Panax Notoginseng Saponins Attenuates the ATP Induced Increases of Intracellular Ca\(^{2+}\) in Sertoli cells: Involvement of PLC signaling pathway

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**ABSTRACT** Panax notoginseng saponin (PNS) is a traditional Chinese medicine. Recent studies showed that Panax ginseng, a member of the same PNS family, improves sperm motility in men with asthenopernia or spermatogenetic disorder. However, molecule mechanisms underlying these effects are not well understood. Here, we explored the effects of PNS on the Ca\(^{2+}\) signals in cultured primary rat Sertoli cell using Fura-2 based calcium imaging techniques. Our results showed that PNS had no effect on the basal [Ca\(^{2+}\)], but depressed the ATP-evoked [Ca\(^{2+}\)]\(_i\) responses by 54 %. A-317491, an inhibitor of P2X receptors, further facilitated inhibitory effects of PNS. Moreover, in Ca\(^{2+}\)-free solution, PNS failed to prevent the stimulating effect of caffeine on [Ca\(^{2+}\)], which functions by activating the ryanodine receptors. However, PNS significantly reduced the elevation of [Ca\(^{2+}\)], induced by Mech, a specific IP\(_3\) receptor activator. Meanwhile, U-73122, an inhibitor of phospholipase C, reversed the reduction of [Ca\(^{2+}\)]\(_i\) level in application of PNS. These results suggest that [Ca\(^{2+}\)]\(_i\) reduction regulated by PNS is attributed to the prevention of Ca\(^{2+}\) release via IP\(_3\) receptor and PLC signaling pathway may be involved in the process.

**1. Introduction**

Panax notoginseng saponins (PNS), chemicals extracted from the root of Panax notoginseng (Burk.) F.H. Chen, is highly valued and important traditional Chinese medicine (Sanqi or Tianqi in Chinese) [1]. Evidences showed that Panax ginseng, a same family member of PNS, improves sperm motility in men with asthenopernia or spermatogenetic disorders [2]. Interestingly, one study in China suggested that PNS relieved the orchiditis induced by LPS through improving the irregular arrangement and hydropsia of Sertoli cell and Leydig cell in rats [3]. However, molecule mechanisms underlying these effects are not clear.

It has been found that inflammatory factor can affect Sertoli cells and resulted in cellular calcium overload or cell death [4]. Naturally, the decrease of extracellular inflammatory factor or stable intracellular calcium concentration may produce partially protective effects on Sertoli cell. Adenosine triphosphate (ATP) an important synergistic transmitter or modulator has involved in various cascade reactions in inflammation [5]. ATP can trigger multiple downstream signaling elements, like cAMP/PKA, IP\(_3\)/PLC pathways through activating purinergic receptors and calcium influx [6]. Moreover, one study reported that purinergic receptors, P2X2 and P2X3, are expressed during...
spermatogenesis in Sertoli cells of the adult rat [7]. In the present study, we investigated the signaling mechanisms of PNS on intracellular Ca$^{2+}$ induced by ATP.

2. Materials and Methods

2.1 Drugs and agents. PNS (Lot No. 08105830) with chemical purity about 97% was provided by Wu-Zhou pharmaceutical (group) Co., Ltd (Wuzhou, China).

2.2 Cell culture. Adult testes were retrieved in sterile conditions from deceased male multi-organ donors. Briefly, seminiferous tubules separated from testes were subjected to a two-step sequential enzymatic digestions at 37 °C with trypsin (Sigma, USA) and DNase (Sigma, USA) for 30 min in the first step and collagenase P and hyaluronidase (Sigma, USA) for 20 min in the second step, followed by RPMI-1640 culture medium containing 100 ml/L fetal bovine serum to terminate the digestion at 4 °C. Sertoli cells were replenished with RPMI-1640 medium supplemented with 100 ml/L FCS and incubated at 37 °C under 5% CO$_2$.

2.3 Measurement of intracellular free calcium. Calcium imaging was performed as described previously [8]. For [Ca$^{2+}$]$_i$, measurements, cultured Sertoli cells were rinsed three times with HEPES-buffered solution containing (in mM) 140 NaCl, 5 KCl, 1 MgCl$_2$, 2 CaCl$_2$, 10 glucose and 10 HEPES (pH 7.3). Then, cells were incubated with 2 μM fura-2 AM for 30 min at 37 °C. The intracellular free calcium concentration was presented as the ratio of the fluorescence signals obtained (340/380 nm).

2.4 Statistical analysis. Statistical significance between the multiple groups was determined using paired t test or one-way ANOVA when appropriate followed by post hoc comparisons (SPSS 11.5 software). Data are presented as means±S.E.M. Differences at the $P<0.05$ level were considered statistically significant.

3. Results

ATP increases [Ca$^{2+}$], in cultured rat sertoli cell

We recorded the transient [Ca$^{2+}$], increases followed 100μM ATP treatment with fast superfusion (approximately 45s). In the majority of tested cells, repeated ATP stimulation produced equal [Ca$^{2+}$], responses and these responses were fully reversed after a 5 min washout with normal extracellular solution (Fig. 1A). Cells were pretreated with perfusion of 30 μM A-317491 for 5 min and repeated ATP stimulation produced reduced [Ca$^{2+}$], responses (54.1±2.2% of the initial control responses, $P<0.05$, $t$-test, $n = 22$, Fig. 1B).

