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Taurine modulates calcium influx under normal and ototoxic conditions in isolated cochlear spiral ganglion neurons

Hai-ying Liu[1,2], Fang-lu Chi[2], Wen-yuan Gao[1]

[1]Department of Physiology, the Second Military Medical University, Shanghai 200433, P.R. China

[2]Department of Otolaryngology, Eye Ear Nose and Throat Hospital of Fudan University, Shanghai 200031, P.R. China

Correspondence: Wen-yuan Gao, e-mail: wygao2005@vnet.citiz.net

Abstract:
The effect of taurine on calcium homeostasis of isolated cochlear spiral ganglion neurons under normal and ototoxic conditions was investigated using fluo-3 calcium imaging. Sole application of taurine (15 mM) induced an increase in intracelluar Ca^{2+} concentration ($[Ca^{2+}]_i$), which was largely inhibited either by the application of an L-type calcium-channel blocker nifedipine or a calcium-free medium. Preincubation with 1 mM gentamicin induced an inhibition of the high K^+-evoked elevation of $[Ca^{2+}]_i$. Short-term exposure to taurine prevented this inhibition. The results suggested that taurine at this concentration was able to increase $[Ca^{2+}]_i$ mainly by calcium influx through L-type calcium channels in isolated spiral ganglion neurons and to antagonize gentamicin-induced inhibition of calcium elevation evoked by high K^+ by its calcium homeostatic effect.

Key words:
taurine, gentamicin, calcium imaging, spiral ganglion neurons, fluo-3, confocal laser scanning microscope

Abbreviations: $[Ca^{2+}]_i$ – intracellular calcium level, CLSM – confocal laser scanning microscope, GM – gentamicin, HBSS – Hank's balanced salt solution, OHCs – outer hair cells, SGNs – spiral ganglion neurons, VGCCs – voltage gated calcium channels

Introduction

Taurine (2-aminoethanesulfonic acid) is the major intracellular free β-amino acid present in most mammalian tissues and the second most abundant free amino acid in the brain. The physiological role of taurine has received considerable attention since the reports that cats fed a taurine-deficient diet developed central retinal degeneration and cardiomyopathy [5]. Now taurine has been shown to be involved in many important physiological functions, such as antioxidant defense, neuroprotection, osmoregulation, membrane stabilization, calcium modulation and so on [16, 19]. In general, there is a consensus that taurine is a powerful agent in regulating the intracellular calcium levels in neurons [5, 16]. Recently, researchers have demonstrated the cellular and subcellular localization of taurine in the inner ear. However, little is known about its role in the inner ear or its calcium-modulating effect in the spiral ganglion neurons (SGNs), the primary auditory neurons responsible for auditory signal transduction to the brain [3, 9].

Several lines of evidence suggest that calcium immobilization is involved in the ototoxicity of aminoglycoside antibiotics [1, 11]. It has been reported that voltage-gated calcium channels (VGCCs) are present in the cochlear SGN membrane which open under depolarization, resulting in calcium influx [10]. Streptomycin, an ototoxic aminoglycoside, has been shown to antagonize calcium elevation under high K^+ depolarization in isolated SGNs of guinea pigs, in which blocking or inactivating of VGCCs was probably involved. This was also thought to be one of the mechanisms of acute ototoxicity of aminoglycoside [1, 11]. However, whether gentamicin (GM), another kind of aminoglycoside, has the similar acute effect on $[Ca^{2+}]_i$ in isolated SGNs is unknown, which will be explored in this study.

We recently found that taurine could attenuate the ototoxicity of GM in guinea pigs *in vivo*, though the underlying mechanism for this protection remains unclear [12]. Furthermore, taurine has been demonstrated to have a calcium modulatory effect on isolated cochlear outer hair cells (OHCs), the auditory sensory cells, and antagonize the inhibition of calcium influx induced by GM under high K^+ depolarization [13]. Therefore, further investigation of the effect of taurine on the calcium mobilization of isolated SGNs under normal and ototoxic conditions will provide more evidence in understanding the mechanisms of GM ototoxicity and the protective role of taurine as well.

