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Original Article

# Effects of intracellular calcium and calcium channel blockers on outer hair cells of guinea pig cochlea

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This study investigated calcium currents in isolated outer hair cells (OHC) of guinea pig cochlea using whole cell patch-clamp and intracellular calcium imaging. A P/Q-type $Ca^{2+}$ channel blocker ω-conotoxin MVIIC (5 μM) similar to nifedipine (30 μM) and $Cd^{2+}$ (1 mM), inhibited both the $Ca^{2+}$ current and $Ca^{2+}$-activated- $K^+$-current. The $Ca^{2+}$ current was inhibited by nifedipine. The residual amplitude was further reduced by the addition of 5 μM ω-conotoxin MVIIC. According to our measurements of $[Ca^{2+}]_i$, both nifedipine and ω-conotoxin MVIIC reduced the effect of $[Ca^{2+}]_i$ induced by external application of the high $K^+$ medium (50 mM). The shortening of OHC was induced by the application of the high $K^+$ medium, while introduction calcium channel blockers or lowering the extracellular calcium concentration did not inhibit the hair cell shortening evoked by high $K^+$ stimulation. It was concluded that the shortening of OHC induced by high $K^+$ medium is independent on the extracellular $Ca^{2+}$, membrane $Ca^{2+}$ and $Ca^{2+}$-activated $K^+$-channels. The possible mechanism underlying the unique property of outer hair cells is discussed.

**Key words:** $Ca^{2+}$ currents, motility, outer hair cells, whole-cell voltage clamp

## Introduction

The outer hair cells (OHC) of the mammalian cochlea exhibit motility, an essential step in the mechanical amplification to normal hearing, in response to changes in membrane potential[3]. Sound wave is transmitted via the ear canal and the middle ear to the cochlea, where the resulting pressure differences across the organ of corti elicit a complex vibratory motion. The bending of the OHC in the direction of the tallest stereocilia causes the opening of non-selective cation channels located near the top of the hair bundle, allowing an ion current to pass through the hair cells, thereby generating receptor potentials. OHC motility is thought to contribute an essential step of mechanical amplification to normal hearing. A voltage dependent inward $Ca^{2+}$ current flows across the plasma membrane of mammalian cochlear OHC during depolarization and contributes to the motile response[11]. L-type $Ca^{2+}$ channels play a role in the OHC of guinea cochlea[16, 17]. The L-type voltage-dependent $Ca^{2+}$ channel, in particular, may be responsible for the influx of $Ca^{2+}$ from the perilymph[16] resulting from membrane

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depolarization. Depolarization of OHCs opens voltage-activated $Ca^{2+}$ channels that in turn cause a localized rise in intracellular $Ca^{2+}$ provoking neurotransmitter release. A fast motile response can be induced by the voltage steps associated with structures in the cell cortex[7]. A slow motile response (shape changes measured in seconds) also can be produced by a high $K^+$ medium[19]. Extracellular high-potassium (high $K^+$) or extracellular high-calcium (high $Ca^{2+}$) solution was able to increase $[Ca^{2+}]_i$, suggesting the importance of cell membrane potential and $Ca^{2+}$ transport across the membrane for $[Ca^{2+}]_i$ regulation. The longitudinal component of the shape change is probably due to activation of myosin light chain kinase and the $Ca^{2+}$/calmodulin-dependent kinase II. Both motor and cytoskeletal proteins may be targets of phosphorylation which could conceivably result in a change of electromotility.

However, external $Ca^{2+}$ is not required for OHC contraction. Furthermore, an intracellular $Ca^{2+}$ does not produce a shortening of OHC; to produces a prolongation[8]. Thus, the aim of this study was to investigate the relation between the $Ca^{2+}$ channel and slow motile response of hair cell induced by high $K^+$ medium.

