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sucrose-gap study

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ABSTRACT cyanobacterial neurotoxin extract of blue-algae (*Ossoillatoria agardhii*) inhibited 100 mM K^+ -membrane potential of *Locusta migratoria* foregut muscles. In single sucrose-gap method, isotonic KCl saline depolarized the foregut muscle in the right chamber up to 6-8 mV and 4-6 mV in the left chamber followed with a slight contraction. Separate addition of cyanobacterial neurotoxin extract induced membrane depolarization of about 0.5 mV, but no mechanical activity was recorded. However, in double sucrose-gap method, 100 mM K^+ -induced an inward current of about 0.4 μA but no tension was seen. Pre-treatment with the extract for 10 minutes prior to 100 mM K^+ -addition induced an outward current of about 0.05 μA , but inhibited 100 mM K^+ -inward current by 50% of the control. The base line tension was lowered and the inhibitory effect was reversible, which may reflect a Na^+ / Ca^{2+} -exchange. This study suggested that the neurotoxin extract would reduce firstly the influx of external Ca^{2+} , and secondly would reduce the trans-membrane Na^+ -gradient so disturbing Na^+ / Ca^{2+} -counterexchange postulated that there is a Na^+ / K^+ -pump involved during membrane depolarization. The cyanobacterial neurotoxin effect could be related to the simultaneous production of both alkaloid and peptide toxins from a single cell of *Anabaena flos-aquae* (NRC-44-1). In addition, this neurotoxic effect could be due to different types of toxins produced at the same time, such as saxitoxin and anatoxin-a, which may cause paralysis by inhibiting the

inward flow of Na^+ -ions in nerve tissue or may caused a depolarizing blockade of the indirectly elicited twitch in muscle.

INTRODUCTION

Animal deaths due to cyanobacterial blooms have been reported from many different parts of the world (for references see Mutwally & Jamel Al-Layl, 1992 & 1993; Ismail & Jamel Al-Layl, 1995; Jamel Al-Layl, 1996). However, in most of these incidents it was difficult to establish the final cause of animal death. On the other hand, cyanobacterial produce microcystins toxic materials, which effect either the liver or the nervous system (Codd & Poon, 1988; Carmicheal *et. al.*, 1990; Kiviranta *et. al.*, 1991; Mutwally & Jamel Al-Layl, 1992 & 1993).

Microcystins are potent and specific inhibitors of protein phosphatase *in vitro* with the same action as the tumor-promoting toxin of diarrhetic shellfish poisoning, okadaic acid (Lawton & Codd, 1988). The larvae of small shrimp and mosquitoes were also affected with the addition of neurotoxin and hepatotoxin cyanobacterial (Lightner, 1978; Turell & Middelbrook, 1988; Kiviranta *et. al.*, 1991).

The bioelectric characteristic of resting muscle cells result from an unequal distribution of ions on either side of the muscle membrane. The sign and size of the potential difference across this membrane is determined mainly by the relative permeability of the inorganic ions Na^+ , K^+ and Cl^- (Mutwally, 1990). Stampfli (1954) introduced the sucrose-gap technique, which has been used for the continuous recording of electrical activity in visceral muscles and muscles exhibiting rhythmic contractile activity (Lennard & Huddart, 1989; Mutwally, 1990). However, a single sucrose-gap method has intrinsic limitations in its application. A double sucrose-gap method first introduced by Berger (1963) is advantageous in that it is possible to polarize a large region of cell membranes in an approximately uniform way. The usage of double sucrose-gap method is to make possible a stable potential record for long periods of time and measurement

of membrane resistance and current.

In the preceding papers of Mutwally and Jamel Al-Layl (1992 & 1993) the extract of blue-algae (*Ossoiillatoria agardhii*) (10^{-5} , 5×10^{-5} & 10^{-4} M), inhibited the spontaneous activity (Spt.act), 100 mM K^+ -contractures and field stimulation- (FS) responses of *Locusta migratoria* foregut and hindgut muscles in dose-dependent manner. The aim of this present study is to extend the knowledge of the neurotoxicity of cyanobacterial (*Ossoiillatoria agardhii*) on locust foregut muscles using sucrose-gap and voltage-clamp techniques.