The effect of taurine on $[Ca^{2+}]_i$ has been investigated in neurons and non-neuronal cells, such as retina and cardiomyocytes, in hypoxia or excitotoxicity [2, 16, 20]. However, so far, little has been known about whether taurine could exhibit calcium modulation in isolated SGNs under normal or ototoxic conditions. In the present study, real-time changes of $[Ca^{2+}]_i$ in freshly isolated SGNs from guinea pig cochleae exposed to taurine, high K^+ and/or GM were recorded with the use of confocal laser scanning microscope (CLSM) in conjunction with the free cytoplasmic calcium indicator fluo-3.

Materials and Methods

Animals

Healthy pigmented guinea pigs (250–350 g) with normal hearing were used. This experiment was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Chemicals

Hank's balanced salt solution (HBSS), collagenase type IV, bovine serum albumin, concanavalin A, gentamicin, taurine and nifedipine were all purchased from Sigma, USA. All other chemicals used were of the highest purity grade available. Drugs were dissolved in HBSS or a calcium-free solution (in mM: NaCl 150; KCl 5; EGTA 2; $MgCl_2$ 1; Hepes 10; D-glucose 5) before use. Nifedipine was dissolved first in DMSO, the final concentration of which in the incubation solution was 15 μM. The constituents of the high K^+ solution in mM were: NaCl 55; KCl 100; $CaCl_2$ 2; $MgCl_2$ 1; Hepes 10; D-glucose 5. All solutions were adjusted to pH 7.4 and osmolarity 300 mOsm before use.

Isolation of SGNs

SGNs were acutely isolated from the guinea pig cochleae as described by Han et al. [7, 8] and Li et al. [11] with a few modifications. The temporal bones were immediately removed after decapitation under anesthesia and placed in HBSS. The bulla was opened and then the bony shell of the cochlea was removed. Then the modiolus was placed in a drop of HBSS containing collagenase (1 mg/ml, type IV) and bovine serum albumin (0.2%) for 10 min at room temperature, and subsequently placed into fresh HBSS. The SGNs were then mechanically isolated and transferred into fresh HBSS in concanavalin A (1 mg/ml)-coated, coverslip-bottomed chambers, and stabilized for 20 min. The SGNs of guinea pig are classified into two types, type I (90–95%, with myelin sheaths) and type II (5–10%, without myelin sheaths) neurons [18]. In this experiment, both types of SGNs were used.

Ca^{2+} imaging

The measurements of $[Ca^{2+}]_i$ were made following our previously established methods [13]. Briefly, cells were incubated for 30 min at 37°C in 5 μM fluo-3/AM (Dojindo, Japan) in HBSS and then washed twice. After loading of fluo-3/AM, the chambers were placed on CLSM (Leica TCS-SP2, Heidelberg, Germany) and superfused with HBSS at a rate of ~1 ml/min at room

temperature. Drugs were administered through the perfusion system.

SGNs were exposed to HBSS first, and then to drugs for about 60 s to 100 s, and finally to HBSS again. The calcium-free solution was used instead of HBSS under calcium-free condition. The time course of drug-induced changes in soma $[Ca^{2+}]_i$ was measured in SGNs. The xyt scan mode was selected (temporal resolution of 1s per scan). One series of images comprised 250–300 sections. All the above and the quantification of the fluorescence intensity were performed using TCS-SP2 software from Leica. $[Ca^{2+}]_i$ was evaluated as the relative changes in the cellular fluo-3 fluorescence intensities in each experiment normalized to the baseline fluorescence recorded prior to stimulation (F/F_0).

Statistical analysis

Data were expressed as the means ± SD and analyzed with one-way ANOVA. All procedures including confocal observation never exceeded 3 h after the isolation of SGNs.

