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Scorpion Venom Activates Both Ca^{2+}-ATPase and Inositol 1,4,5-trisphosphate Receptor in the Microsomes of Tracheal Epithelial Cells

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Abstract : The effects of scorpion (*Leiurus quinquestriatus hebraeus*, Lqh) venom were evaluated on the activities of microsomal Ca^{2+}-ATPase and Ca^{2+} release channel prepared from the epithelial cells of pig airway. Whole venom of Lqh (120 μg/ml) increased the activity of microsomal Ca^{2+}-ATPases about 32% in the tight-sealed microsomes and about 28% in the Triton X-100-treated or Ca^{2+} ionophore A23187-treated leaky microsomes. Thapsigargin, a specific antagonist of Ca^{2+}-ATPase, inhibited 42% of total ATPase activity and also completely blocked the effects of Lqh venom, suggesting that Lqh venom directly activiates the microsomal Ca^{2+}-ATPases. In order to determine if Lqh venom increases the microsomal uptake of $^{45}Ca^{2+}$, Lqh venom was added in the uptake medium. The Lqh venom increased microsomal $^{45}Ca^{2+}$ uptake up to ~20% and the increase was only observed when heparin, an antagonist of $InsP_3$ receptor channel, was added in the uptake medium. Lqh venom in the absence of heparin unexpectedly decreased the rate and the amount of $^{45}Ca^{2+}$ uptake. These results were explained by simultaneous increases in $^{45}Ca^{2+}$ release as well as $^{45}Ca^{2+}$ uptake by Lqh venom. Lqh venom itself increased the release of $^{45}Ca^{2+}$ as much as $^{45}Ca^{2+}$ release by 4 μM $InsP_3$, implying that Lqh venom also activates $InsP_3$ receptor, microsomal Ca^{2+} release channel. Based on these results, we suggest that the Lqh venom consists of at least two components; one activates the $InsP_3$ receptor and the other activates the Ca^{2+}-ATPase. Currently we are investigating the chemical and electrophysiological properties of the active components of Lqh venom.(Received March 30, 1996; accepted April 24, 1996)

Introduction

Scorpion venoms have been widely used to characterize ion fluxes through the biological membranes focusing on the behaviors of ion channels.[1-4] Scorpion venom from a species contains 1~10 different polypeptides which cosist of 35 to 70 amino acid residues.[5,6] The toxicity of scorpion venom appears very effective to insects rather than to mammals; however, the intoxicated animals show the symptoms such as hyperexcitability, salivation, dyspnea, convulsion, paralysis, and death. Although most of scorpion venoms have been poorly characterized, the functional role and molecular structure of charybdotoxin (CTX) have been intensively studied. CTX is purified from *Leiurus quinquestriatus* and inhibits the Ca^{2+}-activated K^+ channel (K_{Ca} channel) protein in skeletal muscle.[2,7] CTX is a basic peptide toxin with high affinity (K_D=~3 nM) and specificity to the K_{Ca} channel. Another scorpion toxin, imperatoxin, was also purified and characterized by Valdivia *et al.*[3] from *Pandinus imperator*. Imperatoxin specifically targeted against the ryanodine receptor, a Ca^{2+} release channel, in both skeletal and cardiac muscle also with a high affinity (K_D=~10 nM). The usage of these scorpion venoms has been succesful to characterize biological functions because of their high-specificity and high-affinity to target proteins.

