สำนักงานกองทุนสนับสนุนการวิจัย (สกว.)
The Thailand Research Fund (TRF)

รายงานวิจัยฉบับสมบูรณ์

โครงการ *ผลของพิษงูเห่าไทย (Naja kaouthia) ต่อการทำงานของเซลล์กล้ามเนื้อหัวใจ: สารประกอบที่มีศักยภาพในการ พัฒนาเป็นยาเพิ่มแรงบีบตัวของหัวใจ*

โดย อ.น.สพ.ดร. กิตติพงษ์ ทาจำปา

เดือน ปี ที่เสร็จโครงการ
ธันวาคม 2558

สัญญาเลขที่ TRG5680085

โครงการ ***ผลของพิษงูเห่าไทย*** *(Naja kaouthia)* ***ต่อการทำงานของเซลล์กล้ามเนื้อหัวใจ: สารประกอบที่มีศักยภาพในการ พัฒนาเป็นยาเพิ่มแรงบีบตัวของหัวใจ***

ผู้วิจัย อ.น.สพ.ดร.กิตติพงษ์ ทาจำปา สังกัด คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

และ จุฬาลงกรณ์มหาวิทยาลัย (ต้นสังกัด)

กิตติกรรมประกาศ

งานวิจัยนี้ได้รับการสนับสนุนเงินทุนวิจัยจากทุนส่งเสริมนักวิจัยรุ่นใหม่ปี พ.ศ. 2556 จากสำนักงานกองทุนสนับสนุนการวิจัย สำนักงานคณะกรรมการการอุดมศึกษาและจุฬาลงกรณ์มหาวิทยาลัย สัญญาเลขที่ TRG 5680085 ให้กับ อ.น.สพ.ดร.กิตติพงษ์ ทาจำปา ทางผู้วิจัยขอขอบคุณเป็นอย่างยิ่ง แก่ผู้ให้ทุนสนับสนุนงานวิจัยนี้

ขอขอบคุณ สถานเสาวภา สภากาชาดไทยผู้เอื้อเฟื้อพิษงูเห่าไทยและเป็นส่วนให้งานนี้สำเร็จลุล่วงไปได้

ขอขอบคุณ Institut de Génomique Fonctionnelle, University of Monpellier, ผู้ให้ความเอื้อเฟื้อในเครื่องมือทดลองบางส่วน

Abstract

Project Code: TRG5680085

Project Title: Effects of Thai Cobra (Naja kaouthia) Venom on Cardiac Myocyte Function: Potential Lead Compounds for New Inotropic Drug.

Investigator: ***Kittipong Tachampa, Faculty of Veterinary Science, Chulalongkorn University***

E-mail Address: Kittipong.T@chula.ac.th

Project Period: June 2013 – June 2015

Naja kaouthia ***is the most commonly venomous snake found in Thailand and causes high mortality rate in most hospitalized cases. Its venom caused profound effect not only on the neuromuscular system but also on the cardiovascular system. Based on our preliminary study, we found that fraction 6 isolated from*** *N. kaouthia* ***venom (NK6) suppressed cardiac contraction in both rat*** *in vivo* ***model and in isolated perfused heart. However, in isolated cardiomyocytes, we found that NK6 perfusion increased calcium transient and enhanced myocytes shortening. Therefore, it has a potential to be a lead compound for developing a new cardiac positive inotropic drug. This study aimed to elucidate mechanism in which NK6 induces changing in cardiomyocytes function (i.e. calcium transient and myocyte shortening). NK6 was isolated and purified by means of ion exchange chromatography. Mouse cardiomyocytes were isolated using enzymatic perfusion in isolated perfused heart system. Morphological changed from rectangular to round shape-cells were observed after incubation with various concentration of NK6 in doses and time dependent manner. Calcium transient and myocyte shortening were measured based on fluorescence photometry and digital cell geometry measurement (Ionoptix^{Tm}), respectively. At high dose of NK6 (>100 uM), calcium transient was significantly increased simultaneously with an initial increase in myocyte shortening followed by losing its contraction ability. The dose dependent effect of NK6 from (0.01-1 uM) on calcium transient revealed two different binding affinities (Km 1 = 2.47 and Km 2 = 165.40) suggesting at least two interaction sites involving calcium homeostasis in cardiomyocytes. Pharmacological approach using nifedipine showed that NK6 was able to attenuate the effect of nifedipine on calcium transient, indicating a competitive binding at the L-type calcium channel. In addition, NK6 increased calcium transient in the presence of ryanodine and the effect of caffeine on calcium transient was augmented by NK6, indicating the second possible interaction site at the ryanodine receptor. Patch-clamp***

experiment revealed that NK6 blocked L-type calcium current in Cav1.2 expressed in HEK cells. A selective decrease in Cav-L currents, without affecting Cav-T conductance by NK6 was also confirmed. There was a decline of the plateau phase of cardiac action potential by NK6 confirming the inhibition of calcium current. The SERCA may not involve with the action of NK6 since the blocking of SERCA by Thapsigargin didn't changed the effect of NK6 inducing calcium overload. This study may benefit in the development of positive inotropic drugs aid to increased contractility of the heart in heart failure patients.

Keywords: Naja Kaothia Venom Cardiomyocyte Myocyte Shortening Calcium Transient

บทคัดย่อ

*รหัสโครงการ: **TRG5680085***

*ชื่อโครงการ: ผลของพิษงูเห่าไทย **(Naja kaouthia)** ต่อการทำงานของเซลล์กล้ามเนื้อหัวใจ: สารประกอบที่มีศักยภาพในการ พัฒนาเป็นยาเพิ่มแรงบีบตัวของหัวใจ*

ชื่อนักวิจัย: อ.น.สพ.ดร.กิตติพงษ์ ทาจำปา สังกัด คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

E-mail Address: Kittipong.T@chula.ac.th

*ระยะเวลาโครงการ: มิถุนายน **2556** ถึง มิถุนายน **2558***

*งูเห่าไทย **(Naja kaouthia)** เป็นงูมีพิษที่พบได้บ่อยในประเทศไทยและเป็นสาเหตุของการตายที่สูงในผู้ป่วยที่เข้ารับการรักษา พิษของงูเห่าออกฤทธิ์ไม่เฉพาะแต่ระบบประสาทและกล้ามเนื้อเท่านั้นแต่ยังมีผลต่อระบบหัวใจและหลอดเลือดด้วยตามผลการศึกษาเบื้องต้นเราพบว่าโปรตีนในแฟรคชั่นที่หกจากพิษงูเห่า **(NK6)** มีผลต่อการกดการบีบตัวของกล้ามเนื้อหัวใจทั้งในหนูทดลองและในหัวใจที่เอาออกมากำซาบด้วยสารละลายนอกตัวหนูทดลอง อย่างไรก็ตามในเซลล์กล้ามเนื้อหัวใจเดี่ยวที่แยกออกมาทางผู้วิจัยพบว่า **NK6** มีผลในการเพิ่มแคลเซียมภายในเซลล์กล้ามเนื้อหัวใจและทำให้เซลล์หดสั้นด้วยความแรงที่มากขึ้นดังนั้น **NK6** จึงเป็นสารที่มีศักยภาพในการพัฒนาต่อเพื่อเป็นยาเพิ่มแรงบีบตัวของกล้ามเนื้อหัวใจสำหรับผู้ป่วยหัวใจล้มเหลว การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อที่จะหากลไกการออกฤทธิ์ของ **NK6** ต่อการทำงานของกล้ามเนื้อหัวใจ ทั้งในแง่ปริมาณแคลเซียมที่เพิ่มขึ้นภายในเซลล์ **(calcium transient)** และการหดตัว **NK6** จากพิษงูเห่าถูกแยกและทำให้บริสุทธิ์ด้วยวิธีการแลกเปลี่ยนไอออนโครมาโตกราฟฟี เซลล์กล้ามเนื้อหัวใจของหนูเมาส์ถูกแยกออกจากหัวใจโดยการใช้เอนไซม์กำซาบหัวใจหนูที่ถูกเอาออกมา เซลล์ที่ถูกแยกออกมามีลักษณะเป็นทรงเหลี่ยมแต่เมื่อนำมาแช่ในสารละลายทีมี **NK6** เซลล์มีการเปลี่ยนแปลงรูปร่างเป็นทรงกลมในลักษณะตามความเข้มข้นและเวลาที่แช่อยู่ในสารละลาย **calcium transient** และการหดตัววัดด้วยวิธี **fluorescence photometry** และ **digital cell geometry (Ionoptix ^{Tm})** ที่ความเข้มข้นสูง **(>100 uM) calcium transient** เพิ่มสูงขึ้น อย่างมีนัยยะสำคัญพร้อมกับการเพิ่มขึ้นในช่วงแรกของการหดตัวของเซลล์กล้ามเนื้อหัวใจตามด้วยการลดความสามารถในการหดตัว ผลของ **NK6** ต่อ **calcium** ขึ้นกับความเข้มข้นโดยมีค่าการจับตัว **(binding affinity)** สองค่าคือ **Km1 = 2.47** และ **Km2** เท่ากับ **165.40** ซึ่งบ่งชี้ว่ามีอย่างน้อย **2** ตำแหน่งที่ **NK6** ออกฤทธิ์ในการกระบวนการรักษาสมดุลย์แคลเซี่ยมภายในเซลล์ เป็นที่น่าสนใจเป็นอย่างยิ่ง เมื่อ **NK6** สามารถลดผลของ **nifedipine** ต่อค่า **calcium transient** ซึ่งบ่งชี้ว่า **NK6** น่าจะออกฤทธิ์ผ่านทางโปรตีนขนส่งแคลเซี่ยมชนิดแอล นอกจากนี้ **NK6** ยังสามารถเพิ่มฤทธิ์ของ ไรยาโนดีนและคาเฟอีนในการเพิ่ม **calcium transient** บ่งชี้ว่า น่าจะมีโปรตีนตัวที่สองที่เป็นเป้าหมายของ **NK6** ซึ่งก็คือตัวรับชนิดไรยาโนดีน การทดลองแพทช์แคลมป์พบว่า **NK6** ยับยังกระแสแคลเซียมผ่านโปรตีนขนส่งชนิดแอลใน **HEK** เซลล์โดยไม่มีผลต่อโปรตีนขนส่งชนิดที พบการลดลงของระยะพลาโทของ **cardiac action potential***