Results

To examine the effect of taurine on SGN $[Ca^{2+}]_i$, microfluorometric $[Ca^{2+}]_i$ measurements were performed before, during, and after superfusion of 15 mM taurine. SGNs perfused with HBSS were used as controls. A marked elevation of fluorescence intensity ($F/F_0 = 0.34 \pm 0.03$, n = 13) was seen in SGNs after the application of taurine. Upon removal of it, $[Ca^{2+}]_i$ declined rapidly. A typical experiment is illustrated in Figure 1A. In contrast, almost no changes in $[Ca^{2+}]_i$ were seen in SGNs perfused with HBSS ($F/F_0 = 0.02 \pm 0.01$, n = 10).

In order to further determine the source of calcium flux caused by taurine, a calcium-free solution and an L-type calcium channel blocker nifedipine were applied. SGNs were first incubated and perfused with calcium-free solution and then followed by 15 mM taurine (dissolved in calcium-free solution) superfusion. It was found that the SGNs showed a small but significant upgrade of $[Ca^{2+}]_i$ ($F/F_0 = 0.09 \pm 0.01$, n = 15) under calcium-free condition compared to HBSS alone. Meanwhile, 15 μM nifedipine inhibited

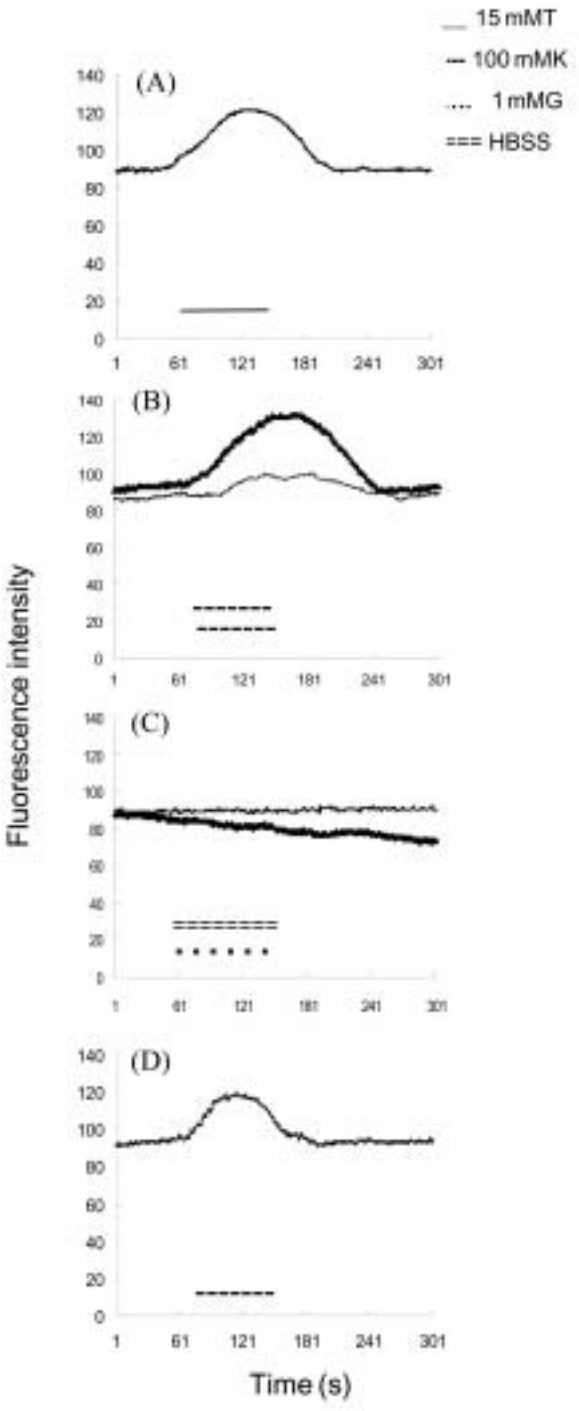

Fig. 1. Time-dependent changes in $[Ca^{2+}]_i$ upon exposure to 15 mM taurine **(A)**, 100 mM K^+(dark-colored curve) and 100 mM K^+ after 1 mM gentamicin preincubation (light-colored curve) **(B)**, 1 mM gentamicin only (dark-colored curve) and HBSS only (light-colored curve) **(C)**, 100 mM high K^+ after 1 mM gentamicin plus 15 mM taurine pretreatment **(D)** in isolated