In the present work, we have used whole venom of the scorpion, *Leiurus quinquestriatus hebraeus* (Lqh) to characterize intracellular Ca^{2+} stores in tracheal epithelial cells. These intracellular Ca^{2+} stores are activated by receptor-mediated activation of an inositol 1,4,5-trisphosphate ($InsP_3$) second messenger system. These $InsP_3$-sensitive Ca^{2+} stores have both $InsP_3$ receptor and Ca^{2+}-ATPase. The $InsP_3$ receptor is a Ca^{2+} release channel which releases the stored Ca^{2+} into cytoplasm and the Ca^{2+}-ATPase is an organellar Ca^{2+} pump which sequesters cytoplasmic Ca^{2+} into the lumen of the stores by the expense of ATP. The $InsP_3$-sensitive Ca^{2+} stores play important roles in transepithelial secretion and mucociliary clearance which are major lung defence mechanisms.[8] Although the details of these two physiological processes have been intensively studied, they are poorly understood. This is partly due to the lack of the characterization of $InsP_3$-sensitive Ca^{2+} stores, more specifically,

Key words : Scorpion venom, Tracheal epithelium, Ca^{2+}-ATPase, $InsP_3$ receptor, $^{45}Ca^{2+}$ uptake and release.
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the lack of the specific modualtors to study the functions of the $InsP_3$-receptors.[9] Heparin is a unique and well-known inhibitor of $InsP_3$ receptor, but its specificity is relative poor and its application is limited in *in vivo* system.[10] Therefore, the purpose of this study is to isolate a new natural ligand of $InsP_3$ receptor and to use it as a tool of research for the characterization of $InsP_3$ receptor in tracheal epithelial cells.

Materials and Methods

Materials

Venom of the scorpion *Leiurus quinquestriatus hebraeus*, inositol 1,4,5-trisphosphate, thapsigargin, heparin, and Ca^{2+} ionophore A23187 were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). $^{45}CaCl_2$ was supplied by Du Pont-NEN Research Products (Boston, U.S.A.). All other reagents were high purity grade. Porcine tracheas of freshly killed pigs were generously supplied by a local slaughterhouse in Cheongju, Chungbuk.

Preparation of microsomes from the epithelial cells of porcine trachea

Membrane vesicles of porcine trachea were prepared by the procedure described previously.[11] Briefly, tracheal mucosa was dissected as described elsewhere[12] and ground by a food processor in a solution containing 130 mM NaCl, 5 mM KCl, 25 mM Hepes (pH 7.2), 1 mM $CaCl_2$, and 5 mM glucose. The homogenate was centrifuged at 3,000 rpm (1,500×g) and the supernatant was centrifuged again for 30 minutes at 10,000 rpm (11,000 ×g) to remove the mitochondrial fractions and cell debris. The suspension of centrifugation was centrifuged again for 1 hour at 24,000 rpm in a type SW 28 rotor (Beckman Instruments, Inc., Fullerton, CA, U.S.A.). The pellet containing membrane vesicles was resuspended in a buffer containing 0.3 M sucrose, 100 mM KCl, 5 mM Hepes (pH 6.8), frozen in liquid N_2, and stored at −80℃ until it is used. The concentration of protein was determined by the Lowry method.[13]

Measurement of microsomal ATPase activity

The activity of microsomal ATPases from the epithelial cells of porcine trachea was measured by an enzyme-coupled method described by Niggli *et al.*[14] Briefly, the activity was spectrophotometrically monitored in an assay medium containing 120 mM KCl, 30 mM Hepes (pH 7.4), 1.5 mM $MgCl_2$, 50 μM $CaCl_2$, 10 mM NaCN, 0.5 mM ATP, 0.4 mM NADH, 2 mM Phosphoenolpyruvate, 1 IU/m*l* pyruvate kinase, 1 IU/m*l* lactate dehydrogenase. The rate of ATP hydrolysis by microsomal ATPases is quantitatively coupled to the rate of NADH oxidation to NAD^+ and the oxidation of NADH decreases the absorbance at 340 nm. The absorbance at 340 nm was continuously monitored and the slope of the decrease in absorbance was used to calculate total ATPase activity. The activity of microsomal Ca^{2+}-ATPase was evaluated by using thapsigargin, a specific antagonist, and the thapsigargin-induced decrease in the microsomal ATPase activity represents the amount of activity mediated by Ca^{2+}-ATPase. The activity of Ca^{2+}-ATPase in the cytoplasmic side-out microsomes will be only measurable. Although mitochondrial contamination was minimized by differential centrifugation, ATPases of contaminated mitochondria were inhibited by cyanide treatment during the analysis.

