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一氧化氮/cGMP 對於聽神經-外毛細胞運動性調控之研究

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## 中文摘要

本實驗主要是研究 L-arginine 對哺乳類耳蝸外毛細胞慢運動性之影響。L-arginine (3 mM) 會導致耳蝸外毛細胞的延長，但 D-arginine (3 mM) 或其他的胺基酸 (L-aspartate or L-glutamate) 並不影響。相似的，cGMP 穿膜相似衍生物，8-(4-chlorophenylthio)guanosine 3':5'-cyclic monophosphate (1 mM) 或 8-bromo-guanosine 3':5'-cyclic monophosphate (1 mM) 也可引發天竺鼠耳蝸毛細胞的延長。L-arginine 引發毛細胞的延長的作用可被一氧化氮合成酵素抑制劑 N<sup>6</sup>-nitro-L-arginine methyl ester hydrochloride (3 mM) or 7-nitroindazole (1 mM) 所阻斷。比較 L-arginine 與 ionomycin 在細胞長度與細胞內鈣的作用，L-arginine 與 ionomycin 皆可造成天竺鼠耳蝸外毛細胞的延長，但 L-arginine 不會引起細胞內鈣離子濃度的變化。將外毛細胞前處理 EGTA (3 mM) 40 分鐘以變降低細胞外鈣濃度並不會影響 L-arginine 的作用。這個實驗證明一氧化氮/cGMP 路徑參與調控哺乳類耳蝸外毛細胞慢運動性的可能性。而 L-arginine 的作用是非鈣依賴性。

關鍵詞：外毛細胞；運動性；L-Arginine；一氧化氮；鈣離子。

## Abstract

The effect of L-arginine on the slow motility of mammalian cochlear outer hair cells was studied in this experiment. L-Arginine (3 mM) but not D-arginine (3 mM) or other amino acids (L-aspartate or L-glutamate) induced length increases of guinea pig outer hair cell. Similarly, the membrane-permeant cGMP analogues, 8-(4-chlorophenylthio)guanosine 3':5'-cyclic monophosphate (1 mM) or 8-bromo-guanosine 3':5'-cyclic monophosphate (1 mM) induced length increases of guinea pig outer hair cells. These length increases induced by L-arginine can be attenuated by a 30 min preincubation of the cells with the nitric oxide synthase inhibitors N<sup>G</sup>-nitro-L-arginine methyl ester hydrochloride (3 mM) or 7-nitroindazole (1 mM). Comparing the effects of L-arginine and ionomycin on cell length and intracellular calcium change in outer hair cells, both L-arginine and ionomycin were able to induce the elongation of outer hair cells but L-arginine did not change the fluorescence intensity of Fluo-3. Preincubation of the cells with EGTA (3 mM) for 40 min to reduce the extracellular calcium concentration did not influence the effect of L-arginine. This experiment demonstrated that nitric oxide/cGMP pathway involvement in regulating the slow motility of mammalian outer hair cells cannot be ruled out. The effect of L-arginine is independent of extracellular calcium concentration.