SGNs. As shown in (A), $[Ca^{2+}]_i$ increased rapidly upon administration of taurine and declined rapidly after removal of the drug. As shown in (B), gentamicin preincubation markedly inhibited the increase in $[Ca^{2+}]_i$ evoked by high K^+. $[Ca^{2+}]_i$ slightly declined at the perfusion with 1 mM gentamicin only, as seen in (C). Taurine (15 mM) pretreatment could antagonize gentamicin-induced $[Ca^{2+}]_i$ decrease during high K^+ perfusion, as seen in (D)

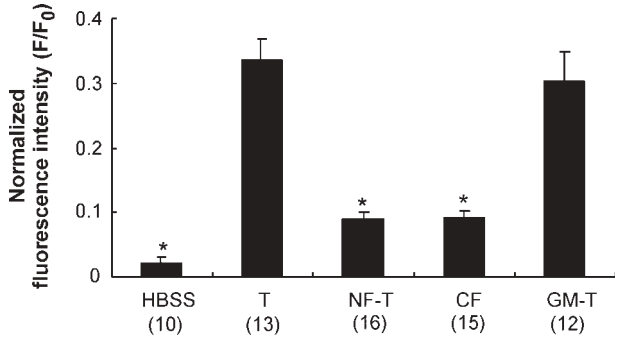

Fig. 2. Effect of taurine on $[Ca^{2+}]_i$ under different conditions. The results are presented as the means ± SD. The number of tested SGNs was shown in brackets. HBSS, Hank's balanced salt solution only; T – 15 mM taurine only; NF-T – nifedipine 15 μM pretreatment before 15 mM taurine perfusion; CF – calcium free condition; GM-T – 1 mM gentamicin pretreatment before 15 mM taurine perfusion. * $p < 0.05$ *vs.* T

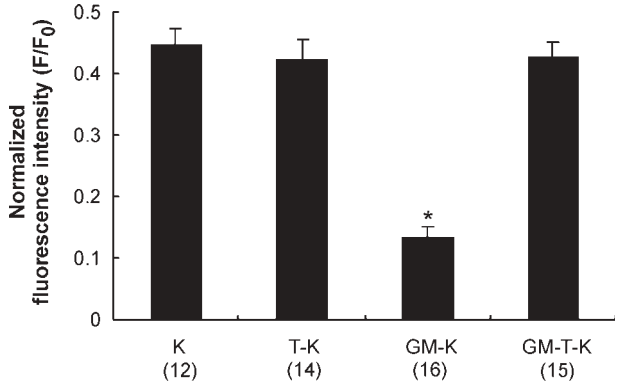

Fig. 3. Effect of taurine and gentamicin on high K^+-induced $[Ca^{2+}]_i$ response in SGNs. The results are presented as the means ± SD. The number of tested SGNs was shown in brackets. K^+ – 100 mM K^+ only; T-K^+ – 15 mM taurine pretreatment before 100 mM K^+; GM-K^+ – 1 mM GM pretreatment before 100 mM K^+; GM-T-K^+ – 1 mM GM 1 h plus 15 mM taurine 10 min before 100 mM K^+. * $p < 0.05$ *vs.* K^+

the taurine-induced $[Ca^{2+}]_i$ increase to 26% of the taurine alone, not significantly different from that under calcium-free condition.