Microsomal $^{45}Ca^{2+}$ uptake and $^{45}Ca^{2+}$ release

Microsomal $^{45}Ca^{2+}$ uptake and release were measured by a modified method of Ghosh *et al.*[10] Briefly, $^{45}Ca^{2+}$ uptake was performed in an uptake medium containing 1.5 μM $^{45}CaCl_2$, 120 mM KCl, 30 mM Hepes (pH 7.0), 1.5 mM $MgCl_2$, 10 mM NaCN. The uptake was initiated by the addition of 1 mM ATP. The microsomes were transfered onto a filter paper (Whatman GF/B) and washed three times with a washing solution containing 30 mM K-Hepes (pH 6.5), 120 mM KCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 100 μg/m*l* heparin. The radioactivity remaining in the microsomes was determined by a liquid scintillation counter. Microsomal $^{45}Ca^{2+}$ release was commenced by adding release-inducing reagents after 7 min of $^{45}Ca^{2+}$ uptake.

Effect of scorpion venom

Whole venom of *Leiurus quinquestriatus hebraeus* was dissolved in a buffer (20 mM Na-Pipes, pH 7.2) and centrifuged to remove the undissolved mucoid material at 3,000 rpm for 15 min (HA-12 rotor, Hanil Science Industrial). The supernatant was gently taken and stored at −20℃ until use. Effects of Lqh venom on the microsomal Ca^{2+}-ATPase and $InsP_3$ receptor were directly measured in the reaction solution.

Result and Discussion

Effect of Lqh venom on microsomal ATPases

The whole venom of Lqh was used to investigate the effect of Lqh venom on the activity of microsomal ATPases. Total activity of microsomal ATPases was 311 nmol/min/mg protein in the absence of venom. The addition of Lqh venom increased the activity in the intact microsomes and the effect of Lqh venom was appeared to be dose-dependent (Fig. 1). The average activities of microsomal ATPases were 332, 396, and 432 nmol/min/mg protein in the presence of venom with the concentrations of 40, 80, and 160 μg/m*l*, respectively (n=2, Fig. 1B). Lqh venom at the concentration of 160 μg/m*l* increased the activity of microsomal ATPases up to 30%.

