude and duration](image-url)
cates the ratio of the maximum post-tetanic twitch tension to the pre-tetanic one while the latter implies the period from immediately after suspension of tetanus to the time when the potentiated twitch tension decreases to just the pre-tetanic level. The PTP was considerably affected by bufalin as has already been reported (1). In the present paper, supplemental experiments were performed and effect of this agent was investigated in greater detail (Fig. 3). In concentrations of $2.5 \times 10^{-9}$ to $1.0 \times 10^{-4}$ g/ml, the increase took place both in the magnitude and especially in the duration. Bufalin, in concentrations of up to $10^{-5}$, had no appreciable effect on the single twitch tension in almost all preparations; whereas, at the higher concentration of $2 \times 10^{-4}$, approx. two-thirds of the preparations showed an increase in single twitch tension, which may explain the incorrect calculation of PTP values. Thus, the effect of the higher concentrations of this agent was studied exclusively using the remaining one-third of the preparations.

Fig. 4 demonstrates typical results of the effects of the higher concentrations of bufalin on the PTP. An enhancement of PTP-magnitude was more striking at a concentration of $2 \times 10^{-4}$, and this effect was relatively slow, even after 30 min, enhancement was still observed. In contrast, the duration of PTP was not obviously potentiated with the higher concentration of bufalin.

At a concentration of $4 \times 10^{-5}$, the neuromyal blockade was seen in some preparations and not in others. Employing the latter, a further experiment with PTP was carried out. Fig. 4-B shows the result of this concentration of bufalin in which the magnitude increased after 10 min, recovered to normal after 20 min, was reduced after 30 min and completely vanished after 40 min; alternatively, the post-tetanic depression was obtained instead of the disappearance of PTP.

3. Effects of bufalin on the muscle twitch response induced by nerve stimulation when applied through two different routes

Slow infusion of bufalin solution through the vena cava was performed using a modi-
Fig. 5. Effects of bufalin on the muscle twitch response induced by nerve stimulation applied via two different routes. (A) and (C): slow infusion of bufalin solution ($5 \times 10^{-5}$ and $1 \times 10^{-4}$) via the vena cava had no appreciable effect on the twitch response. (B) bufalin added to the bath (final concentration: $5 \times 10^{-5}$) blocked the twitch response.

Fig. 6. Effects of MgSO$_4$ and tetrodotoxin on the muscle twitch response induced by nerve stimulation applied via different routes. At dots, drugs were added to the bath. At W, the bath fluid was changed. Period of drug infusion is indicated by the underline. Intravascular infusion of these drugs showed an equivocal effect.
fication of the retrograde injection technique, additionally equipped with an infusion pump in which the constant rate was adjusted to 0.1 ml/min. As mentioned previously (1), bufalin \(4-5 \times 10^{-5}\) added to the bath showed two phases of action on twitch tension of rat diaphragm induced by nerve stimulation: (A) twitch tension was progressively developed, and continued for approx. 20 min. (B) afterwards, a sudden reduction and a complete neuromyal blockade occurred. As shown in Fig. 5-A, unexpectedly, the above mentioned bufalin solution in a continuous infusion given for over 30 min via the vena cava revealed phase A but not that of B. Moreover, as shown in Fig. 5-C, \(1 \times 10^{-4}\) of bufalin caused slight reduction of twitch response but not a complete blockade.

Further experiments with tetrodotoxin, magnesium ions, d-tubocurarine and succinylcholine were carried out in the same manner. The effects of tetrodotoxin and magnesium ions on the muscle twitch response were, as is evident from Fig. 6, significantly weakened when a continuous i.v. infusion was given, whereas in the cases of d-tubocurarine and succinylcholine, such a difference between the two routes of administration was not apparent (Fig. 7).