MATERIALS AND METHODS

Experimental animals: Adult locusts of both sexes reared in laboratory culture were used throughout this study. The colony was maintained at 27°C and fed on green barley or grass daily with water supplied *ad libitum*. The details of the dissection of the isolated foregut muscles, the saline used, the methods to maintain the preparation *in vitro* and the methods to record tension have all been described in the preceding papers of Mutwally and Jamel Al-Layl (1992 & 1993). Single and double sucrose-gap techniques were applied according to Mutwally (1990), Huddart *et. al.* (1992) and Nelson and Huddart (1992).

Chemical and neurotoxin extracts: All chemicals used in this study were obtained from Sigma Chemical Company. All drugs tested were prepared freshly from stock solutions and were added to the organ bath as indicated. However, the extract of blue-algae (*Ossoiillatoria agardhii*) was kindly supplied by Dr. K.M. Jamel Al-Layl, Biology Department, Umm Al-Qura University, Makkah, which was similar to the extracts been used previously by the author.

Isotonic KCl saline (172 mM) (12.8 g / l) and isotonic sucrose solution (172 mM) (58.82 g / l) were made up in distilled water. In the preceding papers of Mutwally and Jamel Al-Layl (1992 & 1993), 5×10^{-5} M cyanobacterial extract gave optimum responses on both locust muscles and thus same concentration is being used in this present study.

Muscle preparations: Locust foregut muscle is about 1 to 1.5 cm long and was used in double sucrose-gap techniques to study the effect of 5×10^{-5} M *Ossillatoria agardhii* extract on membrane potential and 100 mM K⁺-depolarization. For muscle tension recording, Grass Instrument model 79D polygraph and Grass FT 0.03 force-displacement transducers were used to record the effect of the extract on 100 mM K⁺-depolarization of locust foregut muscles.

Sucrose-gap: Muscle tension changes and currents were recorded on an Elonex PC386SXM/16 microcomputer equipped with an Intracell Analogue Data Capture and Display (ADCAD) System via Grass low level d.c. amplifiers. The organ bath was maintained at 20°C with a Grant closed circuit cooler / circulatory system and the bath contents were constantly aerated. Double sucrose-gap apparatus consists of three chambers. Four thin rubber membranes were prepared, two of which were respectively fixed to KCl chamber left and right by using a rubber band and the others were glued to both sides of the central chamber with rubber cement. Small holes of about 0.2 mm were made in the membranes by employing a fine needle. The preparation was mounted in chambers by passing it through four holes of the membranes, and the chambers were set up by a pair of long bolts to form five compartments, *i.e.* two KCl compartments, two sucrose compartments and one central compartment. The central compartment was perfused with insect normal saline or test solutions through inlet and outlet fine tubes and the sucrose compartments were also inflow through a fine tube. The flowrate in the central compartment and sucrose compartments was approximately 0.5 ml / min.

Three flat tip Ag / AgCl IVM electrodes were placed in each of the left, right and central compartments of the gap to allow simultaneous recording of membrane potential and transmembrane currents. These electrodes were connected through a high impedance probe to an HSE microelectrode / voltage clamp amplifier and then through a Grass low level d.c. amplifier to the ADCAD system and onto a microcomputer. The recorded potential differences between

the electrodes in thigh and left represented the compound resting potential of the left side of the muscle. This was usually at a maximum within 2 to 10 minutes of depolarization.