In order to test whether Lqh venom affects the acti-

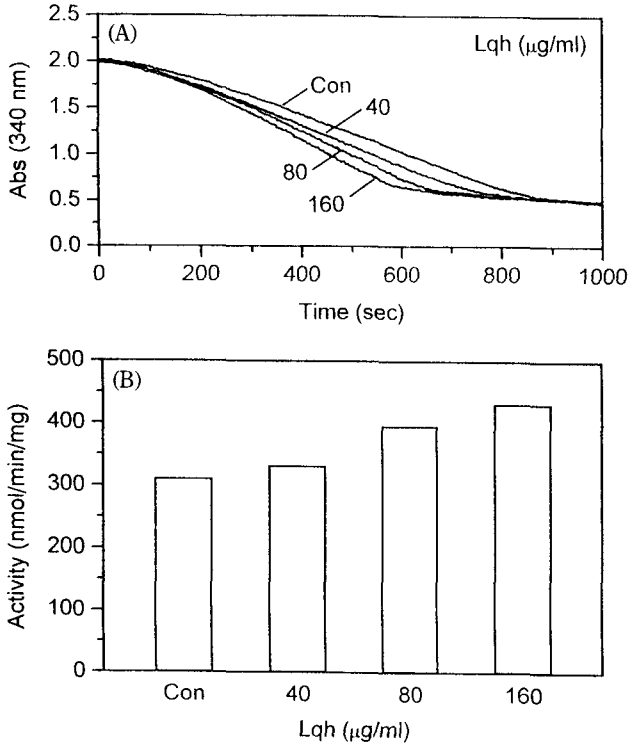

Fig. 1. Dose response of Lqh venom-induced effects on the activity of microsomal ATPase. (A) The activity of total ATPases was monitored by the decrease in absorbance at 340 nm. (B) Average activities of microsomal ATPases in different concentrations of Lqh venom. The average activity obtained from two experiments and was 311 nmol/min/mg protein in the control condition. The activities with Lqh venom of 40, 80, and 160 μg/ml were 332, 396, and 432 nmol/min/mg protein.

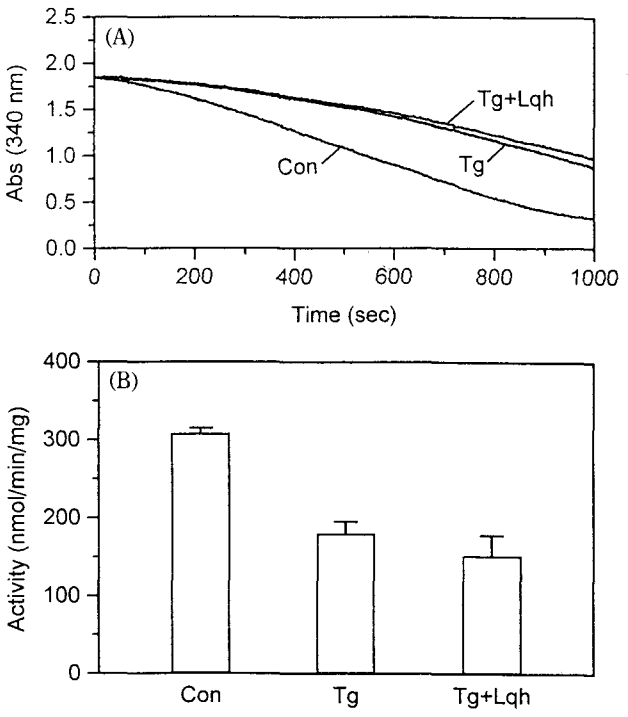

Fig. 2. Thapsigargin, a specific antagonist of microsomal Ca^{2+}-ATPase, blocked the effect of Lqh on the activity of microsomal ATPases. Thapsigargin (Tg, 10 μM) decreased the activity from 308± 7 nmol/min/mg protein (n=8) to 180± 16 nmol/min/mg protein (n=4). Lqh (120 μg/ml) did not increase the activity in the presence of thapsigargin (n=3). The activity was 152± 26 nmol/min/mg protein with simultaneous treatments of Lqh and thapsigargin.

vity of microsomal Ca^{2+} pump, thapsigargin was used to test the effect of Lqh on the microsomal preparation. Thapsigargin is known to selectively inhibit the endoplasmic reticulum isoform of the Ca^{2+}-ATPases,[15] whereas it has little effect on the plasma membrane Ca^{2+}-ATPases of hepatocyte or erythrocyte.[16] Thapsigargin (10 μM) inhibited the total activity of microsomal ATPases up to ~50% from 308± 7 nmol/min/mg protein to 180± 16 nmol/min/mg protein (Fig. 2). This inhibition suggests that ~50% of total ATPase activity is mediated by thapsigargin-sensitive Ca^{2+}-ATPases.[11] Further addition of Lqh slightly decreased the activity to 152± 26 nmol/min/mg protein and no increase in the thapsigargin-insensitive residual activity was observed. The Lqh-induced increase in the microsomal ATPase activity completely disappeared in the presence of thapsigargin. This result implies that Lqh venom somehow activates the microsomal Ca^{2+}-ATPases.