*โดย **NK6** ซึ่งยืนยันการยับยั้งกระแสแคลเซียม โปรตีนขนส่ง **SERCA** ไม่เกี่ยวข้องในกระบวนการนี้ เนื่องจากการใช้ **Thapsigargin** ไม่เปลี่ยนผลในการเพิ่มแคลเซียมภายในเซลล์ การศึกษานี้จะเป็นประโยชน์พื้นฐานในการพัฒนายาช่วยเพิ่มแรงบีบตัวของหัวใจในผู้ป่วยหัวใจล้มเหลว*

คำหลัก : พิษงูเห่า เซลล์กล้ามเนื้อหัวใจ การหดตัวของกล้ามเนื้อหัวใจ การเพิ่มขึ้นของแคลเซียม

เนื้อหางานวิจัย

Introduction

Although snake bites can be fatally, snake venoms become a valuable natural source of biological molecules that potentially to be developed into a novel drug. It is due to the fact that snake venom contains a vast variety of naturally bioactive molecules including nucleotides and proteins in which affecting many vital physiological systems. *Naja kaouthia* ***(NK), the most commonly snake found in Thailand, is unique among venomous snake because it can produce a large quantity of venom. The proteome of NK venom was revealed recently (Kulkeaw et al., 2007) and found to consist of peptides with 61 proteins matched in the database. These proteins were classified into 12 groups according to the differences in their biological activities. Among these toxins, a group of 60–70 amino acid polypeptides known as cytotoxins or cardiotoxins is interesting due to a wide variety of pharmacological actions such as haemolysis, depolarisation of muscles (Dufton and Hider, 1988), muscle fusion, selective killing of certain type of tumour cells, inhibition of protein kinase C and muscle contraction (Kumar et al., 1996). Although the structural and biochemical properties of cardiotoxins are well studied, little is known about its action on cardiac muscle function particularly at cellular level. CTX isolated from*** *Naja simensis* ***(spitting cobra) were studied well*** *in vivo* ***or*** *ex vivo****. In isolated perfused rat heart, CTX seems to induce tonic contraction after a short term augmentation of twitch contractions (Sun and Walker, 1986). Similar results were obtained in the isolated guinea-pig papillary muscle (Harvey et al, 1982). In isolated skeletal and cardiac muscle preparations, purified CTX isolated from*** *N. simensis* ***demonstrated a contracture and depolarization.***

It was proposed that CTX may bind to the membrane and causes depolarization, which leads to calcium influx and/or release of calcium from the sarcoplasmic reticulum (SR) (Fletcher and Jiang, 1993). Moreover, the toxin may form membrane pores, which allow extracellular calcium to enter the muscle and cause contracture, and which cause depolarization and subsequent release of calcium from the SR (Harvey et al., 1982). In contrast, Chen and colleagues (2007) reported that the mechanisms of cytotoxicity by CTX is physically distant from its membrane damaging effect. This result suggests the other possible molecular target(s) on the membrane involved with calcium mobilization. Within the author knowledge, CTX from *N. kaouthia* ***has not been tested yet for its effect and mechanism of action on cardiac myocytes. These resulted in lacking of knowledge and information of this toxin. The present study reported***

for the first time about the action of NK6, a cardiotoxin isolated from Naja kaouthia venom, and its mechanism of action in which blocked the voltage gated calcium channel.

ระเบียบวิธีทดลอง

Materials and methods

Chemicals: All chemicals were purchased from Sigma-Aldrich and were of analytical grade. Acrylamide, Agarose, Bisacrylamide, Carboxymethyl (CM) cellulose, Coomassie brilliant blue, Ethylene diamine tetraacetic acid (EDTA), Fetal calf serum (FCS), HEPES, Heparin, L-glutamine, Glutaraldehyde, Low molecular weight marker (66–6.5 kD), Penicillin, Phyto-hemagglutinin (PHA), Proteinase K, DMEM medium, RIPA buffer, Sodium Dodecyl Sulphate (SDS), Streptomycin, Triton X 100, Tryphan blue, trypsin, Fura-2/AM, type I collagenase, Nifedipine, Ryanodine, Thapsigargin, Caffeine.

Animal: This study will conduct in accordance with the *Guide for the Care and Use of Laboratory Animals* ***and use protocols approved by the Institutional Animal Care and Use Committee, Chulalongkorn University. Rat ventricular myocytes were isolated from male healthy Sprague-Dawley rats weighing between 250-300 grams.***

Purification of toxin: Lyophilized venom (500 mg) kindly provided by Queen Saovabha Memorial Institute (QMSI, Thailand) was dissolved in water and was loaded onto a CM-cellulose column equilibrated with phosphate buffer saline (pH 7.2). NK6 was separated and purified from the fraction six of venom from *Naja kaouthia.*

Single cardiac myocyte isolation: Ventricular myocytes were isolated by modifying AFCS protocol (O'Donnell et al., 2007). Briefly, rat was anesthetized using with 1-3% isoflurane and 100% O_2. Deep pain reflex was checked to ensure that the rat was fully anesthetized. Aseptic technique was applied through the rest of experiment. Peritoneal cavity and chest was opened with a sharp scissor. The heart was cut and immediately placed the heart in a dish containing 10 ml of perfusion buffer at room temperature. Then, the heart was cannulated and perfused with perfusion buffer for 4 min at 3 ml/min to remove the remaining blood clot in the chamber. Then, switch to myocyte digestion buffer containing 0.14 mg/ml trypsin and 12 μM $CaCl_2$ and perfused for 10 minutes. The cell suspension was transferred to a 15-ml polypropylene conical tube and

rinse with 2.5 ml of room temperature myocyte stopping buffer contained serum to inactivate proteases. Rod shape myocytes were reintroduced to calcium to the final concentration of 1mM. Freshly prepared myocytes were subjected for calcium transient and myocyte shortening measurement.

Morphological evaluation: Rod-shaped cardiac myocytes were counted and observed for morphological changes using a hemocytometer and an inverted microscope (Olympus) before and after incubation with various concentration of cobra venom in PBS.

Simultaneously measurement of calcium transient and myocyte shortening: Calcium transient was measured based on fluorescene photometry system (Ionoptix™). Freshly prepared myocytes were loaded with 2 μM Fura-2 AM containing 0.04% Pluronic F-127 for 20 min at room temperature, perfused with Tyrode solution, and stimulated at 1Hz with 30V using a 6 msec pulse duration. Ca^{2+} transient recordings were obtained by measuring fluorescence intensity at excitation and emission wavelengths of 410/485 nm respectively and analyzed using IonOptix software (IonOptix, LLC, Milton, MA). Myocytes shortening, an indirect measurement of cardiac myocyte contractility, was measured based on edge-detection software. Movement of the myocyte during contraction makes the cells become shortening. The displacement of cell membrane then can be detected and recorded using high-speed video microscope. Computer software with edge-detection algorithm (IonOptix, LLC, Milton, MA) analyzed the displacement of the membrane and calculate for sarcomere length changed.

Transient expression of recombinant calcium channels:

HEK cells were cultivated in DMEM supplemented with 10% fetal bovine serum (Invitrogen). Hek cell transfection was performed using Lipofectamine® with a DNA mix containing the plasmid constructs that code for human Cav1.2 channel isoforms. The following cDNA sequences inserted in expression vectors were used (GenBank™ accession numbers are in parentheses): $Ca_V1.2$ (M67515). Two days after transfection, HEK cells were dissociated with Versen (Invitrogen), and plated at a density of ~ 35×10^3 cells per 35 mm Petri dish for electrophysiological recordings.

Electrophysiological recordings:

Macroscopic currents were recorded at room temperature using an Axopatch 200B amplifier (Molecular Devices). For whole-cell experiments on recombinant calcium channel, the

extracellular solution contained the following (in mM): 135 NaCl, 20 TEACl, 2 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES (pH adjusted to 7.25 with KOH, ~330 mOsm) and the internal solution contained the following (in mM): 140 CsCl, 10 EGTA, 10 HEPES, 3 Mg-ATP, 0.6 GTP, and 3 $CaCl_2$ (pH adjusted to 7.25 with KOH, ~315 mOsm). Same solutions were used for outside-out experiments, excepted that the intracellular medium contained no Mg-ATP. Borosilicate glass pipettes have a typical resistance of 1.5–2.5 MΩ. Recordings were filtered at 2–5 kHz. Data were analyzed using pCLAMP9 (Molecular Devices), and GraphPad Prism (GraphPad) software.

Skinned cardiac trabeculae experiments

Right ventricular rat trabeculae were dissected, chemically permeabilized with Triton X-100, and attached to T-clips as described previously (Tachampa et al., 2007). The simultaneous measurement of steady state isometric tension and myofibrillar ATPase activity over a range of free Ca^{2+} concentrations was conducted. Sarcomere length was set at 2.2 μm by laser diffraction. ATP hydrolysis was stoichiometrically coupled to NADH consumption, which was measured in a small cuvette (~25 μl) via UV light absorption (340 nm). Ca^{2+} activation induced force development and concomitant consumption of NADH in the measurement chamber, the rate of which became constant during steady state force development. Trabeculae were activated over a range of free [Ca^{2+}] to measure steady-state isometric tension and ATPase activity. Stiffness of myofilament was calculated from a slope between isometric tension and ATPase activity at a given tension.