A double sucrose-gap system was employed, in collaboration with a HSE voltage-clamp amplifier (Type 309A/310) to record agonist-induced membrane currents. This recording system was based on Coburn *et. al.* (1975), which was modified by Hasimoto and Kobayashi (1981) and further modified to reduce right and left flow chamber volumes to about 800 μ l. Simultaneous electrical and mechanical responses from the test chambers were recorded on Grass model 79D polygraph. Measurement of membrane potential was between the right hand and center compartments and measurement of transmembrane current was carried out indirectly through the HSE amplifier. A voltage clamp current was injected through the left-hand compartment, which created a stable clamp between left and center compartments allowing the muscle to be held at any chosen membrane potential. Any agent that added to the center test compartment would tend to drive the tissue away from the holding potential straining the clamp. To keep the clamp stable the HSE amplifier produces a current exactly equal to but opposite to that produced by the cells. It is this current, after polarity reversal that is seen as the current produced by the muscle cells in response to the test agent.

This study followed three steps in examining the effect of drugs on membrane potential:

- 1- Effect of 100 mM K^+ on foregut muscle electrical and mechanical responses (in single and double sucrose-gap studies).
- 2- Effect of neurotoxin extract (5×10^{-5} M) alone, on the muscle electrical and mechanical responses (in single sucrose-gap studies).
- 3- Effect of 10 minutes pre-treatment of neurotoxin extract (5×10^{-5} M), on subsequent 100 mM K^+ -induced responses (in double sucrose-gap studies).

Each result was a replicate of 8 experiments, run for 20 to 30 minutes and followed by 20 minutes washing period with normal saline.

RESULTS

Single sucrose-gap studies show the depolarization effect of foregut muscle in the right side chamber with isotonic KCl. In eight separate preparations, the compound membrane potential was from 6 - 8 mV accompanied with a slight contracture (Figure 1a). The depolarization effect of the foregut tissue in the left side chamber (test chamber) with isotonic KCl and the compound membrane potential was between 4 - 6 mV, but no contracture was seen (Figure 1b). Separate addition of 5×10^{-5} M cyanobacterial extract induced relatively little depolarization about 0.5 mV, accompanied with slow onset of rhythmic responses, but no mechanical response was recorded (Figure 1c).

When foregut muscle was examined in the double sucrose-gap and voltage clamped at normal resting potential, 100 mM K^+ -application was seen to induce an inward transmembrane current of about 0.4 μA , but no tension was recorded (Figure 2a). Addition of 5×10^{-5} M cyanobacterial extract on the preparation for 10 minutes prior to 100 mM K^+ -application induced 0.05 μA outward current, while the addition of 100 mM K^+ -induced inward current of about 0.15 μA . The muscle base line tension was dropped due to this treatments (Figure 2b). Upon returning to control condition, (addition of 100 mM K^+) at the end of the experiment, the membrane potential induced resting potential of about 0.2 μA inward current, but no muscle response was recorded (Figure 2c). In all experiments, muscle tensions and membrane potentials were returned to normal state after washout with insect normal saline (2 mM Ca^{2+}).

DISCUSSION

In this study, the mean observed compound membrane potential seen was 6-8 mV and the mean depolarization seen with 100 mM K^+ in the test chamber was 4-6 mV. Mutwally (1990) studied the electrical activity of the locust foregut muscle using single sucrose-gap technique applying different concentration of Ca^{2+} -saline, KCl, neuropeptide (LMS) and

different amino acids. The K^+ -contractions and the membrane potentials of this muscle were uncoupled. Moreover, the membrane depolarization increased directly with increasing external K^+ -levels. In addition, this depolarization persisted in Ca^{2+} -free saline, a Na^+ -influx and not a Ca^{2+} -influx probably mediates this depolarization. This may also increase Na^+ -conductance, which is controlled by Ca^{2+} -bound to the membrane (Reilly and Peretz, 1987; Huddart and Hill, 1988; Mutwally, 1990). In this study similar results were found.