Key words: Outer hair cell ; Motility; L-Arginine; Nitric oxide; Calcium

## 1. Introduction

The outer hair cells play a very important role in sound transduction. Outer hair cells seem to act like tiny motors that amplify the movement of the basilar membrane during low-intensity sound stimuli. Recent studies demonstrated that prestin is required for electromotility of the outer hair cell (Liberman et al., 2002). The hair cells' motor is driven by the receptor potential, and it does not use ATP as an energy source (Kachar et al., 1986). It is also extremely fast, as it must be able to keep up with the movements induced by high-frequency sounds. The slow motility responses of outer hair cells can be elicited in response to osmotic pressure changes (Dulon et al., 1987), as well as to a variety of chemical stimuli (Zenner et al., 1986; Flock et al., 1986; Schacht and Zenner, 1987; Ulfendahl, 1987; Dulon et al., 1990). The increase of intracellular calcium level by ionomycin was result in a length increase of mammalian outer hair cell through mechanisms of the activations of myosin light chain kinase and calcium/calmodulin-dependent protein kinase (Puschner and Schacht, 1997; Coling et al., 1998). Furthermore, external calcium was not required for the first outer hair cell contraction but essential for relaxation (Zenner et al., 1985). Due to the high potassium levels extracellularly can cause the shortening of outer hair cell but ionomycin induced the elongation of the cell, there is a need to further studies to clarify the detail for the role of calcium both intra- and extracellularly. As we known NO/cyclic GMP pathway actively regulates vascular smooth muscle involves decreasing the  $Ca^{2+}$  sensitivity (Blatter and Wier, 1994). Nitric oxide synthase and guanylate cyclase are important regulatory enzymes in the cGMP pathway. Nitric oxide-stimulated soluble guanylate cyclase activity was detected in supporting cells but not in outer hair cells (Fessenden and Schacht, 1997, 1998; Tian et al., 1999), but we cannot preclude the possibility in the synapse of outer hair cells. It has been reported the outer hair cell electromotility (fast motility) is modulated by cyclic GMP-dependent pathway (Szönyi et al., 1999). Furthermore, Nuttall et al. (2001) demonstrated the present of nitric oxide in the outer hair cell of guinea pig using the nitric oxide fluorescent dye 4,5-diaaminofluorescein acetate. In these studies, we attempt to reveal whether L-arginine, a precursor of nitric oxide to affect the slow motility of outer hair cells. The results showed that L-arginine but not D-arginine was to increase cell length of outer hair cell. The effect of L-arginine can be prevented by pretreatment of NOS inhibitor  $N^G$ -nitro-L-arginine methyl ester hydrochloride (L-NAME) or 7-nitroindazole (7-NINA). The elongation of outer hair cell induced by L-arginine is not only independent of extracellular  $Ca^{2+}$  concentration but also without the increased effect of the intracellular calcium concentration. The effects of L-arginine were quite different from that of effects of the ionomycin on the mammalian outer hair cell

## 2. Materials and Methods

Outer hair cells were isolated from guinea pig (Hartley, National Laboratory Animal Breeding

and Research Center, Taiwan) after enzymatic and mechanic dissociation as described previously (Tan et al., 2001). Anesthetized guinea pigs (250~400g) were decapitated and temporal bones quickly removed. The bulla and the bony walls of cochlea were immediately opened and the organ of Corti dissected. Tissues and dissociated cells were handled in Hank's balanced salt solution consisting of 137 mM NaCl, 5.4 mM KCl, 1.25 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 0.4 mM MgSO<sub>4</sub>, 0.33 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM D-glucose and 5 mM HEPES. The first two apical turns of organ of Corti were collected and transferred into collagenase (type IV, Sigma) solution (1 mg/ml in Hank's balanced salt solution). After 10 min digestion the pieces of organ of Corti were placed in a cover-slip chamber and the outer hair cell were isolated by gentle titration for 3~5 times with a 20 µl pipette under stereomicroscope. Cell motility (contraction and elongation) was recorded for 5-10 min using 40 X or 100 X oil immersion objective on an inverted Zeiss microscope (Axiovert 25, Germany) fitted with a digital camera AxioCam (Zeiss, Germany). Analysis of length change was performed by the software AxioVision (Zeiss, Germany). Cell lengths were measured from the distance between the cuticular plate and the synaptic ending of the cell. Cells were selected for measurement without obvious sign of damage, swelling, nucleus dislocation or granulation. The pH was adjusted to 7.35~7.40 with NaOH and the osmolarity to 310 ± 3 mosm. Osmolarity (osm) of the medium was controlled before and after each experiment at room temperature (24~27 °C). The length of isolated apical outer hair cells ranged from 60~80 µm were used for this experiment.

The changes of intracellular Ca<sup>2+</sup> concentration in outer hair cells were determined using the fluorescent Ca<sup>2+</sup> indicator fluo-3/AM and a confocal laser scanning fluorescence microscope (Zeiss Confocal LSM410 equipped with an Argon-Krypton Laser). Fluo-3/AM (Molecular Probes, Eugene, USA) was dissolved in DMSO and 3 µM loaded into outer hair cells on cover slip for 40 min. Cells loaded with fluo-3 were observed with an inverted microscope (Zeiss Axiovert 135M, Germany), with excitation at 488nm and emission at 525nm. The changes of intracellular calcium levels of outer hair cells were indicated by the relative Fluo-3 fluorescence intensity.