Lqh venom directly activates the microsomal Ca^{2+}-ATPases

Since there is a tight coupling between the activities of Ca^{2+}-ATPase and Ca^{2+} release channel in intact microsomes,[11] the Lqh venom-induced increase in the activity of microsomal Ca^{2+}-ATPase can be explained by two possibilities; 1) Lqh venom may directly activate microsomal Ca^{2+}-ATPase or 2) it may indirectly activate the Ca^{2+}-ATPase through the activation of Ca^{2+} release channel. Therefore, we prepared leaky microsomes to break (uncouple) the concentration gradient of Ca^{2+} across the microsomal membranes and examined the effect of Lqh on the microsomal Ca^{2+}-ATPases.

Leaky microsomes were successfully obtained by using either Ca^{2+}-ionophore A23187 or Triton X-100. The activity of microsomal ATPases was increased from 307± 7 nmol/min/mg protein to 332± 7 and 340± 12 nmol/min/mg protein by treating the microsomes with 10 μM Ca^{2+}-ionophore A23187 (Iono) and 0.0001% Triton X-100 (Tr), respectively (Fig. 3). The activity increases in the leaky microsomes are explained by an uncoupling mechanism.[11] In the leaky vesicle preparation, Lqh venom was able to increase the activity of Ca^{2+}-ATPase about 18% and 16% in the treatments of Ca^{2+}-ionophore plus Lqh venom and of Triton X-100 plus Lqh venom, respectively. These results indicate that Lqh venom directly activates the microsomal Ca^{2+}-ATPases.

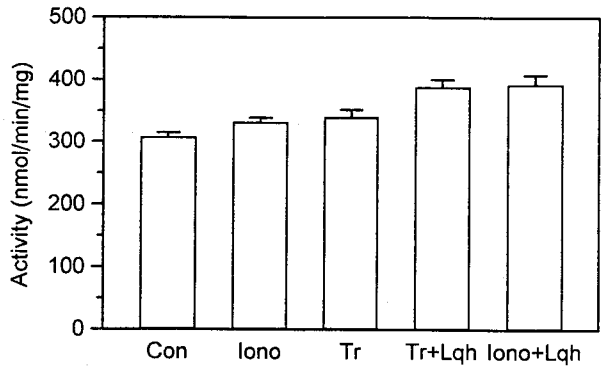

Fig. 3. Lqh increased the activity of Ca^{2+}-ATPase. Leaky vesicles were made by the addition of either Ca^{2+}-ionophore A-23187 (Iono, 10 μM) or Triton X-100 (Tr, 0.0001%) and the effects of Lqh were measured in the leaky vesicles. Both ionophore and Triton treatments increased the activity from 303± 7 nmol/min/mg protein to 332± 7 or 340± 12 nmoles/min/mg protein, respectively (n=7). Lqh (120 μg/ml) in addition to ionophore or Triton X-100 increased the activity to 393± 15 or 389± 12 nmoles/min/mg protein, respectively (n=4).

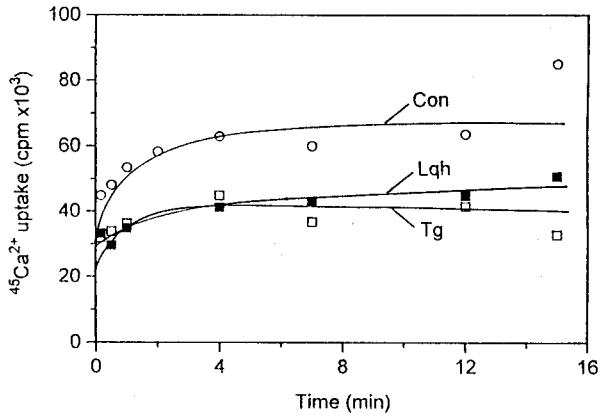

Fig. 4. Lqh decreased microsomal $^{45}Ca^{2+}$ uptake. The uptake experiment was done in the solution containing 1.5 μM $^{45}Ca^{2+}$ and 50 μM Ca^{2+}. Microsomal proteins of 75 μg were used to measure the uptake.