Data and statistical analysis

In the first experiment, exponential fit was applied for various doses of cobra venom vs calcium transient. K_m and EC_{50} will be calculated based on exponential fit. Linear regression was fit for various doses of cobra venom vs myocyte shortening. Student paired t-test will be used to compare pre- and post-treatment of cobra venom. One-way analysis of variance (ANOVA) will be used for comparing the difference among the inhibitors treated groups. P-value < 0.05 will be considered statistically significant. Dunnett's Multiple Comparison Test were used to test the difference from control groups. Electrophysiology data are presented as the mean ± SEM, and compared using student t-tests or ANOVAS for multiple comparisons. n is the number of cells used.

ผลการทดลองและบทวิจารณ์

Results and Discussion

A. Isolation of NK6 from Thai cobra venom

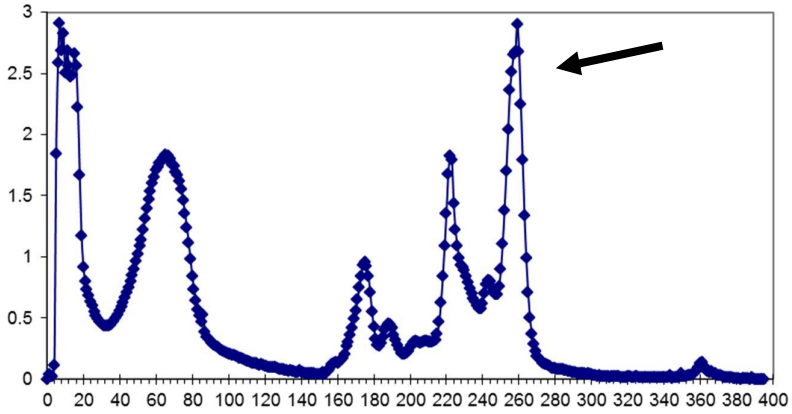


Figure 1. Fractionation of Thai cobra venoms by Resource® S cation exchange chromatography. Ten milligrams of the venom were injected into the column and eluted by a linear gradient (0 to 0.8 M NaCl) Flow rate was 1 ml/minute.

N. kaouthia venom was applied on CM-cellulose column equilibrated with phosphate buffer and further purified by cation exchange chromatography. Chromatogram of N. kaouthia venom is shown Figure 1. Consistently with previous report (Yap et al., 2011), cardiotoxin was characterized by the last fraction (fraction 6) eluted by a linear gradient of NaCl and was named as NK6. Purity of NK6 was confirmed as a single band in 12.5% SDS-PAGE (data not shown) with a molecular weight of 6.7 kDa. This protein is categorized as cardiotoxin reported previously (Kulekew et al., 2007 and Yap et al., 2011)

B. Effect of NK6 on Morphology of Cardiomyocyte

The isolated rat ventricular cardiomyocytes were rod shape (>80%). There were 3 phases of cells in response to NK6. After adding NK6 in various concentration from 0.001- 1 μM, cells were contracted normal at the beginning followed by hyper-contractile state. Eventually, cells were changed from rod shape (Figure 2. left) to round shape cells (Figure 2. right). This result indicates calcium overload event that happen in sarcoplasm. Transformations of cardiomyocytes from rod-shape to round shape myocytes were time and dose-dependent (Figure 3).

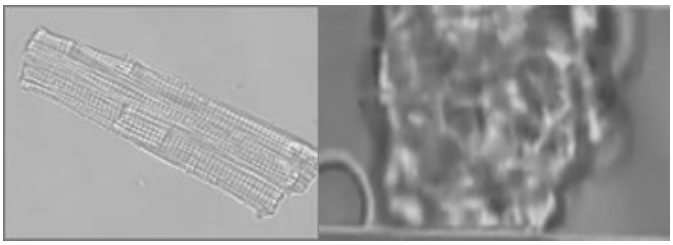


Figure 2. Rod shape cardiomyocyte (Left) and round shape cardiomyocyte after incubation with NK6.

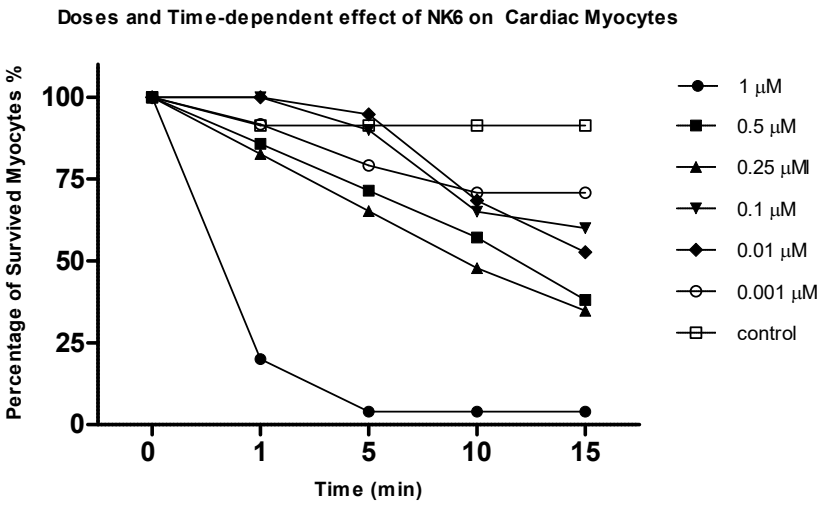


Figure 3. Changes in the percentage of rod-shaped cells during incubation in the presence of various concentrations (as indicated in Figure legend) of NK6 in single ventricular myocytes.

C. NK6 and Cardiomyocyte Function

We speculated that NK6 might cause an increase in intracellular calcium in cardiomyocytes. To test this hypothesis, we simultaneously measured calcium transient and myocyte shortening. In the initial experiment, we added a high concentration of NK6 at 2 mM into the perfusate. Calcium transient was observed to be overshoot and myocyte shortening was increased strongly and eventually undetected (data not shown) due to disruption of the sarcomere pattern (Figure 2, right). Then, a working concentration range of NK6 was established and assessed for kinetic properties of NK6 on calcium transient and myocyte shortening. Figure 4 showed the dose–dependent changed in calcium transient. The relationship between calcium transient and concentration of NK6 was not linear but rather fit well with exponential plot. There were 2 phases of exponential association with Km 1 = 2.47 and Km 2 = 165.4 μM, respectively. This result

suggested that there are two possible binding sites of NK6 to its proteins target(s) in cardiomyocytes. In addition, an effective concentration fifty (EC50) was 0.04 µM. The differences in binding affinities reflexed the different binding sites for NK6 with its target involving with calcium mobilization in cardiomyocytes. One with high affinity and another with lower affinity. To authors' knowledge, there has been no report on the binding affinity of cardiotoxin in cardiomyocyte. Vernon and Roger (1992) reported the calculated affinity constants (Km) from the Scatchard plots ranged from 2.5 to 1.02 µM for the cardiotoxin in erythrocytes. The binding affinity of NK6 in cardiomyocytes were comparable to this previous report although there are differences in study model.

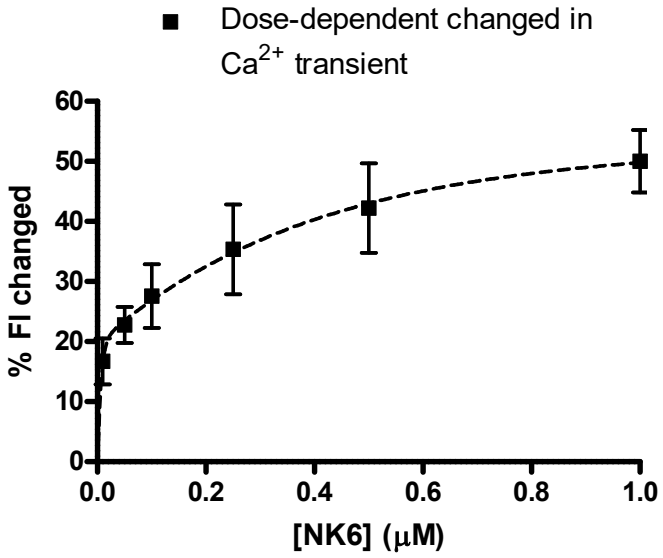


Figure 4: Dose dependent changed in calcium transient of cardiomyocytes (n=10) by NK6. Dose dependent changed in myocyte shortening were also investigated. As shown in Figure 5, percentage of sarcomere changed (i.e. Myocyte shortening) was linearly increased with an increased concentration of NK6. This result concomitant with an increase in calcium transient, since the strength of contraction is a function of calcium binding to myofilament proteins.

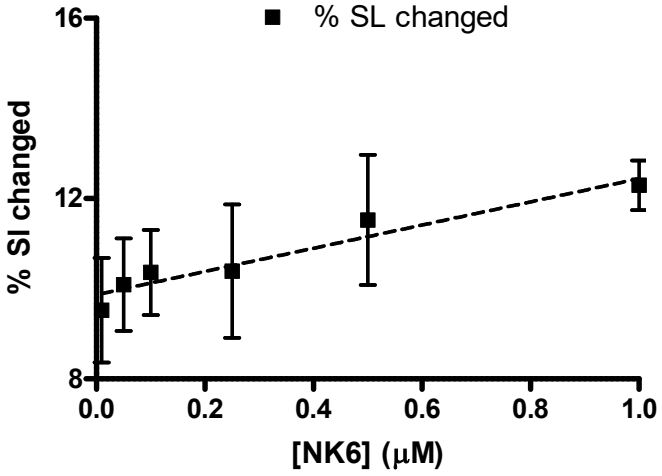


Figure 5: Dose dependent changed in myocyte shortening of cardiomyocytes (n=10) by NK6.