Mutwally and Jamel Al-Layl (1992 & 1993) reported that K^+ -contractures and FS-responses of locust foregut and hindgut muscles were inhibited with pre-treatment of (10^{-5} , 5×10^{-5} & $10^{-4}M$) blue-algae (*Ossoillatoria agardhii*) cyanobacterial neurotoxin extracts. This inhibitory action was dose-dependent and was stronger on foregut muscles. They also concluded that the neurotoxin effect was stronger on the tonic responses than on the phasic one and the site action of this extract affected the neuronal plexus driving both muscles. However, the extract indirectly inhibited the transmembrane Ca^{2+} -transport, which could be through Na^+ -channel activity or Na^+ / K^+ -pump affecting Na^+ / Ca^{2+} -counterexchange Mutwally and Jamel Al-Layl (1993). The extract did not affect the K^+ -contractures because of isotonic K^+ -addition, which may depolarizes both muscle compartments fully and ion-channels are open. These results suggested that this extract have strong effect on non-depolarized muscles than on the depolarized one. Mutwally (1990 & 1993) concluded that spontaneous activity and K^+ -contractures are dependent upon $[Ca^{2+}]_o$.

The toxic effect of cyanobacterial could be related to the simultaneous production of both alkaloid and peptide toxins from a single cell of *Anabaena flos-aquae* (NRC-44-1) (Jamel Al-Layl *et. al.*, 1988) and to different types of toxins produced at the same time (Sivonen *et. al.*, 1989). The recent work of Ismail and Jamel Al-Layl (1995) and Jamel Al-Layl (1996) were focused on the toxicity effect of cyanobacterial on mice liver tissues and on blood of albino rats, which damaged both cells respectively.

Single and double sucrose-gap voltage clamp studies show that control dose of 100 mM K^+ , used in this study, induced a transmembrane inward current of about 0.4 μA , leading to a membrane depolarization of about 4 mV accompanying a weak contracture. K^+ -induced depolarization is thought to directly activate voltage operated Ca^{2+} -channel *via* voltage sensor sub-unit (Reuter, 1983). This may suggest voltage-dependent events as described in mollusc (Huddart *et. al.*, 1992; Nelson & Huddart, 1992). K^+ -induced depolarization may release bound cellular Ca^{2+} needed for contraction by different membrane transduction routes, or it may even access different cellular Ca^{2+} -stores (Reilly & Peretz, 1987). When depolarization persisted in Ca^{2+} -free saline, a Na^+ -influx and not a Ca^{2+} -influx probably mediates this depolarization. This may increase Na^+ -conductance, which is controlled by Ca^{2+} -bound to the membrane (Reilly & Peretz, 1987; Huddart & Hill, 1988; Mutwally, 1990). In *Busycon* muscle, Hill and Lici (1981) suggested that there is a Na^+ / K^+ -pump involved during depolarization. In addition, Dorsett and Evans (1989) concluded that in molluscan muscle, there is a Cl^- -transport involved in membrane potential development. This observation may reflect differences in the way that $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ are mobilizes to activate muscle contractions.

Some members of the *Gonyaulax* complex produce Saxitoxin, a tetrahydropurine. Saxitoxin was found to cause paralysis by inhibiting the inward flow of Na^+ -ions in nerve tissue (Steidinger & Baden, 1984). Anatoxin-a (AnTX-a) is an exotoxin isolated from filamentous, freshwater, blue-green algae *Anabaena flos-aquae* (Carmichael *et. al.*, 1975). Spivak *et. al.* (1980) reported that AnTX-a, a semirigid, bicyclicamine, caused a depolarizing blockade of the indirectly elicited twitch in frog sartorius muscle, and when endplate regions of this muscles were voltage-clamped, AnTX-a induced endplate currents and concomitant increases in endplate current noise.

In squid giant axon membrane, the aphantoxin was found to be very potent and specific

inhibitor of voltage-dependent Na^+ -channels but with no effect on the K^+ -conductance (Adelman *et. al.*, 1982). The aphantoxin was also found to be as effective as saxitoxin at equivalent concentrations and had the same dose-response relationship as tetrodotoxin (Cuervo & Adelman, 1970).