![Fig. 7. Effects of succinylcholine (SCh) and d-tubocurarine (d-Tc) on the muscle twitch response induced by nerve stimulation applied via two different routes. The left tracing: samples added to the bath. The right tracing: samples infused intravascularly. These results contrast with these Figs. 5 and 6.](image)

4. Modification of effects of d-tubocurarine and succinylcholine on the neuromuscular junction of rat diaphragm by pretreatment with bufalin

In a previous experiment (1), it was observed that pretreatment with bufalin \(2 \times 10^{-5}\) reduced the d-tubocurarine effect and enhanced the succinylcholine effect on the twitch response induced by nerve stimulation. From these observations, it is hypothesized that bufalin is a depolarizing agent on the neuromuscular junction of rat diaphragm. In the present paper, these experiments were again performed using the preceding infusion method. In other words, effects of the pretreatment of bufalin on the d-tubocurarine or
the succinylcholine effects were studied via two routes in which bufalin was added either to the bath or infused into the vena cava. When bufalin was infused continuously into the vena cava, d-tubocurarine or succinylcholine was added to the bath and vice versa.

The pretreatment with bufalin \( (2 \times 10^{-5}) \) reduced the d-tubocurarine effect and enhanced the succinylcholine effect on the twitch response induced by nerve stimulation only when bufalin was added to the bath. Bufalin, when infused via the vena cava, showed little effect on the inhibitory actions of d-tubocurarine and succinylcholine.

5. Effects of bufalin on acetylcholine release by nerve stimulation

In order to verify whether the neuromuscular blockade by bufalin was due to its inhibitory action on acetylcholine release at the nerve terminal, the following experiments were carried out. The amount of acetylcholine released by nerve stimulation at 50 Hz for 10 min \( (10) \) ranged from 30 to 70 ng per intact diaphragm when using guinea-pig assays. According to Meretrix heart assays, however, the amount of acetylcholine released under these conditions was to some extent less. Fig. 8 shows the results of these experiments when using guinea-pig ileum assays. The amount of acetylcholine released varied from preparation to preparation but, in the same preparation, was fairly constant when the nerve was stimulated every 15 min at 50 Hz for 10 min.

The diaphragm was incubated with bufalin (final concentration: \( 10^{-4} \)) for 30 min at a room temp. (25–27°C) and afterwards, immediately washed out and stimulated. As

![Fig. 8. Effect of bufalin on ACh release from the phrenic nerve of the rat by the stimulation at frequency of 50 Hz for 10 min. ACh release before addition of bufalin is shown in the first two open columns. Muscle preparations were incubated with bufalin \( (10^{-4}) \) for 30 min, washed out and immediately stimulated. The ACh release in these conditions is shown in the dotted column. The lined column shows the ACh release in the presence of bufalin after incubation. Between successive collections of ACh, the muscle was left still for 10 min. Vertical bars indicate standard errors.](image-url)
shown in Fig. 8, the amount of acetylcholine released was approx. twice that of the control level. On the other hand, when the stimulus was given in the presence of bufalin after the incubation, the amount of acetylcholine released was reduced to less than 50% that of control level.

**DISCUSSION**

As has been reported in a previous paper (1), neuromuscular blocking actions of cardiotonic steroids were much weaker than those observed by Greeff and Westermann (11), and on the isolated rat diaphragm, in so far as were examined, only bufosteroids such as bufalin and resibufogenin caused a complete block of the response to nerve stimulation without affecting the response to direct stimulation when the concentration was increased. Greeff and Westermann (11), however, also observed that ouabain blocked the responses to both direct and indirect stimulations simultaneously; hence, the effect of bufalin or resibufogenin appears to be qualitatively and quantitatively different from that of ouabain.

In the present report, the mechanism of bufalin-induced blockade of the response to nerve stimulation was studied using rat diaphragm. In the previous report (1), bufalin was classified into a depolarizing type blocker, but from the results of experiments concerning the PTP etc, the possibility was advanced that the pre-junctional effect of bufalin involves the neuromyot blockade in addition to the post-junctional effect.