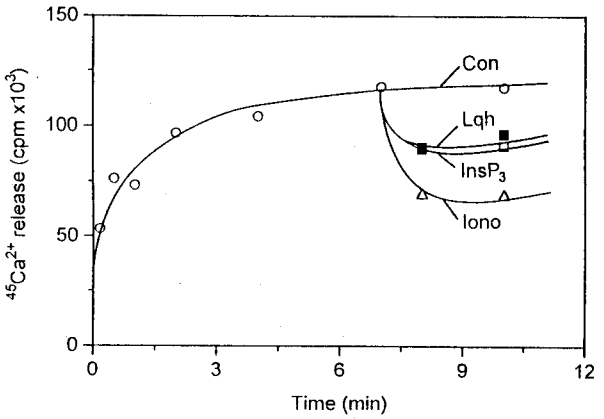

Fig. 5. Lqh-induced release of microsomal $^{45}Ca^{2+}$. The release of $^{45}Ca^{2+}$ was initiated after 7 minutes of active uptake in different conditions. Lqh- and IP_3-induced releases were similar level and were about 24% and 25% of the stored $^{45}Ca^{2+}$, respectively. Ca^{2+} ionophore-induced release was ~42%.

Effect of Lqh venom on the microsomal $^{45}Ca^{2+}$ uptake and $^{45}Ca^{2+}$ release

Since Lqh venom increases the activity of microsomal Ca^{2+}-ATPase, we expected that Lqh venom would increase the microsomal $^{45}Ca^{2+}$ uptake; however, microsomal $^{45}Ca^{2+}$ uptake was remarkably inhibited by Lqh venom (Fig. 4). In the time course of $^{45}Ca^{2+}$ uptake a saturation was observed within 2 min and the level of maximal $^{45}Ca^{2+}$ uptake was 23.6 pmol/μg protein. In the presence of Lqh, the amount of $^{45}Ca^{2+}$ uptake was decreased to 16.1 pmol/μg protein, ~30% inhibition, and this was a similar level of inhibition in the thapsigargin-inhibited $^{45}Ca^{2+}$ uptake.

To explain this unexpected inhibition in the Lqh-induced $^{45}Ca^{2+}$ uptake, we hypothesized that Lqh might also activate microsomal Ca^{2+} release channel, resulting in the release of uptaken luminal $^{45}Ca^{2+}$. In order to demonstrate whether or not Lqh venom activates any Ca^{2+} release channel in the microsomes, we measured Lqh-induced microsomal $^{45}Ca^{2+}$ release (Fig. 5). After active uptake of 7 min, the additions of inositol 1,4,5-trisphosphate ($InsP_3$) and Ca^{2+}-ionophore A23187 released microsomal $^{45}Ca^{2+}$ about 20% and 40%, respectively. $InsP_3$ releases microsomal $^{45}Ca^{2+}$ by the activation of microsomal $InsP_3$ receptor, an intracellular Ca^{2+} release channel. Interestingly, Lqh venom released ~20% of stored $^{45}Ca^{2+}$ which was similar level of release to the $InsP_3$-induced $^{45}Ca^{2+}$ release.

Lqh may release luminal $^{45}Ca^{2+}$ by working as either of an Ca^{2+}-ionophore or a Ca^{2+} channel activator. Since both $InsP_3$ and Lqh released similar amounts of stored $^{45}Ca^{2+}$, a close functional relation between these two ligands may exist. In Fig. 6, the ATPase activity in control was 307± 7 nmol/min/mg protein. When $InsP_3$, Lqh, and $InsP_3$ plus Lqh were added, the activities were 344± 7, 405± 7, and 425± 12 nmol/min/mg protein, respectively. $InsP_3$ increased the activity of Ca^{2+}-ATPase about 15% by releasing luminal $^{45}Ca^{2+}$ through the activation of $InsP_3$ receptors by an uncoupling mechanism as shown in Fig. 3. The activity of Ca^{2+}-ATPase in the presence of $InsP_3$ plus Lqh appeared to be not significantly different from that with Lqh alone, postulating that both Lqh and $InsP_3$ activate the $InsP_3$ receptor. Otherwise, the effects of these two ligands will be additive. These results as well as the results shown in Fig. 5 may also suggest that Lqh alone can activate the $InsP_3$ receptor in the absence of $InsP_3$. If this is true, Lqh will be very valuable to study the structure of $InsP_3$ receptor since no endogenous activity of $InsP_3$ receptor has been reported in the absence of $InsP_3$.[17-19]