The SGN $[Ca^{2+}]_i$ response at high K^+-induced depolarization and the effect of taurine on it was also explored. SGNs were preincubated with 15 mM taurine for 5 min before 100 mM K^+ perfusion. Some SGNs were perfused with K^+ directly, in which an obvious increase in $[Ca^{2+}]_i$ was seen ($F/F_0 = 0.45 \pm 0.03$, n = 12) followed by a decline of fluorescence upon removal of K^+ (Fig. 1B). The SGNs preincubated with taurine also showed an upgrade of fluorescence ($F/F_0 = 0.42 \pm 0.03$, n = 14) during K^+ perfusion, not significantly different from that with K^+ alone.

Based upon the above results, the effect of GM pretreatment on K^+-/taurine-induced $[Ca^{2+}]_i$ response was further observed in SGNs which were preincubated with 1 mM GM for 1 h before 100 mM K^+ or 15 mM taurine perfusion. Some SGNs without any pretreatment were perfused with 1 mM GM directly. It was found that GM inhibited high K^+-induced $[Ca^{2+}]_i$ increase to 29% of the K^+ alone. In contrast, an increase in $[Ca^{2+}]_i$ was seen in other GM-preincubated SGNs when purfused with taurine ($F/F_0 = 0.30 \pm 0.04$, n = 12), slightly but nonsignificantly lower than that perfused with taurine directly. In addition, the non-stimulated SGNs perfused directly with 1 mM GM revealed a slight decline or no change in $[Ca^{2+}]_i$ ($F/F_0 = -0.11 \pm 0.06$, n = 15) as shown in Figure 1C.

Finally, the effect of taurine pretreatment on GM-induced inhibition of $[Ca^{2+}]_i$ elevation during high K^+ depolarization was investigated. Some SGNs were first preincubated with 1 mM GM for 1 h and then treated with taurine (15 mM) for an additional 10 min, just before K^+ perfusion began. It showed that most SGNs revealed an increase in $[Ca^{2+}]_i$ ($F/F_0 = 0.43 \pm 0.03$, n = 15/18) during K^+ depolarization, just like those non-GM treated SGNs (Fig. 1D), with other three SGNs remaining unchanged in $[Ca^{2+}]_i$.

Figure 2 summarizes the effect of taurine on SGN $[Ca^{2+}]_i$ under different conditions. Figure 3 presents the effect of taurine and gentamicin on high K^+-induced $[Ca^{2+}]_i$ response in SGNs.

Discussion

The calcium modulatory roles of taurine in several kinds of cells have been reported. Zhao et al. [20] found that taurine application produced a transient increase in $[Ca^{2+}]_i$ in hippocampal neurons. Investigators also observed $[Ca^{2+}]_i$ increases in heart cells, retinal cells and cochlear OHCs *in vitro* [2, 13, 16]. In the present study, we found for the first time that a single application of taurine induced an increase in $[Ca^{2+}]_i$ in isolated SGNs. Upon removal of taurine, $[Ca^{2+}]_i$ declined rapidly. These results imply that, as in neurons, in some non-neuronal cells and OHCs, a sole application of taurine was able to trigger an increase in $[Ca^{2+}]_i$ in SGNs.

The mechanism by which $[Ca^{2+}]_i$ increased after a single taurine application was not completely known. This elevation of $[Ca^{2+}]_i$ presumably came from the mobilization of intracellular calcium stores or from extracellular space or both. Satoh et al. [15] summarized that taurine could regulate the $[Ca^{2+}]_i$ level by modulating the activity of VGCC, Na^+ channels, and Na^+-taurine cotransport in cardiomyocytes directly or indirectly. With respect to the pathway of $[Ca^{2+}]_i$ increases provoked by taurine in OHCs, the Ca^{2+} influx through VGCCs was thought to be involved [13]. Moreover, taurine also has the ability to modulate mitochondrial calcium homeostasis [4]. In our present study, $[Ca^{2+}]_i$ was elevated in the tested SGNs when 15 mM taurine was applied. However, a significantly small taurine-triggered elevation of $[Ca^{2+}]_i$ was seen in most SGNs when either a calcium-free solution or a calcium channel blocker was applied. Therefore, it was speculated that taurine-induced $[Ca^{2+}]_i$ increase in SGNs mainly came from the extracellular space and that the membrane calcium channels were involved in that process. On the other hand, the entry of taurine into cytoplasm may also trigger a small amount of calcium discharge from the intracellular storage [2, 4], which will need further investigation.