In skeletal and smooth muscles, it was postulated that there are two different Ca^{2+} -binding sites: First, a superficial site (bind Ca^{2+} -loosely), which were releases by excess of K^+ -ions, and second, a sequestered site (bind Ca^{2+} -tightly), which were releases by ACh (Frank, 1963; Tashiro & Yamamoto, 1971). It was postulated that, by depolarization produced by electrical stimulation or by ACh, Ca^{2+} -ions are released from the plasma membrane and also from the sarcoplasmic reticulum (SR) (Tashiro & Yamamoto, 1971).

Mahmood and Charmichael (1987) concluded that the inhibition of electric eel acetylcholinesterase (AChE) and horse serum butyrylcholinesterase (BuChE) by AnTX-a (s) was time- and concentration-dependent, which was irreversible inhibition. Charmichael *et. al.* (1979) concluded that AnTX-a is a potent depolarizing neuro-muscular blocking agent possessing both muscarinic and nicotonic activity. Biggs and Dryden (1977) suggested that, in frog muscles, the major effect of AnTX-a is on the post-synaptic part of the neuro-muscular junction but there is also a pre-synaptic action, which leads to a reduction in the frequency of miniature end plate potentials. Eriksson *et. al.* (1986) suggested that the death of muskrat might have caused by the accumulation of microcystins in mussel's tissues.

In single sucrose-gap study, separate addition of cyanobacterial neurotoxin extract induced membrane potential depolarization, but no mechanical activity was seen. However, in double sucrose-gap studies, the extract caused 0.05 μA outward current and reduced 100 mM K^+ -inward current transmembrane up to 50% as compared to the control. The base line tension was lowered due to this treatment and the inhibitory effect was a reversible. This may reflect a

Na⁺ / Ca²⁺-exchange, since both ion fluxes in many smooth muscles are strongly inter-dependent (Mangel *et. al.*, 1982).

Moreover, In this study, a noticeable feature of this toxic extract was the induction of rapid spike-like membrane oscillations. This extract may decreased membrane potential and Na⁺ / K⁺-ATPase due to fall in membrane potential which initially increases the excitability of muscle fibers, but as the membrane potential falls further, excitability begins to decrease. Mutwally and Jamel Al-Layl (1992) suggested that the neurotoxin extract would reduced firstly the influx of external Ca²⁺, and secondly would reduced the transmembrane Na⁺-gradient so disturbing Na⁺ / Ca²⁺-counterexchange.

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أثر المستخلص السمي للطحلب الأزرق المخضر على عضلات الأمعاء الأمامية للجراد الرحال: دراسة باستخدام غشاء

السكروز (العازل الكهربائي)

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الملخص:

أظهرت هذه الدراسة أنه باستخدام تجربة غشاء السكروز المفرد (العازل الكهربائي) وعند إضافة محلول كلوريد البوتاسيوم الأيزوتوني في الجهة اليمنى من الجهاز قد تسبب في أحداث (depolarization) لاستقطاب الغشاء الخلوي لعضلة الجراد الرحال الأمامية. بجوالي ٦-٨ مليفولت و ٥-٦ مليفولت للجهة اليسرى من الجهاز، تبعها أنقباض بسيطة للعضلة. وعند إضافة المستخلص السمي لطحلب أوسيلانوريا أقارديا بمفرده، أحدثت هذه الأضافة لاستقطاب الغشاء الخلوي بمقدار ٠.٥ مليفولت، ولكن بدون تسجيل أي أنقباض عضلية. هذه النتيجة أوحى بأن المستخلص قد أثر على النهايات العصبية المنتشرة على العضلة.