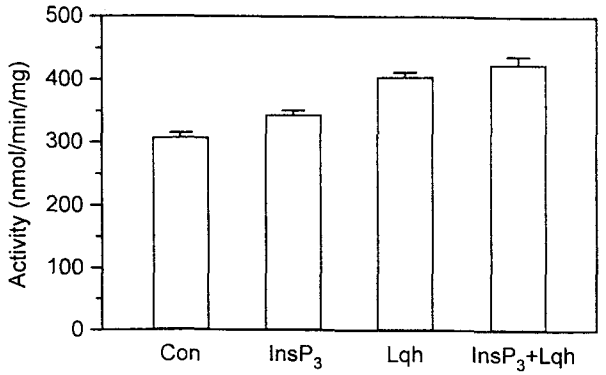

Fig. 6. Lqh and $InsP_3$ both increased the activity of Ca^{2+}-ATPase. $InsP_3$ (4 μM) increased the activity to 344± 7 nmol/min/mg protein. Lqh (120 μg/ml) also increased the activity to 405± 7 nmol/min/mg protein. However, the addition of both Lqh and $InsP_3$ did not significantly increase the activity.

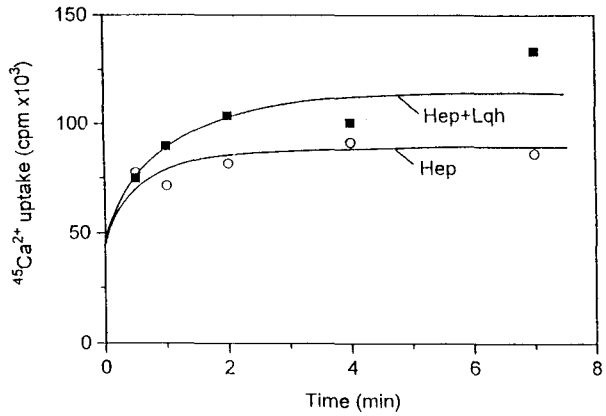

Fig. 7. Heparin blocks the Lqh-induced $^{45}Ca^{2+}$ release. The uptake experiment was done in the solution containing 1.5 μM $^{45}Ca^{2+}$ and 50 μM Ca^{2+}. Heparin (20 μg/ml) was used to block the Lqh effect.

Heparin blocks only Lqh-induced $^{45}Ca^{2+}$ release

The effect of Lqh venom on $^{45}Ca^{2+}$ uptake was measured in the presence of heparin, an antagonist of $InsP_3$ receptor.[20,21] $^{45}Ca^{2+}$ uptakes as a function of time were shown in Fig. 7. Although heparin at 100 μg/ml inhibited slightly the activity of microsomal $^{45}Ca^{2+}$-ATPase (unpublished observation), heparin at 20 μg/ml did not inhibit the microsomal $^{45}Ca^{2+}$ uptake (Fig. 7, Hep) and the level of $^{45}Ca^{2+}$ uptake at saturation was 30.8 pmol/μg protein. In the presence of heparin plus Lqh, the uptake increased to 39.0 pmol/μg protein, ~27% increase, while Lqh alone decreased the uptake by ~30% as shown in Fig. 4. This result demonstrats that heparin completely blocks the Lqh-induced $^{45}Ca^{2+}$ release and strongly suggests that Lqh targets the $InsP_3$ receptor. As a result, the uptake was increased by 30% because the whole venom of Lqh still activated the microsomal Ca^{2+}-ATPases.