<u>D. Molecular targets of NK6 and Calcium homeostasis</u>

Regulation(s) of $[Ca^{2+}]_i$ are the key step of cardiac muscle contraction and function. Under physiological condition, $[Ca^{2+}]_i$ is kept at very low concentration (~ 0.1 μM) by storing in the sarcoplasmic reticulum (SR). Upon depolarization of sarcolemma membrane in T-Tubule, Ca^{2+} enters the cardiac cells through the voltage-gated calcium channel. The small amount of Ca^{2+} that entering the cell will result in the release of more Ca^{2+} from the SR through calcium released channel called "Ryanodine receptor" (RyR). This process is known as calcium-induced calcium release. Relaxation of the cardiac muscle happen when $[Ca^{2+}]_i$ fall down by pumping back to SERCA2 or extrude out of the cell in exchanged with Na+ by the sodium-calcium exchanger (NCX). Because NK6 was shown to interfere with calcium homeostasis in cardiomyocytes; therefore, we used pharmacological approach to test the indirect interaction of NK6 with channel proteins involved the calcium induced calcium released mechanism. As we proposed earlier that there might be at least two binding sites of NK6 on cardiomyocytes, we first focused on the voltage gated calcium channel, the main responsible channel for cardiomyocyte calcium mobilization. Firstly, an inhibitor of voltage-gated calcium channel, nifedipine, were used to block this channel in the presence of NK6. A representative tracing was shown in Figure 6. and 7. Nifedipine at concentration of 10 uM was shown to inhibit both calcium transient and myocyte shortening as expected. Interestingly, the effect of nifedipine was attenuated by continuing perfusion with 1uM of NK6. This result suggested that NK6 may competitively interact with voltage gated calcium channel with nifedipine. In this regard, it was proposed that cardiotoxin may

interacts extracellularly with the plasma membrane at the level of the calcium channels in the rat aortic smooth muscle (Kwan et al., 2002). Our findings are strengthening the evidence that the target of cardiotoxin is the calcium channels. In addition, Crotoxin from South African rattle snake was reported to potentiate the L-type calcium currents and modulates the action potential of neonatal rat cardiomyocytes (Zhang et al., 2010) indicating the of nature of toxin from snake venom that may acts on calcium channels.

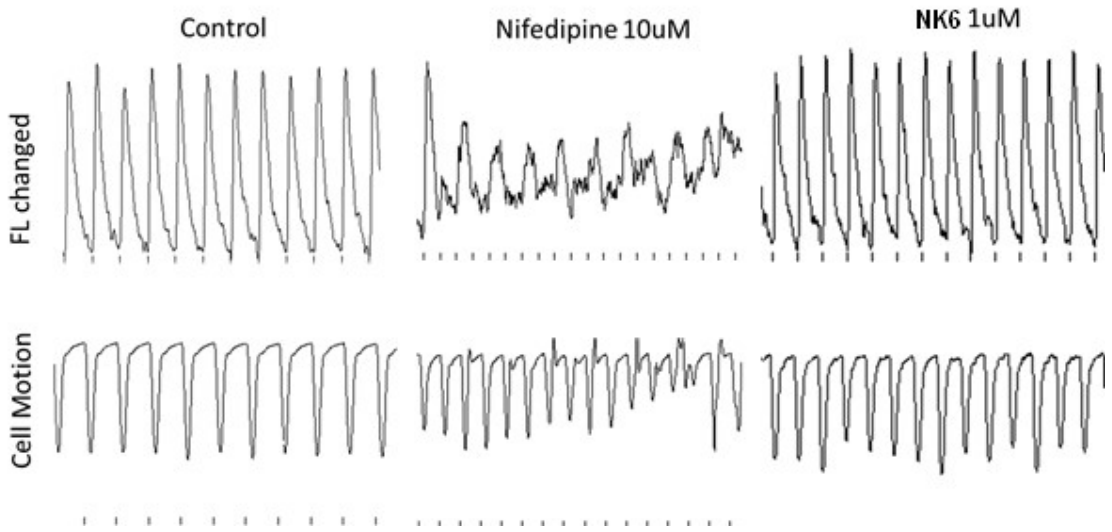


Figure 6. A representative tracing of calcium transients (upper) and myocyte shortening (lower). Cell was perfused with Tyrode for 3 minutes as control then switched to Nifedipine 1 μM in Tyrode solution. After an effect of Nifedipine was observed, cell was further perfused with 1 uM NK6 until the effect was observed.

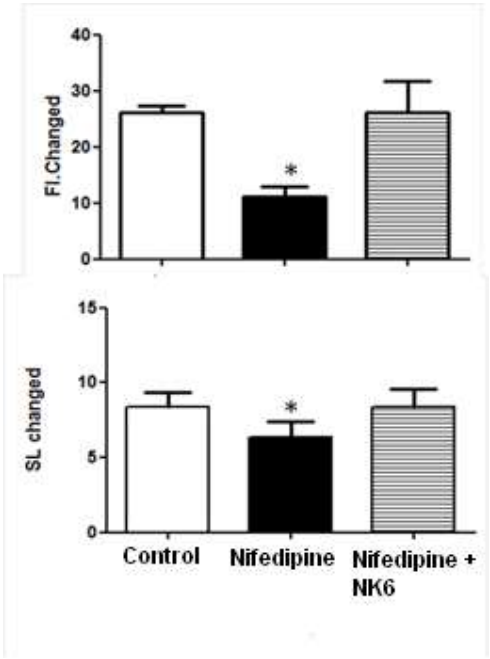


Figure 7. NK6 attenuated the effect of 10 uM Nifedipine on calcium transient (upper) and myocyte shortening (lower). Ten myocytes were recorded in each groups. Data were presented as mean ± SEM. * P< 0.05.

Further investigation was performed to test whether NK6 has an action on the second possible target of NK6, the calcium release channel, we perfused myocytes with either a stimulant (Caffeine) or inhibitor (Ryanodine) in the presence with NK6. The effect of caffeine at 5 μM in the presence of NK6 1 μM was shown in Figure 8. It is well known that caffeine could open calcium release channel on the SR and cause and overshoot of calcium transient. This result is confirmed in Figure 8 (upper). Caffeine, however, affected the myocyte shortening by causing a contracture (Figure 8 lower-middle). A contracture of myocytes was due to too much of calcium existing in the sarcoplasm, hence the crossbridges were fully activated. Interestingly, after switching the solution from caffeine to 1 μM of NK6 in Tyrode, the calcium transient even further rises up. This result suggests that there might be other sources for calcium besides the sarcoplasmic reticulum and NK6 might induced the released of calcium from these sources. The average data was not shown since the calcium transient after CTX was overshoot and was unable to be numbered.

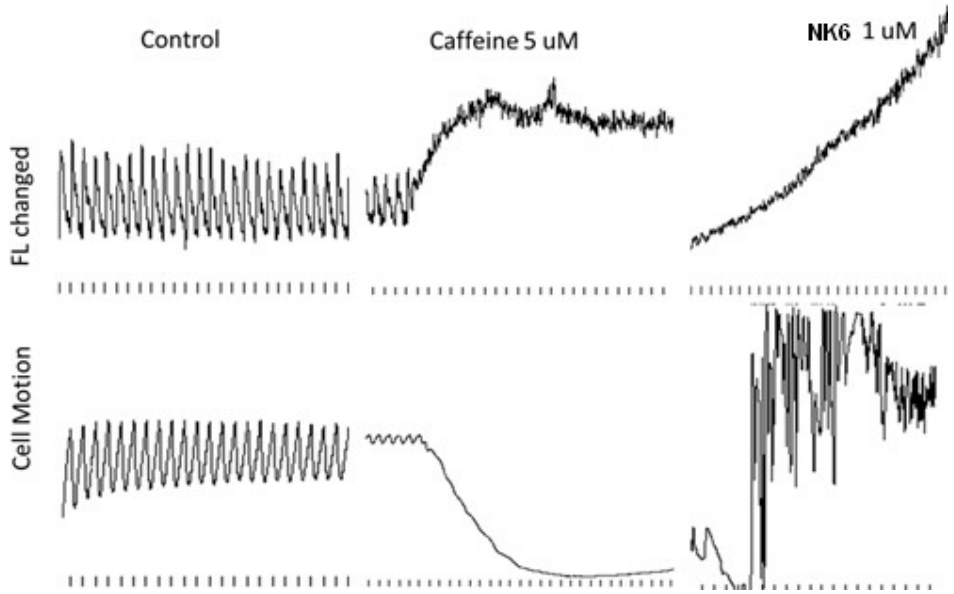


Figure 8. A representative tracing of calcium transients (upper) and myocyte shortening (lower). Cell was perfused with Tyrode for 3 minutes as control then switched to Caffeine 5 μM in Tyrode solution. After an effect of caffeine was observed, cell was further perfused with 1 μM NK6 until the effect was observed.

On the other hand, the effect of Ryanodine, inhibitor of Ryanodine receptors, was also tested in the presence of NK6. Figure 9 shown representatives tracing of calcium transient and myocyte shortening after treated with Ryanodine (10 μM) and NK6 (1 μM). As expected, Ryanodine blocked calcium released from the SR and resulted in diminishing the calcium transient. Consequently, Myocyte shortening was also attenuated due to lack of calcium to activate the cross-bridges. Interestingly, although the Ryanodine receptors were blocked, continuing perfused myocyte with NK6 still resulted in a calcium transient overshoot. This result also confirmed that a rise up in intracellular calcium during NK6 perfusion might due to extra source of calcium storage. Our study further support previous study in which CTX from N. kaouthia was reported to modulate calcium released from terminal cisternae prep in human and equine skeletal muscle (Fletcher et al., 1993). There are two possibilities to explain a rise in calcium transient. The first one is the Ryanodine receptor might be one of the target of NK6 because NK6 might potentiate the calcium release through opening of Ryranodine receptor. The latter possibility is there are addition sources of calcium that might be activated by NK6 either from outside or inside the cells. Myocyte shortening were unmeasurable due to calcium overload. This result is consistent in 10 myocytes tested but the average data was not shown.