وعند استخدام تجربة غشاء السكروز الثنائي (عازلين كهربائين)، وجد ان اضافة ١٠٠ مليمول بوتاسيوم (الظابط) سجلت (**Depolarization ; inward current**) لاستقطاب الغشاء الخلوي بمقدار ٠.٤ ميكروأمم ولكن لم تسجل أي أنقباض عضلية. وبأضافة المستخلص الطحلي السام على نفس العضلة وقبل اضافة محلول البوتاسيوم بعشرة دقائق، لاحظنا أن المستخلص قد أحدث (**Hypopolarization ; outward current**) فوق استقطاب الغشاء الخلوي بجوالي ٠.٠٥ ميكروأمم، وفي نفس الوقت ثبتت هذه الأضافة اللااستقطاب المستحدث بالبوتاسيوم للنصف (٠.٢ ميكروأمم) مقارنة بالظابط. مستوى الشد العضلي ارتقى للأسفل، كما أن أثر الشيط كان رجعيا للحالة الطبيعية بعد غسل العضلة بالمحلول الفسيولوجي الطبيعي، هذه النتائج أوحى بوجود مضخة للصوديوم / الكالسيوم تتحكم في تبادل هذه الأيونات عبر الغشاء الخلوي.

نتائج هذه الدراسة تقترح بأن المستخلص السمي قد أثر على النهايات العصبية بشيطه أولا، دخول الكالسيوم المتواجد خارج الخلية، وثانيا بشيطه لمستوى أيونات الصوديوم المتواجدة على غشاء الخلية مما يؤدي إلى اختلال عملية تبادل أيونات الصوديوم / الكالسيوم، على أفراض أن لمضخة الصوديوم / البوتاسيوم تدخل في عملية كهربائية الغشاء الخلوي. كما وأنها قد تؤكد بأن للمستخلص المستخدم أثرا سمي على النهايات العصبية المنتشرة على هذه العضلة. قد يكون سبب ذلك هو الأفرزات الناتجة عن التحولات السريعة في الطحلب لكلا النوعين السامين: القاعدي والبيتيدي المستخلصان من خلية طحلبية واحدة، أو لأنواع سامة أخرى مختلفة ولكن منتجة في نفس الوقت من الخلية الطحلبية مثل ساكستوكسين و أناتوكسين-أ، اللذين يحملان سميها شلل للنهايات العصبية وذلك بشيط أيونات الصوديوم الداخلة للخلية أو أنها تحدث تشيط غير مباشر للأنقباضات العضلية والعصبية.

FIGURE LEGENDS

Figure 1. Effect of (5×10^{-5} M) cyanobacterial extract and 100 mM K^+ - addition on locust foregut muscles membrane potential in single sucrose-gap. Upper traces represent membrane potential recordings while the lower traces indicate mechanical activities. (a) Addition of isotonic K^+ -saline ($\bar{\quad}$) in the right chamber, induced membrane potential, (b) Recording of the left chamber membrane potential induced with the addition of 100 mM K^+ -saline (control, $\bar{\quad}$), (c) Membrane potential recordings of the muscle in the left chamber induced with the extract alone ($\bar{\quad}$), and (-) represent the addition of normal saline for washing in all experiments. The membrane potential, force and time calibrations were standardized for each experiment reported in all experiments as indicated on the right hand side of the Figures.

Figure 2. Effect of (5×10^{-5} M) cyanobacterial extract and 100 mM K^+ - addition on locust foregut muscles membrane potential in double sucrose-gap. Upper traces represent current membrane potential recordings while the lower traces indicate mechanical activities. (a) show the inward current developed in response to 100 mM K^+ -saline ($\bar{\quad}$) under voltage clamp conditions were the clamp potential was set at natural resting potential, (b) the extract induced slight initial outward current (first arrow) followed by an inhibition of 100 mM K^+ -induced current (2nd $\bar{\quad}$), (c) at the end, after washing with normal saline the addition of 100 mM K^+ -induced an inward current which stabilized to the control level under voltage clamp conditions were the clamp potential was set at natural resting potential. Upper (-) represent the addition of normal saline for washing in all experiments. The current, tension and time scales were identical in all the experiments above as indicated on the right hand side of the Figures.