### *Chemicals*

L-arginine, D-arginine, N<sup>G</sup>-nitro-L-arginine methyl ester hydrochloride (L-NAME), 8-(4-chlorophenylthio) guanosine 3':5'-cyclic monophosphate (pCPT-cGMP), 8-bromo - guanosine 3':5'-cyclic monophosphate (8-br-cGMP) were obtained from Sigma (St Louis, MO, USA). 7-nitroindazole was obtained from Tocris (MO, USA). All drugs were dissolved in Hank's balanced salt solution except Fluo-3 AM and pCPT-cGMP, which were dissolved in dimethyl sulphoxide (DMSO). The final total DMSO concentration did not exceed 1% in the bath medium. The osmolarity of all mediums for these experiments were controlled to be 310 ± 3 mOsm.

### *Statistics*

The values given are means ± SEM. The significance of differences was evaluated by the Student's *t*-test. When more than one group was compared with one control, significance was evaluated according to one-way analysis of variance (ANOVA). Probability values of < 0.05 were considered to be significant.

### 3. Results

#### 3.1 *L-arginine but not D-arginine induced cell elongation*

A typical isolated outer hair cell with bundle of cilia (*c*) on the top and auditory nerve terminal (synapse, *s*) attached on the bottom of cell was shown in Fig. 1A, and the picture after 120 sec the addition of L-arginine (3 mM) was shown in Fig. 1B. Application of L-arginine (3 mM) was unable to induce a morphological change of outer hair cell except the cell elongation. Perfusion with L-arginine (3 mM) but not D-arginine 3 mM induced the elongation of outer hair cell (Fig. 1C). The effect of L-arginine was reversible after washout. L-arginine (3 mM) but not L-glutamate (3 mM) and L-aspartate (3 mM) induced the elongation of outer hair cell. The effect of cell elongation reached a maximal about after 100 sec ( $102.8 \pm 0.6\%$ ,  $n = 11$ ; Fig. 1 C) L-arginine was applied. Other amino acids, D-arginine (3 mM;  $99.5 \pm 0.3$ ;  $n = 4$ ) L-glutamate (3 mM;  $99.6 \pm 0.4$ ;  $n = 6$ ) or L-aspartate (3 mM;  $99.7 \pm 0.4$ ;  $n = 4$ ) or vehicle ( $99.7 \pm 0.3$ ;  $n = 4$ ) was not to change the cell length significantly (Fig. 2A). The dose response curve showed that 2 mM to 3 mM of L-arginine produce a significant increase effect of the length of cells (Fig. 2B). The higher concentration level of L-arginine (10 mM) was to induce a maximal length increases (114% of before addition of L-arginine), and then accompanied by a significant morphological change (e.g., cell swelling, nucleus dislocation). Therefore a reasonable concentration of L-arginine (3 mM) was without to produce the cell toxicity was used throughout the experiment.

#### 3.2 *Nitric oxide synthase inhibitors attenuate the effect of L-arginine*

Preincubation of cells with NOS inhibitors, N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME, 3 mM, Fig.3A and 3B) or 7-nitroindazole (7-NINA, 1 mM; Fig. 3B) for 30 min were to reduce significantly the cell elongation induced by L-arginine (3 mM). The maximal length increases produced by the pretreatment of L-NAME ( $n = 7$ ) were  $100.5 \pm 0.3\%$  ( $P < 0.05$  as compared with L-arginine alone;  $102.8 \pm 0.6$ ,  $n = 11$ ), and by the pretreatment of 7-NINA ( $n = 5$ ) were  $100.7 \pm 0.4\%$  ( $P < 0.05$  as compared with L-arginine alone) (Fig. 3B).

#### 3.3 *Membrane permeant Cyclic GMP analogues -induced cell elongation*

Cell-permeable cyclic GMP analogues, 8-(4-chlorophenylthio) guanosine 3':5'-cyclic monophosphate (pCPT-cGMP, 1 mM) or 8-bromoguanosine 3',5'-cyclic monophosphate (8-br-cGMP, 1 mM) induced a cell elongation reached a maximum at 4 min (Fig. 4). The maximal length induced by pCPT-cGMP was  $101.5 \pm 0.3\%$  of control ( $P < 0.05$  as compared with before the drug is applied,  $n = 7$ ) and induced by 8-br-cGMP was  $101.10 \pm 0.30\%$  ( $P < 0.05$  as compared with before the drug is applied,  $n = 5$ ). In the vehicle alone experiment showed that without produce a significant effect on the length change of outer hair cell (maximal length change was  $99.4 \pm 0.5\%$  of before treatment,  $n = 4$ ). The effects induced by cyclic GMP analogues were significant but lesser potent than L-arginine on the outer hair cell elongation.