## Materials and Methods

Apical OHCs were isolated from the cochlea of guinea pigs (250-450 g) euthanized using sodium pentobarbital. The organ of corti was microdissected in phosphate external solution (in mM, NaCl 142, KCl 4, $CaCl_2$ 1.5, $MgCl_2$ 1, $Na_2HPO_4$ 8, $NaH_2PO_4$ 2, D-glucose 10, pH=7.3). Two apical turns of the organ of corti were transferred into 80 µl of external solution containing 0.5 mg/ml of trypsin (type III, Sigma) for 15 min, and then the apical hair cells were mechanically dissociated in the enzyme-free phosphate external solution. Cells were used within 3 h of the dissection. Isolated apical OHC (about 50-80 µm long) were placed in a disc chamber and visualized at 400X magnification using a Zeiss inverted microscope at room temperature (24-28℃). The conventional whole-cell recording techniques were made using an Axopatch-200A amplifier (Axon Instruments). Recording pipettes were pulled using a flaming micropipete puller (Model P-87, Sutter Instrument co., USA), which had an access resistance between 7-12 MΩ in the bath. The internal solution for the measurement of the outward current contained the following (in mM): KCl 144, $MgCl_2$ 2, $Na_2HPO_4$ 8, $NaH_2PO_4$ 2, D-glucose 10, ATP 1, GTP 0.1, EGTA 0.5. The external and internal solutions for the measurement of $Ca^{2+}$-current contained the following (in mM): choline-Cl 140, CsCl 5, $CaCl_2$ 10, HEPES 10, glucose 10 and N-methyl-D-glucamine chloride (NMDG) 30, N-methyl-D-glucamine aspartate 90, Tetraethylammonium chloride 30, $CaCl_2$ 0.5, $MgCl_2$ 5, EGTA 5, ATP 5, cAMP 0.2, GTP 1, HEPES 10, respectively, as described by Nakagawa et al (1992). The pH of the external and internal solutions were adjusted to 7.3 and 7.2, respectively, using Tris-OH. Leakage of the current was subtracted by adding those currents generated during equal amplitude steps of opposite polarity. The recording signals were monitored and analyzed using a Macintosh G3 computer with a digital-to-analog converter and Maclab software (Powerlab/4s, AD Instruments).

Measurement of $[Ca^{2+}]_i$ was performed as previously described using microspectroflurorimetry and the $Ca^{2+}$ -sensitive indicator fluo-3 AM (Calbiochem., USA) [15]. Briefly, cochlear OHC placed on 22 mm diameter coverglasses were loaded for 30 min at 37 ± 0.5 ℃ with fluo-3 AM (1 µM) applied in Leibovitz's L-15 medium. Coverslips were placed in a round chamber (~0.5 ml) in modified Kreb's solution (120 mM NaCl, 5 mM KCl, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 13 mM Glucose) on an Axiovert 135M inverted microscope (Zeiss). Excitation of fluo-3 occurred at 480 nm and emitted light monitored at 540 nm. Cell-derived fluorescent images were visualized using a 100x, 1.3NA oil-immersion objective. Digitized images were then used to calculate ratio images using LSM 4 software (Zeiss). The measurement of cell length was performed using an Axiocam CCD camera and Axiovision software. The outer hair cell length change recorded using a CCD camera (Macrofire), with the Zeiss microscope (×63 objective). Cell electromotile length changes were determined

by tracing cell boundaries manually using NIH Imaging software (ImageJ 1.45, NIH, USA). Data are given as mean ± S.E. Comparisons of sample means were analyzed using the Student's t-test. A $P < 0.05$ was considered significant.