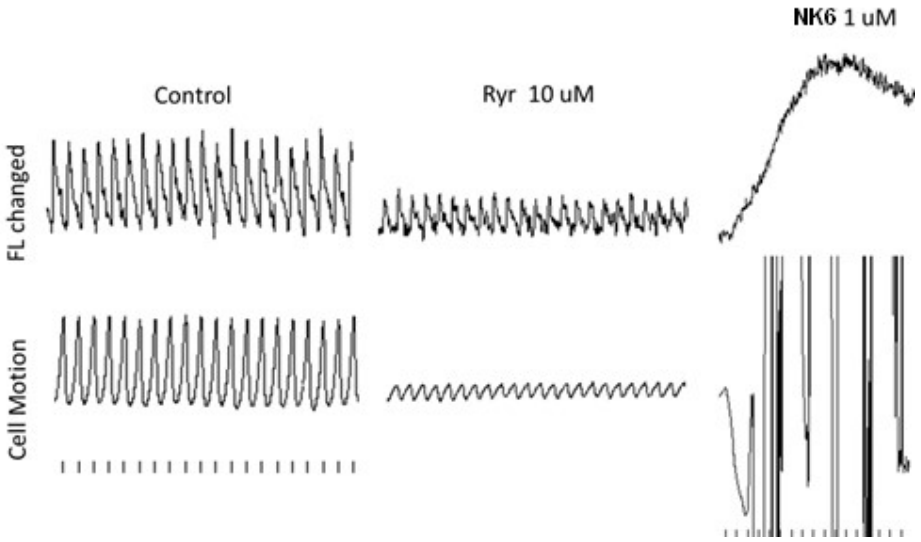


Figure 9. A representative tracing of calcium transients (upper) and myocyte shortening (lower). Cell was perfused with Tyrode for 3 minutes as control then switched to Ryanodine 10 μM in Tyrode solution. After an effect of Ryanodine was observed, cell was further perfused with 1 μM NK6 until the effect was observed.

In order to test whether calcium pump on the SR (SERCA) involved with the action of NK6, Thapsigargin was used to test for the NK6 on calcium transient and myocyte shortening. The decline of calcium transient is known by the major action of SERCA in which pump calcium back to store in the SR. Thapsigargin is known as an inhibitor of SERCA activity. The effect of Thapsigargin and NK6 perfusion on myocyte was shown in Figure 10. Perfusion the myocyte with 5 μM of Thapsigargin resulted in a diminished of calcium transient. This result can be explained by lacking of calcium stored in the SR due to a blockage of SERCA. Myocyte shortening was also diminished since calcium was not transported back into SR and may extrude out of the cell via Na^+/H^+ exchanger. When NK6 was perfused into the cell, calcium transient was abruptly increased and myocyte shortening was disorganized due to calcium overloaded in the cells. This result imply that NK6 either block SERCA or NK6 has no effect on SERCA but the calcium transient increased because of the release of calcium from other sources. To investigate this speculation, we also measured time to 70% calcium decay (Tau). The decline phase of calcium transient mainly comes from the uptake of calcium into SR via SR Ca^{2+}-ATPase (SERCA). Tau represents how fast calcium will reuptake into the SR. As shown in Figure 11., dose-responses curve of Tau by NK6 fit well with one-phase exponential decay plot with a single Ki of 22.37

msec. This suggests that NK6 might interact with SERCA and this interaction resulted in an increase speed of calcium reuptake by SERCA.

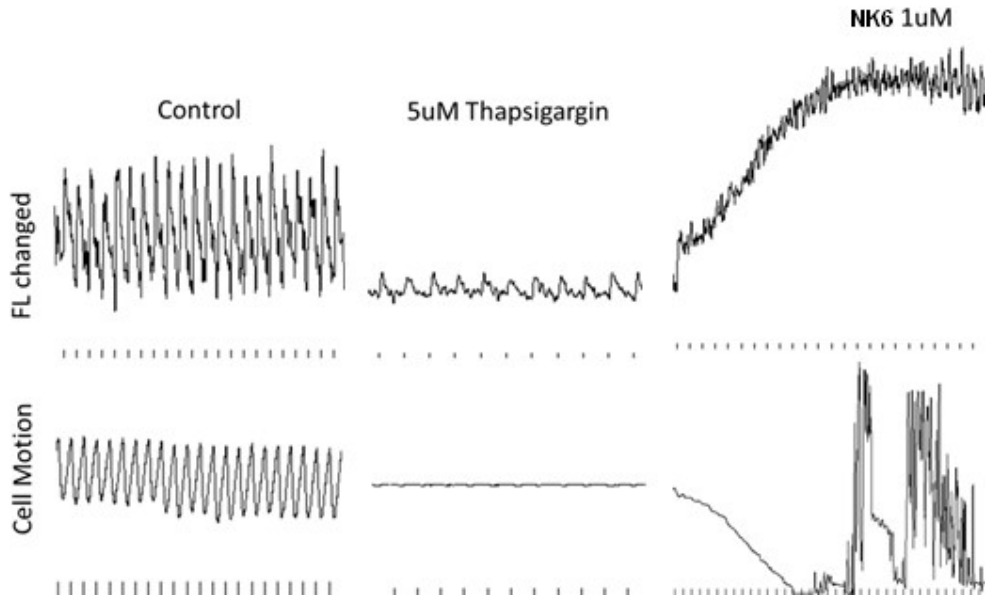


Figure 10. A representative tracing of calcium transients (upper) and myocyte shortening (lower). Cell was perfused with Tyrode for 3 minutes as control then switched to Thapsigargin (5 µM) in Tyrode solution. After an effect of Thapsigargin was observed, cell was further perfused with 1 µM NK6 until the effect was observed.

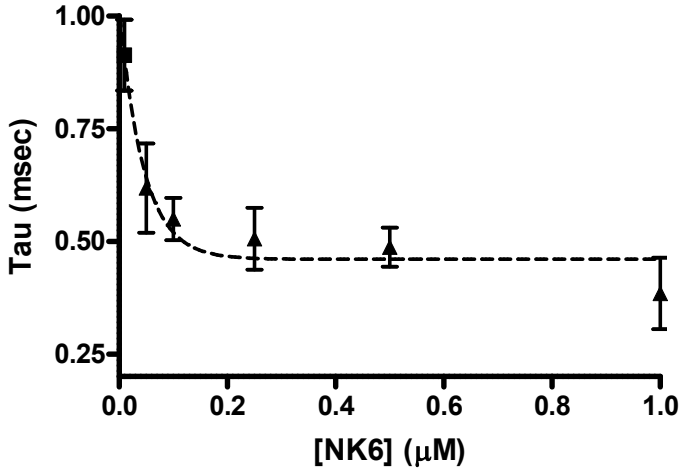


Figure 11. Dose-dependent changed in time to 70% calcium decay (Tau) by NK6.

E. NK6 Inhibit Voltage-Gated Calcium Channel

To test whether NK6 directly interact with voltage gated calcium channel, we employed patch-clamp experiment in HEK cells transiently expressed Cav1.2. As shown in Figure 12, NK6 inhibit calcium current through L-type calcium channel in dose dependent manner and almost totally block the current at 1 μM. This is the first evidence ever to report the direct action of cardiotoxin on calcium channel in cardiomyocyte. The effect of NK6 on L-type calcium current was also compared with cardiotoxin 3 (CTX3) from *N. mosambiqueca*. ***NK6 has more potent action when compare at also 1 μM (Figure 12; Triangle).***

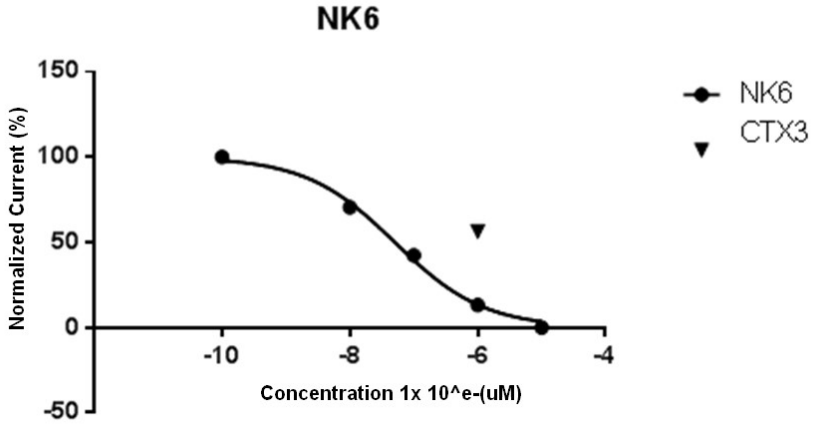


Figure 12. Dose response of L-type calcium current in percentage. NK 6 @ various concentration was applied on HEK cells expressed human L-type current Cav1.2 (n =5).