#### 3.4 *L-arginine does no change in the intracellular calcium level.*

Addition of 3 mM L-arginine had no effect on the change of Fluo-3 fluorescence intensity (Fig. 5A). However, the Fluo-3 fluorescence intensity was increased after by the addition of high potassium medium (75 mM KCl replacing with equal molar NaCl) and can be furthered enhanced by the subsequent addition of ionomycin (10  $\mu$ M) (Fig. 5B). The levels of intracellular calcium were not correlative with the cell length changes. Fig. 5C showed that the

time course of cell length changes, L-arginine or ionomycin induced elongation but high  $K^+$  induced shortening of outer hair cell.

### *3.5 Effect of L-arginine is independent of extracellular calcium concentration*

The outer hair cell was incubated in calcium free Hank's balanced solution (supplemented with 3 mM EGTA) for 40 min, the addition of 3 mM L-arginine still produced an elongate effect of outer hair cells (Fig. 6 A) but the level of intracellular calcium concentration remained unchanged (Fig. 6 B).

## **4. Discussion**

The present study showed that L-arginine but not D-arginine or other amino acids (L-aspartate or L-glutamate) to induce the effect of extension of outer hair cell. Due to the effect of L-arginine can be attenuated by pretreatment with nitric oxide synthase inhibitors (L-NAME or 7-NINA), the NO/cGMP pathway involving in the regulation of slow motility of outer hair cell cannot be ruled out. Furthermore, these results revealed that a  $Ca^{2+}$ -independent pathway in the regulation of cell slow motility was quite different from that of the effect of ionomycin (Coling, et al., 1998).

The specific effect of L-arginine

It has been reported that electrostatic interactions among the fibers comprising the pillars could lead to changes in the cell length (Steele, 1990). Furthermore, electrostatic interaction between stereocilia may influence the mechanical properties of the hair bundle (Dolgobrodov, et al., 2000). We tested the effect of four polar amino acids (L-arginine, D-arginine, L-aspartate or L-glutamate) individually on the isolated outer hair cell in this study. The results showed that all polar amino acids except the L-arginine were unable to induce the elongation of outer hair cell. The effect of L-arginine was independent of its polar property. Due to the D-form of arginine without effect on the elongation of outer hair cell, the effect of L-arginine was to be considered as specific.

Comparison between the effect of L-arginine and ionomycin

The movement of outer hair cells can be induced by potassium (Zenner, 1985), electric stimulation as well as by ATP (Schacht and Zenner, 1987; Flock et al., 1986) and ionomycin (Dulon et al., 1990). The elevation of intracellular calcium by ionomycin in turn generates circumferential or cortical forces leading to a reduction of cell diameter and an elongation of the cell. The length increases induced by ionomycin can be inhibited by preincubation of the cells with the myosin light chain kinase inhibitors or an inhibitor of  $Ca^{2+}$ /calmodulin-dependent protein kinase II (Pushner and Schacht, 1997; Coling et al., 1998). These data suggested that protein kinase activity regulates calcium-dependent processes that effect shape changes of outer hair cells. High potassium medium, is similar to ionomycin, has been shown to increase intracellular calcium concentration but to cause a length shortening (contraction) of outer hair cells (Fig. 5B and 5C). Although the high potassium medium and ionomycin produce an increase of intracellular calcium level, their actions must be via a different mechanism. L-arginine did not change the concentration of intracellular calcium but still to produce an elongate effect of outer hair cell in the present studies. It has been demonstrated that external calcium was required for relaxation of the



contracted outer hair cells (Zenner et al., 1985; Dulon et al., 1990), the effect of L-arginine was independent of extracellular calcium provide an another possible pathway to induced length increases of the outer hair cell.