## Results

Three different $Ca^{2+}$ channel blockers ($Cd^{2+}$, nifedipine and ω-conotoxin MVIIC) were tested on the $K^+$ current of hair cell. After the whole-cell recording conditions were established, the average value of the membrane potential was found to be –52.3 mV (SE = 3.4 mV, n = 12). Fig 1A shows a typical series of current and voltage recordings obtained from one OHC. The outward current was activated at potentials more positive than –30 mV (Fig 1A and 1B, -●-; holding potential = -50 mV). The I-V relationship of outward current is shown in Fig. 1 B (n = 3-5). $Ca^{2+}$ channel blockers, ω-conotoxin MVIIC (5 μM, -○-), nifedipine (30 μM, -▽-) and $CdCl_2$ (1 mM, -▼-) significantly depressed the outward current. The inhibitory effects caused by reduction in $Ca^{2+}$ influx from the $Ca^{2+}$ channel were, therefore, depressed the amplitude of the $Ca^{2+}$-activated $K^+$ outward current. To investigate the effects of these blockers on $Ca^{2+}$ current, we used the external choline solution and internal pipette NMDG solution. The inward currents activated between –20 mV and –30 mV step from the –70 mV holding potential. They increased with further depolarization peak nearing +10 mV in 10 mM $Ca^{2+}$ containing solution (Fig 2, -●-). The peak amplitude of the inward current was significantly decreased after adding 5 μM ω-conotoxin MVIIC (Fig 2). Application with nifedipine (30 μM), a L-type $Ca^{2+}$ channel blocker, inhibited the amplitude of inward current significantly, too (n =3, Fig 2, -○-). Subsequent addition of ω-conotoxin MVIIC (5 μM) further inhibited the inward current (Fig 2, -▼-). The $Ca^{2+}$ influx into living OHC was visualized by a visible wavelength $Ca^{2+}$ probe fluo-3. Superfusion with high $K^+$ solution (50 mM) increased the fluorescence intensity as shown in Fig. 3A. Pretreatment with ω-conotoxin MVIIC (5 μM; -▼-) or nifedipine (30 μM; -○-)

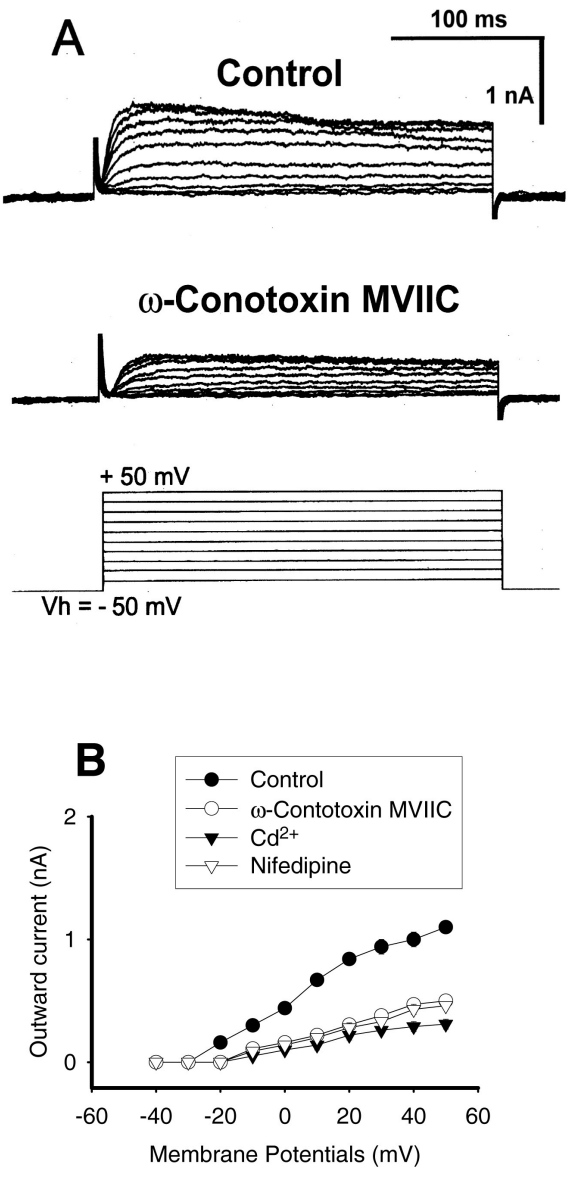


Fig. 1. Effects of $Ca^{2+}$ channel blockers on the outward potassium current. Holding potential (Vh) was –50 mV and potassium outward current were elicited by 300 ms depolarizing pulses from holding potential to +50 mV. A, the representative outward potassium current with (lower trace) or without (upper trace) 5 μM ω-conotoxin MVIIC B, $Ca^{2+}$ channel blockers on the I-V relationships of the outward potassium current. $Ca^{2+}$ channel blockers, 5 μM ω-conotoxin MVIIC (-○-), 30 μM nifedipine (-▽-) or 1 mM $Cd^{2+}$ (-▼-) significantly inhibited the outward potassium current (control current: -●-). Each point is the average of 3-5 cells and the vertical bars show ± SEM. Calibration in A: 100 ms, 1 nA.