In the first experiment, effects on the post-junctional membrane were studied by the retrograde injection technique, in which bufalin, in the concentration causing the blockade of the response to nerve stimulation, also made the response to acetylcholine injection disappear. As this phenomenon was observed even in the diaphragm preparations from denervated animals, it can be considered to be purely a post-junctional effect. Moreover, the denervation supersensitivity to bufalin as well as to acetylcholine has been brought about, which may be explained by the fact that bufalin reduced acetylcholine sensitivity to the motor end-plate by causing prolonged depolarization. These findings appear to be supported by the fact that the effect of bufalin on direct stimulation was considerably enhanced even when using denervated preparations (1) because it is generally considered that, after nerve section, extra-junctional receptors appear, spreading with time after denervation over the entire surface of the muscle, so that a depression of direct stimulation is seen with depolarizing agents (5).

Next, effects of bufalin on the PTP were studied. No direct evidence has been obtained to explain the mechanism of PTP, but the hypothesis that PTP is accounted for by the acceleration of the transmission mechanism in the pre-synaptic terminals is generally supported (12). As mentioned previously (1), bufalin, in concentrations not affecting single twitch response, increased both the magnitude and the duration, whereas other cardiotonic steroids studied, increased the duration without affecting the magnitude. Segawa, Kojima and Takagi (7) have investigated the effects of six anticholinergic agents on PTP and have observed that these six showed an abolition of PTP which may be explained by the depressive action observed on cholinceptive sites of the nerve terminals. Reports,
however, concerning cases of increase in PTP are usually not seen at present when this technique is employed. Moreover, in addition to cardiotonic steroids, chemicals reported to facilitate motor nerve terminals, such as phenol (13), guanidine (14), and others, had also little or no effect on PTP-magnitude (1). Consequently it cannot be considered that effects of bufalin on PTP were due to the facilitating action on the nerve terminals, but, at least, it can be postulated that bufalin has a greater effect on the motor nerve terminals than the other chemicals studied.

These increases in PTP were observed following application of bufalin ranging from $2.5 \times 10^{-6}$ to $2 \times 10^{-3}$ g/ml, while higher doses of this agent, on the contrary, caused a disappearance of PTP, which was thought to be attributed to the inhibition of the pre-synaptic terminals.

These tendencies were investigated more directly according to the determinations of the amount of acetylcholine released. After a relatively mild treatment of bufalin, acetylcholine was increased, whereas a high concentration of bufalin markedly decreased acetylcholine release. These findings suggest that bufalin has both stimulant and depressant phases of action on the motor nerve terminals.

When bufalin was infused via the vena cava, the neuromuscular blockade did not occur. This phenomenon was quite similar to that observed with tetrodotoxin and magnesium ions, which were generally thought to be the blocking agents acting mainly on the presynaptic nerve terminals.

On the other hand, blocking agents such as succinylcholine and d-tubocurarine, which may be considered to act on the post-synaptic membrane, showed the blockade with intravascular infusion. These observations suggest that the pre-synaptic effect of bufalin is essential for its blockade at the neuromuscular junction. Moreover, regarding the results of the PTP effect, it is assumed that the blockade by bufalin is due to the decline in transmitter release.

As mentioned in Results (4), bufalin, in the concentration not causing a blockade, reduced the effect of d-tubocurarine and enhanced the effect of succinylcholine, only when bufalin had been added to the bath. Bufalin infused via the vena cava had no appreciable effect on inhibitory actions of d-tubocurarine and succinylcholine. These results suggest that this pretreatment effect of bufalin is due to a pre-synaptic effect rather than a post-synaptic one.

From a different point of view, there is the possibility of a neuromuscular block as a result of an increase in the transmitter release. In other words, when a great many transmitters gain access to the motor end-plates, does a blockade result? This possibility can be contradicted by the following observations: (A) Neuromuscular blockade in rat diaphragm was not observed with eserine (7); (B) Bufalin had little or no effect on acetylcholinesterase (1); (C) Bufalin appears to reduce acetylcholine sensitivity at the motor end-plates; (D) With the concentrations causing neuromuscular blockade, bufalin is considered to be responsible for the final diminution in acetylcholine release.

Thus, the neuromuscular blocking action of bufalin is probably due both to the dec-
line in transmitter release from the nerve terminals and to the reduction of acetylcholine
sensitivity at the post-synaptic membrane.

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