#### Nitric oxide/cGMP –signaling pathway

The nitric oxide/cGMP-signaling pathway is important in several physiological functions including vascular smooth muscle relaxation, neuronal signal transduction and inhibition of platelet aggregation (Robbins and Grisham, 1997). It has been suggested that overstimulation of soluble guanylate cyclase may mediate NO-induced cochlear toxicity (Dais et al., 1996). Recent studied have proven that the localization of nitric oxide synthase to nerve fibers in the organ of Corti (Michel et al., 1999). The target enzymes of nitric oxide, guanylate cyclase and protein kinase G, are seen in the supporting cells but not in the sensory hair cells. Nitric oxide/cyclic GMP pathway could play a role in controlling calcium homeostasis of supporting cells and regulating cochlear blood flow (Fessenden and Schacht, 1998). In contrast, nitric oxide signal was observed in the outer hair cell, afferent nerves and their putative endings near inner hair cells and efferent nerve endings near outer hair cells using the fluorescent dye, 4,5-diaminofluorescein diacetate as indicator (Shi et al., 2001). The role of nitric oxide in the regulating on the motility of outer hair cell is need to further investigation. Since the effects of L-arginine on the slow motility of outer hair cell can be attenuated by the pretreatment of nitric oxide synthase inhibitors, we cannot rule out a possible role of nitric oxide/cGMP pathway in the regulating on slow motility of outer hair cell. Furthermore, outer hair cell electromotility is modulated by a cGMP-dependent pathway has been reported (Szönyi et al., 1999). Several possible mechanisms might produce the slow motility of outer hair cells induced by L-arginine. First, the nitric oxide synthase and guanylate cyclase exist in auditory nerve terminals and the nitric oxide as retrograde message to act on the postsynaptic membrane of outer hair cell. Second, the total amount of nitric oxide synthase and guanylate cyclase is too low to be detectable. Third, L-arginine may act as a regulatory role of the membrane motor proteins of outer hair cell directly. While the precise molecular mechanism remains to be established. Take together; the present study demonstrates a calcium- independent cell elongation induced by L-arginine in outer hair cell. The effects of L-arginine revealed that a possible calcium-independent pathway could be involved in the regulation of outer hair cell motility.

#### **Acknowledgments**

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## 6. Figures:

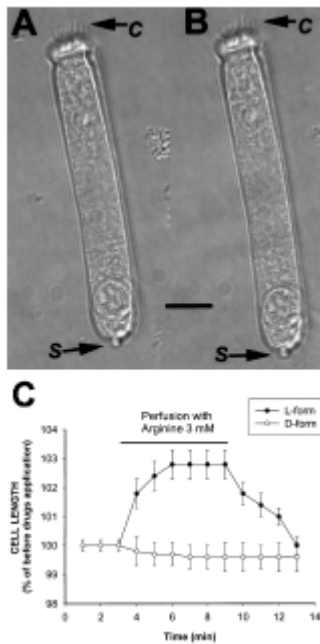


Fig.1 Effect of L- or D-form arginine on the cell length change of isolated outer hair cell of guinea pig. (A) The picture showed a typical intact isolated outer hair cell with clearly visible bundle of cilia (c) on the top and synapse (s) on the bottom of cell. The elongation of cell length can be induced by after the application of L-arginine 3 mM (B, picture showed 130 sec after the application of L-arginine). (C). The time course of the cell length change with perfusion of 3 mM L- or D-arginine ( $n = 4\text{--}6$  for each curve) on the outer hair cells. Cell length increment was shown as a percentage of before the addition of arginine. Scale bar in A and B:  $12\ \mu\text{m}$ . Values are mean  $\pm$  S.E.M. \*  $P < 0.05$  as compared with before the addition of L- or D-arginine.

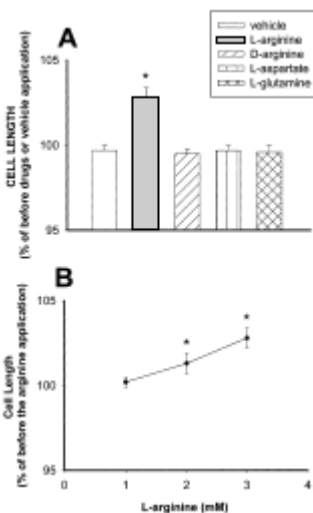


Fig. 2 Compare effects of L-arginine, D-arginine, L-glutamate and L-aspartate in cell length change of outer hair cell. (A) The percentage of maximum length change of outer hair cells produced by four amino acids individually, L-arginine, D-arginine, L-glutamate or L-aspartate. L-arginine (3 mM;  $n = 11$ ) but not other amino acids, D-arginine (3 mM;  $n = 4$ ), L-glutamate (3 mM;  $n = 6$ ), L-aspartate (3 mM;  $n = 5$ ) or vehicle ( $n = 4$ ) was to induce the length elongation of outer hair cell. Length change is shown as percent change from cell length at time zero (before the addition of 3 mM amino acids). (B) Dose-response curve of L-Arginine (1 to 3 mM). Values are mean  $\pm$  S.E.M. \*  $P < 0.05$  as compared with vehicle (in A) or before the arginine applied (in B).