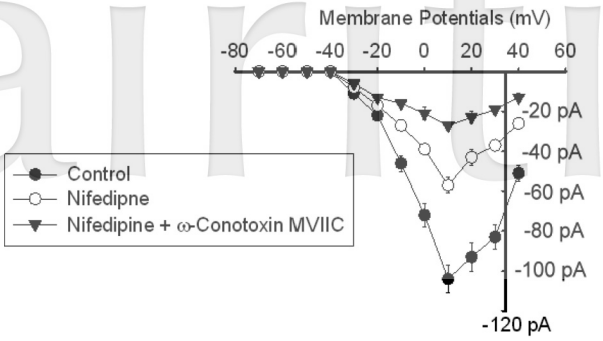


Fig. 2. Effects of $Ca^{2+}$ channel blockers on $Ca^{2+}$ current. Holding potential (Vh) was –70 mV and $Ca^{2+}$ current were elicited by 300 ms depolarizing pulses from holding potential to +40 mV. I-V relationships of $Ca^{2+}$ current: -●-: control $Ca^{2+}$ current; -○- : nifedipine 30 μM; -▼-: nifedipine plus 5 μM ω-conotoxin MVIIC. Each point is the average of three cells and the vertical bars show ± SEM. Calibration in A and B: 100 ms, 0.1 nA.

for 10 min significantly inhibited the increase of intracellular calcium induced by high $K^+$ solution (Fig. 3B). Cell shortening (motility) was induced by depolarization or by application of a high $K^+$ solution. Application of the high $K^+$ medium (50 mM) caused a shortening of the cells (Fig 4B; Fig 4A, before application); the effect was reversible by washout (cell length recovery, Fig 4C). $Ca^{2+}$ channel blockers, nifedipine, ω-conotoxin MVIIC, or the addition of EGTA (2 mM) to extracellular solution did not significantly prevent the shortening of cell induced by high $K^+$ solution (Fig 4D).

## Discussion

Our results show that the calcium channel blockers can inhibit the calcium current or fluorescence intensity of fluo-3 induced by high $K^+$ stimulation. Interestingly, neither calcium channel blockers nor lowering the extracellular calcium concentration (with EGTA) were able to prevent the cell length change induced by high $K^+$ stimulation. These findings demonstrate that the calcium channel or calcium influx to cytoplasm is not essential for high $K^+$-induced cell contraction of OHC to occur. The mechanism underlying the response is clearly different from most other forms of cell motility[7, 8]. OHC shortening induced by