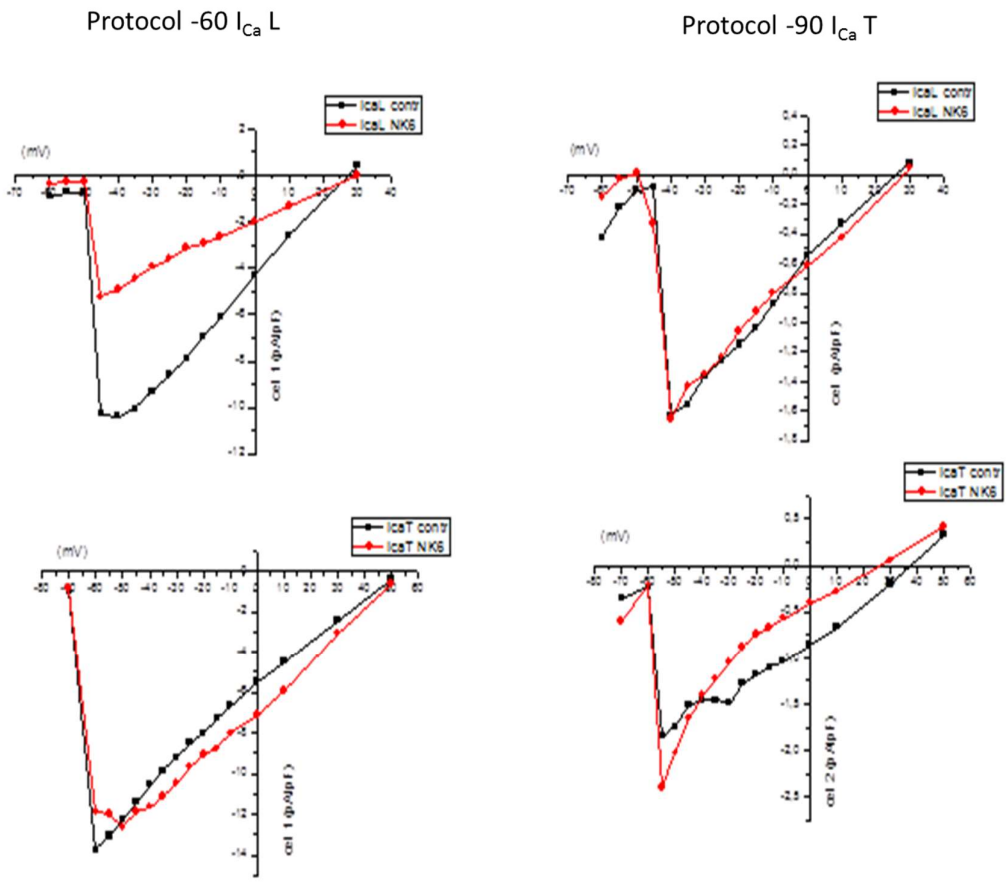


Figure 13. Voltage-clamp experiment: The -60 I_{Ca}-L protocol (left), cell was pre-pulse at -60 mV before changed to different voltages (from -60 mV to +30 mV). Black line represent control current and Red line represent calcium current with NK6. Calcium currents from L-type Cav channels were mostly activate. The -90 I_{Ca}-T protocol (right) cell were prepulse at -90 mV and mostly Ca currents from T-type Cav channels was activate. Upper panel is L-type calcium current and lower is T-type calcium channel.

We further utilized voltage-clamp experiment to extend the mechanism of NK6 on calcium channel. Figure 13, I-V plot of L-type calcium current only was recorded with -60 I_{Ca}-L protocol. It was clearly shown that NK6 inhibit L-type calcium current. (Figure 13: upper left panel). On the other hand, NK6 had no effect on T-type calcium current as shown in Figure 13. (Lower panel). This result suggest that NK6 selectively decrease of Cav-L currents, without affecting T-type calcium channel.

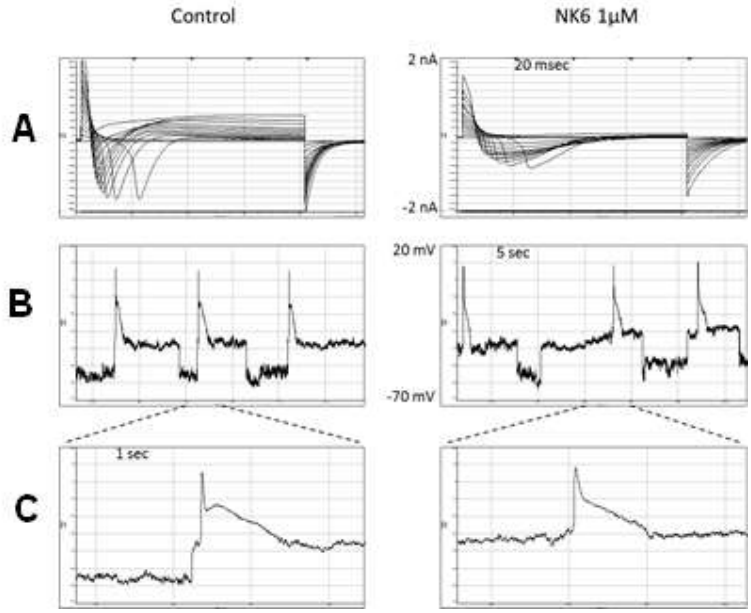


Figure 14. Current-clamp configuration, (A) steps of current injections in order to hyperpolarize the cell. (B) Action potential was measured after the end of the hyperpolarizing step (after the induced depolarization). (C) A representative of single action potential. Control (left) was compared with NK6 treated cells at 1 µM (right).

Measurement of action potential in current clamp experiment confirmed the selective blocking of L-type calcium channel. Figure 14 (B) demonstrated a delay in action potential time after the cells were treated with NK6 at 1 µM due to a less excitability as it took more time to initialize the action potential. In addition, a plateau phase of action potential was also declined because of the inhibition of calcium current.

F. Effects of NK6 on myofilament maximum tension, maximum ATPase, and Stiffness

To test whether NK6 has direct effect on cardiac myofilament, a skinned fiber experiment following by simultaneously measurement of maximum force and ATPase was performed. Basically, plasma membrane of cardiac trabeculae was removed by detergent; therefore, the only remaining in the experiment chamber was the myofilament. The effect of NK6 was tested by adding NK6 at various concentrations into the calcium solution in which bathing the skinned cardiac trabeculae. Figure 15(A) showed a decrease in normalized maximum force of cardiac trabeculae by 5-8% at the dosage of NK6 from 5-100 ug/ml. This result indicated that NK6 has a direct effect albeit, slightly on cardiac myofilament. However, NK6 has no effect on maximum ATPase rate activity as shown in figure 15 (B). The stiffness of myofilament was also obtained

and show on figure 15 (C). The stiffness of myofilaments was decreased in dose dependent manner from 9-11% at the dosage of NK6 from 5-100 ug/ml. This indicate less crossbridges were activates resulted from a decreased in maximum force development. It has been widely known that most of the toxin from snake have effect on ion channels. Interestingly, this is the first evidence demonstrate that NK6 from cobra has a straight effect on non-ion channel intracellular proteins. Further study need to elucidated this pathway.

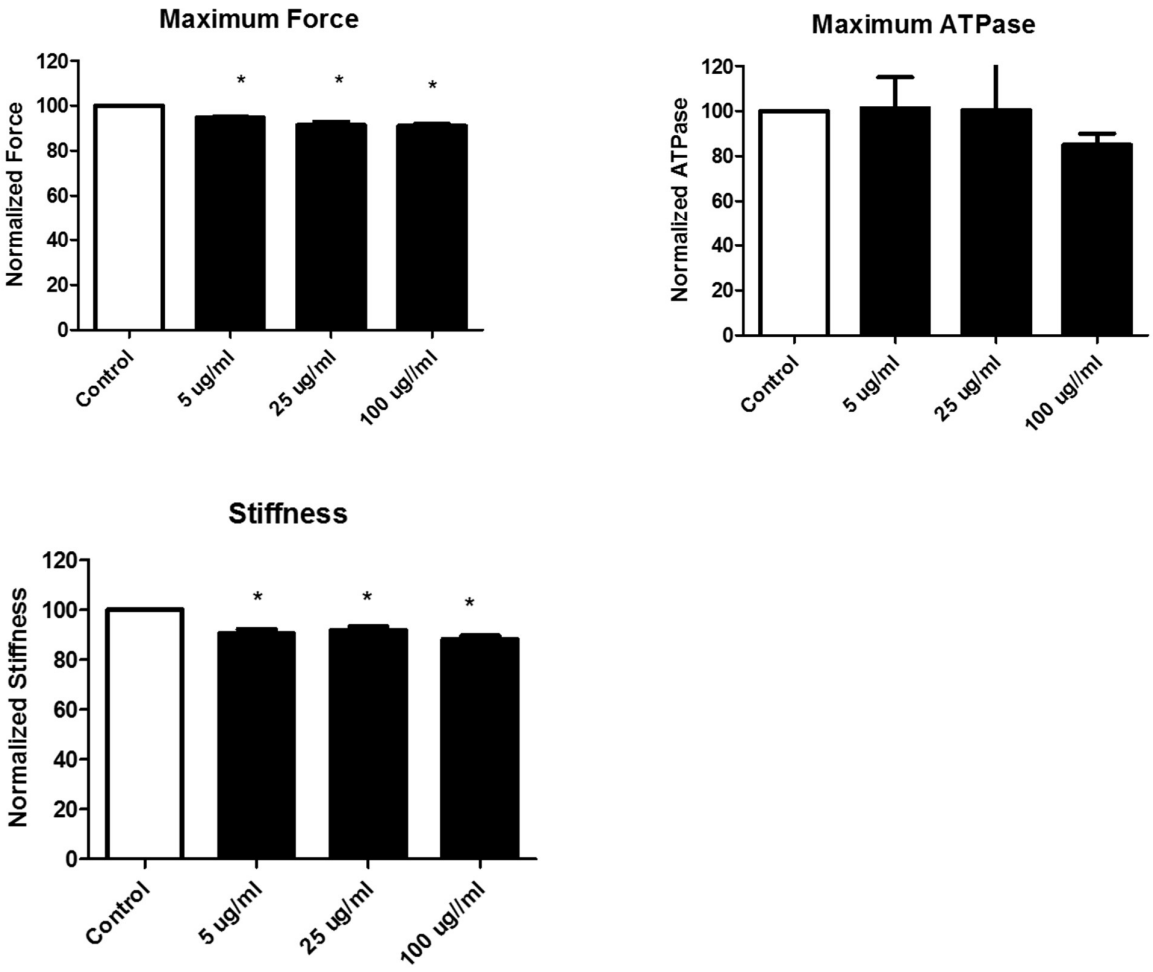


Figure 15: Effect of various concentration of NK6 on (A) Maximum force, (B) Maximum ATPase, (C) Stifness (n=3 in each groups; * P<0.05)