Data have shown that VGCCs exist in the SGN membrane [10]. The high K^+ depolarization *in vitro* may induce the opening of this kind of calcium channels. The influx of calcium plays many important physiological roles, such as activating the calcium-gated potassium channels, triggering vesicle fusion and releasing of transmitters [6]. We observed the $[Ca^{2+}]_i$ increase in SGNs during high K^+ perfusion, consistent with the results Han reported [7, 8]. Meanwhile, taurine pretreatment was found to have no significant impact on K^+-induced $[Ca^{2+}]_i$ increase in SGNs, indicating that taurine probably had the ability to maintain the normal activity of VGCCs.

As primary auditory neurons, SGNs play an important role in hearing and may also be a target of ototoxins. Gentamicin has been known to be an ototoxic agent, which has been found to block calcium channels. Researchers found that GM could reduce presynaptic calcium influx in the motor nerve terminal at the neuromuscular junction [14], and in the nerve terminal of the medial olivocochlear efferent fiber to OHC [17]. Moreover, GM was shown to reduce $[Ca^{2+}]_i$ in nonstimulated OHCs and to inhibit the increase in $[Ca^{2+}]_i$ under high K^+ depolarization [13]. In the present study, preincubation with GM was also shown to induce an inhibition of $[Ca^{2+}]_i$ elevation in isolated SGNs evoked by high K^+ depolarization. Therefore, it was thought that VGCCs were probably involved in the GM-induced inhibition of calcium influx from extracellular space.

The calcium-modulating role of taurine has been extensively studied in terms of its inhibitory effect on $[Ca^{2+}]_i$ overload under excitotoxic conditions [5, 19, 20]. However, little is known about whether it could reverse calcium decrease in SGNs exposed to GM. In our study, GM preatment induced a significant inhibition of $[Ca^{2+}]_i$ increase in SGNs at high K^+, but not that much inhibition at taurine perfusion. Furthermore, additional 10 min of taurine treatment after GM triggered a similar $[Ca^{2+}]_i$ increase at high K^+ as perfused with taurine alone. These results further implied that taurine had the ability to antagonize GM-induced inhibition of $[Ca^{2+}]_i$ elevation in SGNs, possibly by its ability to increase $[Ca^{2+}]_i$. We recently found that taurine could attenuate the acute ototoxicity induced by combined single intravenous administration of GM and furosemide [12]. It was thus postulated that taurine might provide a protective effect against acute ototoxicity through its modulation of calcium influx. Functions other than this modulation may also be involved in the protection, such as osmoregulation, anti-excitatory and antioxidant activity [3, 16, 19].

During glutamate-induced excitotoxicity, taurine attenuates $[Ca^{2+}]_i$ overload in neurons. During ototoxicity, the inhibition of GM-induced calcium influx was antagonized by taurine in the present study. Thus, it is implied that taurine can provide regulation of $[Ca^{2+}]_i$ in both directions with respect to homeostasis: It attenuates calcium influx when there is a calcium overload in excitotoxicity; it promotes calcium influx when the calcium channels are blocked by ototoxic drugs.

In conclusion, we showed that taurine induced a $[Ca^{2+}]_i$ increase in isolated SGNs under normal conditions, in which VGCCs might mainly be involved. Furthermore, taurine could antagonize the inhibition of calcium influx induced by GM under high K^+-induced depolarization. The data of our study provide further evidence that taurine is an important calcium modulator and a potential protector against ototoxic insult in the auditory system. Further studies are necessary to better understand this mechanism and the role of taurine in the inner ear.

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