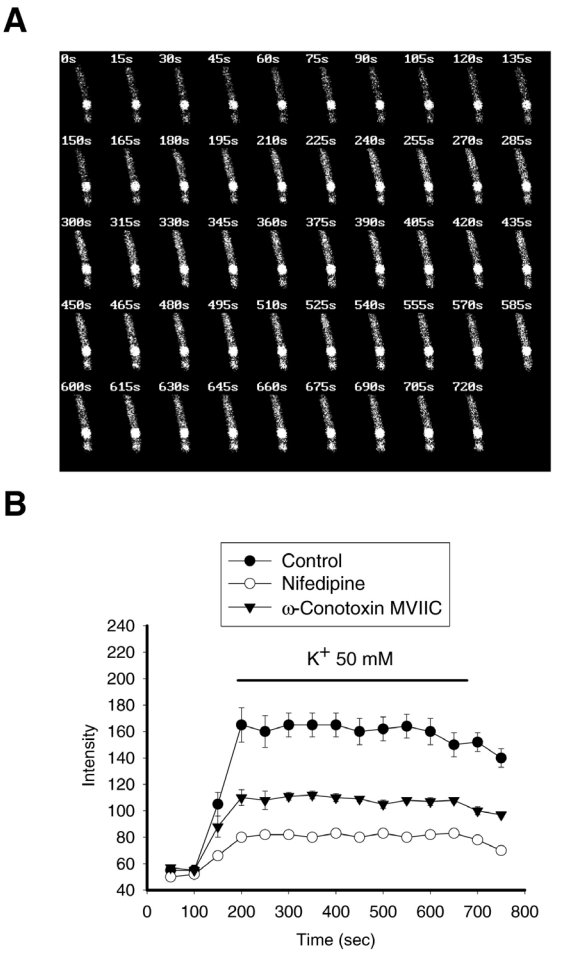


Fig. 3. Effects of calcium channel blockers on the $[Ca^{2+}]_i$ of cochlear hair cell induced by high potassium ($K^+$). A, the typical experiment found that an increase of Fluo-3 intensity after the application of high $K^+$ (50 mM) at the time 90 s. B, application of high $K^+$ medium (-●-) in the bath produced a significantly increased effect of $[Ca^{2+}]_i$. Pretreatment with nifedipine (30 μM; -○-) or ω-conotoxin MVIIC (5 μM; -▼-) significantly reduced the $[Ca^{2+}]_i$ induced by external application of the high $K^+$ medium. The osmolarity of the high $K^+$ medium was adjusted to 317~323 mosmol/l with D-glucose. Each curve is the average of measurement from 3-5 cells.

high $K^+$ or caffeine is independent of extracellular calcium[19]. Previous study suggests that protein kinases (MLCK and CaMKII) mediate the slow motility of outer hair cells. This is in agreement with earlier studies proposing that protein phosphorylation is involved in $Ca^{2+}$ -dependent

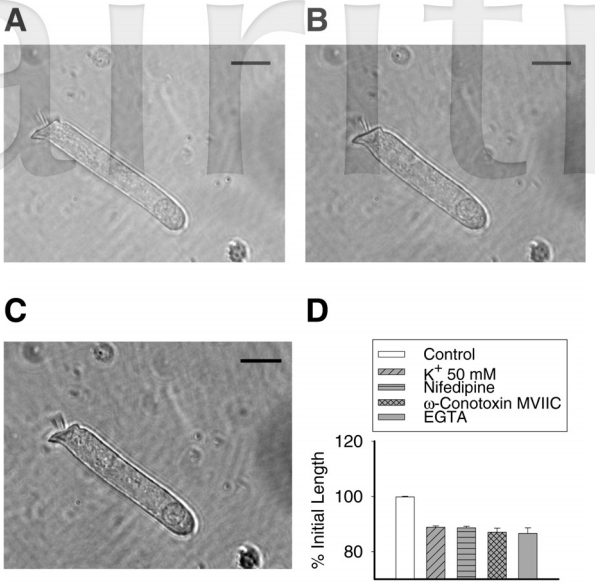


Fig. 4. Effects of calcium channel blockers on the outer hair cell length changes induced by high potassium (K+). A-C show the typical experiment of OHC shortening induced by high K+ medium (50 mM). A, before the perfusion of high K+ medium. B, after perfusion with the high K+ medium. C, washout. D, application of high K+ medium (bar #2) via a micropipette to the cell produced significant cell length changes but not the control solution (bar #1). Pretreatment with nifedipine (30 μM; bar #3), ω-conotoxin MVIIC (5 μM; bar #4) or EGTA (2 mM; bar #5) did not inhibit cell length changes induced by high potassium. The data is presented as percentage maximum length changes of control ± S.E.M. Each bar represents the average of measurement from 5-8 cells. NS: no significance, compared with high potassium (bar #2). The scale bar represents 10 μm.