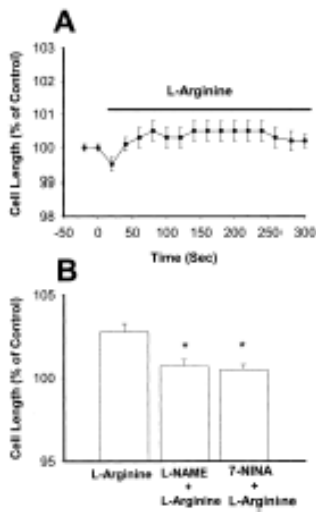


Fig. 3 Pretreatment with nitric oxide synthase (NOS) inhibitors, N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) (L-NAME) or 7-nitroindazole (7-NINA) caused decrease in the effect of cell length elongation induced by L-arginine. The cells were preincubated with NOS inhibitor L-NAME (3 mM) (A) or 7-NINA (1 mM) (B) for 30 min, L-arginine (3 mM) do not cause significant effect on the extension of outer hair cells. Values are mean  $\pm$  S.E.M. \* P < 0.05 as compared with control.

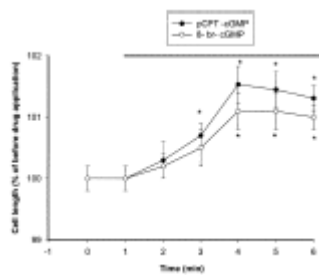


Fig.4 Effect of cyclic GMP membrane-permeable analogues, 8-(4-chlorophenylthio) guanosine 3':5'-cyclic monophosphate (pCPT-cGMP) or 8-bromo cyclic GMP (8-Br-GMP) on the cell length change of outer hair cell. Application of pCPT-cGMP (1 mM) or 8-Br-GMP (1 mM) was significant to induce the cell elongation of outer hair cell. Values are mean  $\pm$  S.E.M. \* P < 0.05 as compared with before the addition of pCPT-cGMP or 8-Br-GMP.

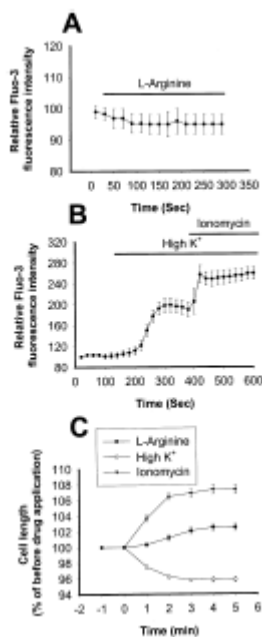


Fig.5 Effect of L-arginine, high potassium or ionomycin on changes of intracellular fluo-3

fluorescence intensity (A; B) and length (C) of outer hair cells. (A) L-arginine (3 mM) cannot cause a significant change in the intracellular calcium level of outer hair cell. (B) Extracellular perfusion with high  $K^+$  (75 mM) medium alone, or the subsequently application of ionomycin (10  $\mu$ M) produced a significant increase of the concentration of intracellular calcium. (C) A comparison of effects of L-arginine, high potassium and ionomycin on the cell length change of outer hair cell. L-arginine or ionomycin was to increase but high potassium to decrease the cell length of outer hair cells. Note that there are without relation between the intracellular calcium level and the change of cell length. Values are mean  $\pm$  S.E.M. \*  $P < 0.05$  when compared to before treatment.

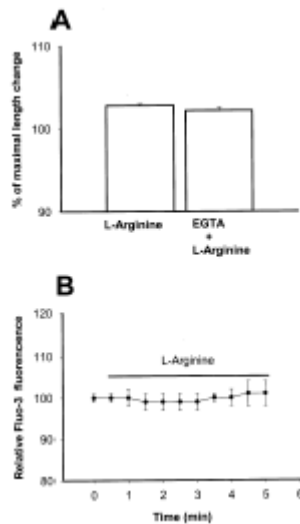


Fig. 6 Reducing the extracellular calcium concentration with EGTA (3 mM) for 40 min cannot to alter the effect of L-arginine on the percentage of maximal elongation of outer hair cell (A). In the fluo-3 calcium imaging studied, L-arginine still cannot to produce a change in the intracellular calcium level when pretreated with EGTA (3 mM) for 40 min (B). Values are mean  $\pm$  S.E.M.

7. 計畫成果自評: 此報告部份已發表在: Hearing Research 178:52-58 (2003).