It may be concluded that NK6 purified from Thai *N.kaouthia* ***venom is a multi-action toxin in which a report here identified at least three targets. First, results in this report pointed out to the voltage gated calcium channel. We showed that NK6 interacted with this calcium channel. In***

addition, NK6 was proofed to inhibit the exclusively to the L-type but not the T-type calcium channel. The ryanodine receptor are the second possible targets of NK6 in which NK6 may opened this channel as shown by an increase of calcium transient either with activator or inhibitor. However, NK6 may also open another unidentified yet source of calcium storage because although caffeine already emptied the SR, calcium transient was still being able to augmented by NK6. In this regards, extracellular source of calcium may be the candidate because there are some previous reports proposed that cardiotoxins may act as an ionophore on the plasma membrane (Fletcher et al, 1993 and Kumar et al.,1997). In contrast, Chen et al., 2007 reported also that the membrane damaging effect of cardiotoxin is not the main mechanism of cytotoxicity and there must be protein(s) target on the membrane. Our results fits well with this assumption. Eventually, the SERCA may be another target for the action of NK6. However, it required to be further studied. Another possible sources in which did not test in the present study is the mitochondria. Cardiotoxin III from Taiwan cobra was reported to disrupted mitochondrial membrane and released calcium in H9C2 cells (Wang et al., 2005). Whether this finding valid with our study need to be verified since there are a different in a study model. Consequently, further investigation is needed to elucidate the unknown additional source of calcium in myocytes. Interestingly, besides the effect on ion channels, NK6 was found here to effect directly on the myofilament proteins (i.e., action and myosin) resulted in decreased maximum force and stiffness without affecting maximum myosin ATPase activity. However, we do not know if this effect required phospholyration of myofilament protein. Further experiments need to elucidated. Since this molecule has multifunction, it might be interesting to establish linking mechanism of each activity. This experiment will provide the basis of NK6 action on cardiomyocyte that may benefit in the development of cardiovascular drugs aid to relieve the symptom in heart failure patient.

Acknowledgements

This work was supported by Grant TRG 5680085 from Thailand Research Fund and the grant from Young Scientist Award (to Kittipong Tachampa) by Franco-Thai Scholarship Program.

เอกสารอ้างอิง

References

Chen KC, Kao PH, Lin SR, Chang LS. The mechanism of cytotoxicity by Naja naja atra cardiotoxin 3 is physically distant from its membrane-damaging effect. Toxicon. 2007 Nov;50(6):816-24.

Wang CH, Wu WG. Amphiphilic beta-sheet cobra cardiotoxin targets mitochondria and disrupts its network. FEBS Lett. 2005 Jun 6;579(14):3169-74. .

Debnath A, Saha A, Gomes A, Biswas S, Chakrabarti P, Giri B, Biswas AK, Gupta SD, Gomes A. A lethal cardiotoxic-cytotoxic protein from the Indian monocellate cobra (Naja kaouthia) venom. Toxicon. 2010 Sep 15;56(4):569-79.

Dufton MJ, Hider RC. Structure and pharmacology of elapid cytotoxins Pharmacol. Ther, 1988;36, pp. 1–40.

Fletcher JE, Jiang MS. Possible mechanisms of action of cobra snake venom cardiotoxins and bee venom melittin. Toxicon. 1993 Jun;31(6):669-95.

Fletcher JE, Tripolitis L, Beech J. Species difference in modulation of calcium release by Naja naja kaouthia snake venom cardiotoxin in terminal cisternae from human and equine skeletal muscle. Toxicon. 1993 Jan;31(1):43-51.

Kwan CY, Kwan TK, Huang SJ. Effect of calcium on the vascular contraction induced by cobra venom cardiotoxin. Clin Exp Pharmacol Physiol. 2002 Sep;29(9):823-8.

Harvey AL, Marshall RJ, Karlsson E. Effects of purified cardiotoxins from the Thailand cobra (Naja naja siamensis) on isolated skeletal and cardiac muscle preparations. Toxicon. 1982;20(2):379-96.

Kumar TKS, Lee CS, Yu C.A case study of cardiotoxin III from the Taiwan cobra (Naja naja atra). Solution structure and other physical properties. Natural Toxins II, Plenum Press, New York., 1996, pp. 115–129.

Kulkeaw K, Chaicumpa W, Sakolvaree Y, Tongtawe P, Tapchaisri P. Proteome of the venom of the Thai cobra, Naja kaouthia. Toxicon. 2007 Jun 1;49(7):1026-41.

O'Connell TD1, Rodrigo MC, Simpson PC. Isolation and culture of adult mouse cardiac myocytes. Methods Mol Biol. 2007;357:271-96.

Sun JJ, Walker MJ. Actions of cardiotoxins from the southern Chinese cobra (Naja naja atra) on rat cardiac tissue. Toxicon. 1986;24(3):233-45.

Tachampa K, Kobayashi T, Wang H, Martin AF, Biesiadecki BJ, Solaro RJ, de Tombe PP Increased cross-bridge cycling kinetics after exchange of C-terminal truncated troponin I in skinned rat cardiac muscle. J Biol Chem. 2008 May 30;283(22):15114-21.

Vernon LP, Rogers A. Binding properties of Pyrularia thionin and Naja naja kaouthia cardiotoxin to human and animal erythrocytes and to murine P388 cells. Toxicon. 1992 Jul;30(7):711-21.

Yap MKK, Tan NH, Fung SY. Biochemical and toxinological characterization of Naja sumatrana (Equatorial spitting cobra) venom. J Venom Anim Toxins incl Trop Dis. 2011:17(4): 451-459.

Zhang P, Lader AS, Etcheverry MA, Cantiello HF. Crotoxin potentiates L-type calcium currents and modulates the action potential of neonatal rat cardiomyocytes. Toxicon. 2010 Jun 15;55(7):1236-43.

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. *ตีพิมพ์ใน* ***Toxicon: Proceedings of the 18th World Congress of the International Society of Toxinology***

   ผู้แต่ง: ***Kittipong Tachampa, Orawan Khow, Narumon Pakmanee, Christian Barrere, Mattia Di Francesco, Joel Nargeot , Narongsak Chaiyabutr, Visith Sitprija***

   ชื่อเรื่อง: ***Disturbance of Intracellular Calcium Homeostasis and Cardiomyocyte Function by a protein in fraction six of Naja kaouthia Venom***

   ปี ***2015 Vol. 103S หน้า 123-124***

2. *การนำผลงานวิจัยไปใช้ประโยชน์*

   ด้านวิชาการ โดย ผู้วิจัยและภาควิชาสรีรวิทยา คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

   โครงการนี้เป็นการสร้างองค์ความรู้ใหม่ซึ่งเป็นพื้นฐานที่สำคัญในการต่อยอดเพื่อพัฒนาสารพิษจากพิษงูมาใช้เป็นยาเพื่อรักษาโรคในผู้ป่วยหัวใจล้มเหลวทางผู้วิจัยได้เป็นตัวแทนภาควิชาฯ ไปเผยแพร่ผลงานและความรู้ที่ ***International Society of Toxinology World Congress 2015*** *ณ เมือง* ***Oxford*** *ประเทศสหราชอาณาจักรอังกฤษ ซึ่งเป็นงานประชุมระดับโลกในสาขางานวิจัยที่เกี่ยวกับพิษจากสิ่งมีชีวิต โดยทางผู้วิจัยได้นำเสนอผลงานในรูปแบบโปสเตอร์และตีพิมพ์ใน* ***Proceeding*** *ของงานประชุมดังกล่าว นอกจากนี้ยังได้แลกเปลี่ยนความรู้และความคิดเห็นกับนักวิจัยชั้นนำของโลกในสาขานี้และทำให้เกิดความร่วมมือเพื่อต่อยอดงานวิจัยกับ* ***National Natural Toxins Research Center , Texas A& M University-Kingsville*** *ประเทศสหรัฐอเมริกา ซึ่งเป็นศูนย์ผลิตและทดสอบพิษงูที่ใหญ่ที่สุดในประเทศสหรัฐอเมริกา ในด้านงานวิจัย และทางสถาบันดังกล่าวยินดีที่จะให้ข้าพเจ้า ส่งนิสิตปริญญาเอกไปทำวิจัยที่สถาบันดังกล่าวในภายภาคหน้า*

   โครงการนี้ยังทำให้เกิดความร่วมมือและแลกเปลี่ยนทางงานวิจัยระหว่างผู้วิจัยกับ ***Institute de Genomique and Fonctionalle, University of Montpellier,*** *ประเทศฝรั่งเศส โดยผู้วิจัยได้ไปนำเสนอผลงานวิจัยในเบื้องต้นพร้อมทำวิจัย ระยะสั้น ที่สถาบันดังกล่าวในช่วงเดือนกันยายน ถึง ธันวาคมปี ค.ศ. 2013*

INTERNATIONAL SOCIETY ON TOXINOLOGY

ELSEVIER

25-30 September 2015

IST 2015 Oxford

The 18th World Congress of the International Society on Toxinology

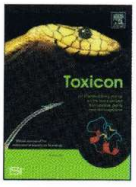
The Examination Schools & The Sheldonian Theatre
Oxford, United Kingdom

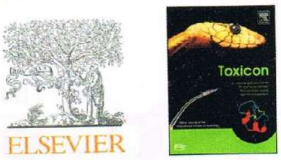
Web: http://LPMHealthcare.com/ist2015
Email: ist2015@LPMHealthcare.com

GLOBAL SNAKEBITE INITIATIVE

BTG

Sfet





Proceedings of the 18th World Congress of the International Society on Toxinology

COVER IMAGE: Eastern Green Mambas (*Dendroaspis angusticeps*) whose venom is the source of dendroaspis natriuretic hormone (Copyright David Warrell – photographed by courtesy of Bio-Ken, Watamu, Kenya).