motility (Dulon et aI., 1990; Coling and Schacht, 1995). Extracellular high-potassium (high $K^+$) can induce the increase of intracellular calcium of OHC. Does intracellular calcium plays a role in OHC shortening? The calcium ionophore ionomycin and ACh have been shown to induce length increases of guinea pig outer cells[4, 6]. The action of ACh on OHC required extracellular $Ca^{2+}$, and is accompanied by changes of intracellular free $Ca^{2+}$ concentration[4]. The elongation produced in OHC by the $Ca^{2+}$ ionophore ionomycin has been attributed to a $Ca^{2+}$/calmodulin-dependent phosphorylation of cytoskeletal proteins[2]. Therefore the increase of intracellular $Ca^{2+}$ alone cannot explain the OHC shortening induced by high $K^+$ medium. The rising intracellular calcium concentration induces a slow shape change in isolated OHCs (circumferential shortening and longitudinal elongations). The longitudinal component of the shape change is probably due to activation of phosphorylating enzymes (myosin light chain kinase and the $Ca^{2+}$/calmodulin-dependent kinase II). The molecular targets of these $Ca^{2+}$ mediated intracellular cascades remain difficult to identify because OHC axial stiffness depends also on the transmembrane potential [9]. Together, these findings suggests that the motor output of OHC can be modulated by regulatory mechanisms that target both cytoskeleton and membrane motor proteins. Although it has been reported that L-type and N-type $Ca^{2+}$ channels coexist in bullfrog saccular hair cells[5, 18], the subtypes of $Ca^{2+}$ channels may be different in different animal species[13, 14]. These two $Ca^{2+}$ channels seem to cooperate to generate the slow contraction of the OHC and the movement of cochlear [12]. In previous study, the $Ca^{2+}$-activated $K^+$ current was not only blocked by the $K^+$ channel blocker tetraethylammonium chloride, but also by the $Ca^{2+}$ channel blockers, which blocked the $Ca^{2+}$ influx through the $Ca^{2+}$ channels, preventing the further depolarization and activation of $Ca^{2+}$-activated $K^+$ current[1, 10]. However, neither the ion channel blockers, $Ca^{2+}$ channel blockers nor $Ca^{2+}$-activated $K^+$ current inhibitors, were unable to inhibit the electromotility of OHC. While the calcium channels of outer hair cell may play a role in exocytosis, they may not be associated with hair cell contraction (motility). The slow motility of OHC appears driven by $Ca^{2+}$-dependent protein kinases (CaMKII and possibly MLCK). An increased activity of $Ca^{2+}$-dependent protein kinases due to the elevation of intracellular calcium is expected to lead to phosphorylation of myosin light chain or a yet unknown target. The phosphorylation of these substrates can initiate the cellular response of cell elongation. High extracellular $K^+$ concentrations produce membrane depolarization or an increase in $Ca^{2+}$ concentration resulting in the shortening of the OHCs. In this study, calcium and calcium

channel blocker are unable to change the slow motility of OHCs. Therefore, the elevation of extracellular $K^+$ concentrations appears to be a direct effect of elevated potassium on plasma membrane of OHC. Our present study shows that the OHC shortening induced by high $K^+$ probably requires only the plasma membrane machinery but not the intracellular calcium concentration, which is in agreement with previous reports[7]. Another possible mechanism for this response is the existence of functional intracellular $Ca^{2+}$ stores in close proximity to the OHC electromotile machinery.

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Original Article

# 細胞內鈣離子與鈣離子管道對於天竺鼠耳蝸外毛細胞的影響

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我們使用細胞膜電位鉗定與鈣離子細胞影像分析研究天竺鼠內耳外毛細胞鈣離子電流的功能角色。海洋錐螺毒素 ω-Conotoxin MVIIC（5 μM）是一選擇性P/Q-型鈣離子管道阻斷劑與nifedipine（L-型，30 μM）與鎘離子（非選擇型，1 mM）相似，可抑制鈣離子電流與鈣離子活化型鉀電流。實驗中外毛細胞鈣電流可被nifedipine 抑制一部份。而剩餘的部份可被 ω-Conotoxin MVIIC（5 μm）所抑制。依據細胞內鈣離子影像分析得知nifedipine 和 ω-Conotoxin MVIIC皆可抑制由高鉀溶液（50 mM）引發的細胞內鈣離子上升作用。高鉀溶液可以縮短外毛細胞的長度，然而給予鈣離子管道抑制劑或降低細胞外液鈣離子濃度皆不能對抗高鉀所引發的毛細胞縮短的現象。總之，高鉀所引發的毛細胞縮短的現象與細胞外液鈣離子濃度，鈣離子管道本身與鈣離子活化的鉀管道無關。此外毛細胞獨特性質的機轉於此文章中討論。

**關鍵詞：**鈣離子電流、運動性、外毛細胞、全細胞電壓鉗定

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