Why does Lqh venom activate both $InsP_3$ receptor and Ca^{2+}-ATPase?

We have shown that the whole venom of Lqh contains at least two different components; one activates the $InsP_3$ receptor and the other activates the Ca^{2+}-ATPase. The activation of the Ca^{2+}-ATPase sequesters cytosolic Ca^{2+} into the lumen of the store while the activation of $InsP_3$ receptor releases the luminal Ca^{2+} into cytoplasm, resulting in Ca^{2+} cycling between cytosol and Ca^{2+} store. The increase in Ca^{2+} cycling utilizes ATP and increases heat production. A similar phenomenon has been known as hyperthermia and will lead a cell to a potentially lethal condition.[22] The hyperthermic effect may be a working mechanism of Lqh venom as a toxin.

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전갈독소에 의한 호흡기 상피세포 마이크로솜 Ca^{2+}-ATPase와 Inositol 1,4,5-trisphosphate 수용체의 활성촉진

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초록 : 세포의 활성을 좌우하는 세포질내의 칼슘농도 조절기작을 이해하기 위하여, 전갈 *Leiurus quinquestriatus hebraeus* (Lqh) 에서 얻은 전갈독소의 세포내 칼슘농도 조절기능과 관련된 효소들의 활성변화에 미치는 효과를 조사하였다. 칼슘펌프와 칼슘채널 효소활성은 돼지의 호흡기 상피세포에서 분리된 마이크로솜에서 측정되었으며, 전갈독소 Lqh는 온전한 마이크로솜 시료의 경우 총 ATPase의 활성을 약 32% 증가시켰고, Triton X-100나 Ca^{2+} ionophore A23187을 처리한 마이크로솜 시료에서는 총 ATPase의 활성을 약 28% 증가시켰다. Lqh 독소에 의한 ATPase의 활성증가는 Ca^{2+}-ATPase의 특이적 저해제인 thapsigargin의 처리로 완전히 저해되었으며, 이것으로 전갈독소 Lqh가 마이크로솜에 위치한 Ca^{2+}-ATPase의 활성을 특이적으로 증가시킴을 확인할 수 있었다. 이러한 결과는 결국 Lqh 독소가 Ca^{2+}-ATPase의 활성화에 의해 마이크로솜의 $^{45}Ca^{2+}$ uptake를 증가시킬 것이라는 예상을 가능하게 하나, 실제로는 Lqh의 처리가 마이크로솜의 $^{45}Ca^{2+}$ uptake를 오히려 약 30% 감소시켰다. 예상되었던 Lqh에 의한 $^{45}Ca^{2+}$ uptake의 증가는 오직 $InsP_3$ 수용체 칼슘채널의 저해제인 Heparin의 존재시에만 얻어졌다. 이것은 Lqh 독소가 Ca^{2+}-ATPase의 활성화에 따른 $^{45}Ca^{2+}$ uptake를 증가시킴과 동시에 또한 $^{45}Ca^{2+}$ release를 유발시킴을 의미하며, Lqh 독소는 실제로 $InsP_3$에 의한 칼슘채널 활성화시에 얻어지는 것과 같은 정도의 $^{45}Ca^{2+}$ release를 유발시킴이 확인되었다. 위의 결과들로부터 Lqh 독소에는 적어도 두가지의 활성성분이 포함되어 있음을 알 수 있었고, 그중 하나는 $InsP_3$ 수용체 칼슘채널의 활성을, 다른 하나는 칼슘펌프의 활성을 촉진시킨다는 결론을 얻었다. 현재 Lqh 독소의 활성성분에 대한 화학적, 전기생리학적 특성들이 연구되어지고 있다.

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