This event is being managed by LibPubMedia Ltd (Oxford) and Congress Oxford Ltd (Oxford).

[2]Rothamsted Research, West Common, Harpenden, Hertfordshire, UK

Ladybird beetles (Coleoptera:Coccinellidae) represent a diverse group of chemically defended predators. Toxicity of whole beetles and extracts has been demonstrated towards birds, other ladybirds and *Daphnia*. Structurally unique alkaloids, found in within the haemolymph, are thought to be responsible. Ecological data suggests that ladybirds are intraguild predators, regularly predated upon by insects but rarely by vertebrates. As defensive toxins are often honed to the taxa most regularly encountered as predators; we hypothesize that ladybird alkaloids may show selectivity for insect targets. Recent research has shown that several ladybird alkaloids target nicotinic acetylcholine receptors (nAChRs) to achieve toxicity. We have used patch-clamp electrophysiology of human TE671 and insect (*Schistocerca gregaria*) neuron cells expressing mammalian muscle-type and insect neuronal-type nAChRs. All ladybird alkaloid extracts and synthetic analogues antagonised vertebrate and insect nAChRs in a concentration dependent manner, but some compounds showed a high level of differential toxicity. (-)-adaline, produced by the 2-spot ladybird (*Adalia bipunctata*) was an antagonist of nAChRs in both human and insect cells. (-)-adaline was between 15-19 fold more potent to the insect nAChR with IC_{50} values of 24.4 and 1.28 μM for human and locust cells respectively at V_h -75 mV. Two-electrode voltage clamp of *Xenopus* oocytes expressing recombinant mammalian and insect neuronal-type nAChRs was also used to compare the selectivity of the alkaloids. The acetylcholine response of recombinant rat α4β2 nAChRs was also inhibited by (-)-adaline with an IC_{50} of 13.2 μM for the peak current at V_h -75 mV. All the compounds tested so far show non-competitive activity and voltage dependence, consistent with open-channel block. Given the results of this study, we propose that ladybird alkaloids represent an as yet untapped source of novel nAChR antagonists that show promise as insecticide leads.

29-5-10: Mapping the residues that mediate interaction of the spider-venom peptide μ-TRTX-Hd1a with the analgesic target $Na_V1.7$

Jennifer J Smith and Glenn F King

Institute for Molecular Bioscience, The University of Queensland, St. Lucia, QLD 4072, Australia

Chronic pain is a serious worldwide health issue, with current analgesics having limited efficacy and dose-limiting side effects. Humans with loss-of-function mutations in the voltage-gated sodium channel $Na_V1.7$ ($hNa_V1.7$) are insensitive to pain, making $hNa_V1.7$ a promising target for analgesic development. We recently described a spider venom peptide named Hd1a (full name μ-TRTX-Hd1a) that inhibits $hNa_V1.7$ with a high level of selectivity over other hNa_V subtypes, including $hNa_V1.4$, $hNa_V1.5$ and $hNa_V1.6$. To determine the residues important for activity at $hNa_V1.7$, alanine-scanning mutagenesis of non-cysteine residues was performed. Testing of these mutant toxin peptides on $hNa_V1.7$ reveal the presence of key residues involved in activity of Hd1a at $hNa_V1.7$. The results obtained greatly improve our understanding of the molecular interactions of the Hd1a:$hNa_V1.7$ complex, and should facilitate the development of more potent and selective $hNa_V1.7$ antagonists.

29-5-11: Disturbance of Intracellular Calcium Homeostasis and Cardiomyocyte Function by a protein in fraction six of *Naja kaouthia* Venom.

Kittipong Tachampa[1], Orawan Khow[2], Narumon Pakmanee[2], Christian Barrere[3], Mattia Di Francesco[3], Joel Nargeot[3], Narongsak Chaiyabutr[2], Visith Sitprija[2]

[1]Department of Physiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand
[2]Queen Saovabha Memorial Institute, the Thai Red Cross Society, Bangkok, Thailand
[3]Institut de Genomique Fonctionnelle, Universites de Montpellier, Montpellier, France

A protein in fraction six (NK6; mol. wt. 6.7 kDa) has been purified from the Thai cobra (*Naja kaouthia*) venom by ion-exchange chromatography and HPLC. In a preliminary study, NK6 suppressed cardiac contraction in rat *in vivo* model. However, perfusion of NK6 in isolated mouse cardiomyocytes caused a rise in calcium transient with an initial increase in myocyte shortening followed by losing its contraction ability. The present study aimed to further investigate the effects of NK6 on calcium homeostasis and myocyte shortening in cardiomyocytes. Morphological changes of cardiomyocytes were observed after incubation with various concentrations of NK6 in a dose and time dependent manner. Calcium transient and myocyte shortening were measured simultaneously based on florescence photometry and edge-detection software. The dose dependent effects of NK6 on calcium transient showed the existence of two binding sites with different affinity (Km_1 = 2.47 μM and Km_2 165.40 μM, respectively), suggesting two interaction sites for NK6 involved calcium handling in myocytes. EC_{50} on myocytes was 0.04 μM. Pharmacological approach using nifedipine showed that NK6 was able to attenuate the effect of nifedipine on calcium transient, indicating a competitive binding at the L-type calcium channel. In addition, NK6 increased calcium transient in the presence of ryanodine and the effect of caffeine on calcium transient was augmented by NK6, indicating the second possible interaction site at the ryanodine receptor. Patch-clamp experiment revealed that NK6

blocked L-type calcium current in Cav1.2 expressed in HEK cells. A selective decrease in Cav-L currents, without affecting Cav-T conductance by NK6 was also confirmed. There was a decline of the plateau phase of cardiac action potential by NK6 confirming the inhibition of calcium current. In summary, action of NK6 may relate to the two calcium channels. Further details of its mechanism of actions at the molecular level are in progress.

29-5-12: Chemical synthesis of mambalgin-1 toxin and binding analysis to Acid Sensing Ion Channels using two-Photon Fluorescence Microscopy

Changlin Tian[1,2], Ming Wen[1], Xiqoqi Guo[1,2], Yao He[1], Longhua Zhang[1], Ying Xiong[1]

[1]School of Life Sciences, University of Science and Technology of China, Hefei, P. R. China
[2]High Magnetic Field Laboratory, Chinese Academy of Sciences, Hefei, P. R. China

Mambalgin-1 is a cysteine rich, 57 residue polypeptide isolated from venom of black mamba snake. Recently, mambalgin-1 was demonstrate to abolish pain through inhibition of acid-sensing ion channels (ASICs) either in central or peripheral neurons. To illustrate the inhibition mechanism of mambalgin-1 to the ASICs channels, the toxin peptide was chemically synthesized through SPPS and chemical ligation methods. The Mmabalgin-1 was shown to have three fingers with richness of charge residues, which might interact with surface cavity of ASIC channels. A small fluorescent unnatural amino acid probe was incorporated into specific site of the ASIC1a channel for two-photon fluorescence microscopy analysis. The observed fluorescence spectrum revealed different structural and dynamic responses of several residues in the ASIC1a channels upon binding of the mambalgin-1 toxin.

Session 6: Conotoxins and other Pain-Inducing Toxins – 29th September

29-6-1: Natural compounds as inhibitors of acid sensing channel ASIC3

Yaroslav A Andreev, Dmitry I Osmakov, Sergey A Kozlov, Eugene V Grishin

Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, 117997, Russia

Proton-gated channels (ASICs) are involved in perception of local pH changes. They play important role in generation of pain signal during tissue injury, ischemia and inflammation and considered to be a perspective target for development of new drugs. To search ASICs modulators in venoms of different creatures and extracts of medicinal plants we used electrophysiology test based on *Xenopus laevis* oocytes injected with different ASICs cRNA. We found a peptide modulator of ASIC3 in extract of nematocysts of sea anemone *Heteractis crispa*. The peptide was named as Hcr1b-1 and inhibited only peak component of ASIC3 current with IC50 5.5 μM and turned out to be close homologue of peptide APeTx2 that is well known and much more potent inhibitor of ASIC3. Another novel peptide named Ugr9-1 was isolated from the venom of the sea anemone *Urticina grebelnyi*. It completely blocked the transient component and partially inhibited the amplitude of the sustained component. Ugr 9-1 significantly reversed inflammatory and acid-induced pain when administrated intravenously in mice at doses 0,1-0,5 mg/kg. Low molecular weight inhibitor of ASIC3 channel was identified in the acidic extract of *Thymus armeniacus*. This compound was named "sevanol", it is a new lignan built up of epiphyllic acid and two isocitryl esters in positions 9 and 10. Sevanol completely blocked the transient component and partially (~45%) inhibited the amplitude of the sustained component. Among other ASIC channels only ASIC1a was also inhibited by sevanol, but six times less efficiently compared to ASIC3 inhibition. Intravenous administration of sevanol in mice significantly reversed thermal hyperalgesia induced by CFA injection and reduced pain response to acetic acid. Therefore sevanol plays significant role in known analgesic and anti-inflammatory properties of thyme. Novel natural modulators of ASICs could be useful for developing new pharmacological substances targeting ASICs, as well as for drug design.

29-6-2: Member of gamma-conotoxin family isolated from *Conus princeps* displays novel molecular target

Johanna Bernáldez[1], Samanta Jimenez[1], Luis J González[2], Jesús Noda[2], Enrique Soto[3], Emilio Salceda[3], Daniela Chávez[1], Manuel Aguilar[4] and Alexei Licea[1]

[1]Dept of Biomedical Innovation, Center of Scientific Research and Higher Education at Ensenada, México
[2]Dept of Proteomics, Mass Spectrometry Laboratory, Center of Genetic Engineering and Biotechnology, Cuba
[3]Institute of Physiology, Meritorious Autonomous University of Puebla, Puebla, México
[4]Institute of Neurobiology, National Autonomous University of Mexico, Querétaro, México