**Fig. 1.** ATP increases [Ca$^{2+}$], in cultured rat Sertoli cells. (A) [Ca$^{2+}$], responses to repeated ATP stimulation under control condition with time interval of 5 min between stimuli. Repeated stimulation with KCl by fast superfusion (100μM, 45 s) produced an equal transient Ca$^{2+}$ increase in Sertoli cells ($n = 22$). (B) Representative experiment showing pretreatment with A-317491 (30 μM, 5 min) reduced the ATP-induced [Ca$^{2+}$], peak ($n = 22$).

PNS reduces ATP-evoked [Ca$^{2+}$], rise in cultured rat sertoli cells
We first pretreated the cells with PNS and have not found any influence on the basal \([Ca^{2+}]_i\) (Fig. 2A). However, pretreatment with PNS (30 μM) for 10 min caused a reduction in ATP induced \([Ca^{2+}]_i\) responses (57.4±1.8% of the initial control responses, \(P<0.05\), t-test, \(n = 20\), Fig. 2B). Repeated ATP stimulation produced equal \([Ca^{2+}]_i\] responses and these responses were fully reversed after a 5 min washout with normal extracellular solution (Fig. 2B). A-317491, a inhibitor of P2X receptors, further decreased the PNS induced \([Ca^{2+}]_i\] responses by 80.9±1.8% compared with control group (\(F_3, \gamma_0=63.2\), ANOVA, \(p<0.001\), Fig. 2C and Fig. 2D, \(n=20\)). The inhibitory effect of PNS (1–300 μg/L) was in a dose-dependent manner (Fig. 1E). The EC\(_{50}\) for potentiation effect was 30 ± 2.5 μg/L.

![Image](image-url)

**Fig. 2.** PNS depressed ATP-evoked \([Ca^{2+}]_i\] rise in cultured rat Sertoli cells. (A) [Ca\(^{2+}\)]_i responses to PNS (n = 12). (B) Representative experiment showing pretreatment with PNS (30 μg/L, 10min) depressed the peak of ATP-induced [Ca\(^{2+}\)] responses (n = 20). (C) A-317491 further decreased the PNS induced [Ca\(^{2+}\)] responses (n=20). (D) Summary data revealed that PNS and A-317491 have synergistic effect on the ATP-evoked [Ca\(^{2+}\)] responses. (E) This inhibitory effects of PNS on ATP-evoked [Ca\(^{2+}\)] response was in a dose-dependent manner. *\(P < 0.05\) compared to control.

**PNS reduces Mch-evoked [Ca\(^{2+}\)], increase in the Ca\(^{2+}\)-free medium**

Pretreatment with PNS caused no change in [Ca\(^{2+}\)]_i in Ca\(^{2+}\)-free medium (Fig. 3A, n=12). As shown in Fig. 3B, in the Ca\(^{2+}\)-free medium, repeated stimulations with caffeine (5 mM, 45 s) evoked equal [Ca\(^{2+}\)] changes when the interval between applications was more than 10 min (n = 20). Meanwhile, in the absence of extracellular Ca\(^{2+}\), pretreatment with PNS (30 μg/L) didn’t influence the caffeine-induced [Ca\(^{2+}\)] level. However, in the absence of extracellular Ca\(^{2+}\), pretreatment with PNS (30 μg/L) did depress the Mch-evoked [Ca\(^{2+}\)] increase by 17.2 ± 1.8% compared with control.
Fig. 3. PNS reduced Mch-evoked \([\text{Ca}^{2+}]_i\) transients. (A) \([\text{Ca}^{2+}]_i\) responses to PNS \((n=12)\). (B) Addition of caffeine \((5 \text{ mM, } 45 \text{ s})\) produced equal \([\text{Ca}^{2+}]_i\) responses with a 10 min interval between stimuli in Sertoli cells \((n=20)\) in the absence of extracellular \(\text{Ca}^{2+}\). (C) Addition of Mch \((5 \text{ mM, } 45 \text{ s})\) produced increase of \([\text{Ca}^{2+}]_i\) that was depressed by PNS in Sertoli cells \((n=20)\) in the absence of extracellular \(\text{Ca}^{2+}\).

**PLC pathway contributes to the inhibitory effect of PNS on \([\text{Ca}^{2+}]_i\)**

Membrane-associated P2X receptor is thought to be linked to activation of cyclic AMP/PKA and phospholipase C (PLC)/IP3 signaling cascades. A membrane-permeant PKA inhibitor H89 and a PLC inhibitor U-73122 were used. As shown in Fig. 4A and 4B, pretreatment with H89 \((10 \mu\text{M, } 10 \text{ min})\) failed to reverse this inhibitory effects of PNS \((F_3, 79=48.6, \text{ ANOVA, } p<0.001, n=20)\). In contrast, pretreatment with U-73122 \((10 \mu\text{M, } 10 \text{ min})\) prevented the inhibitory effects of PNS \((30 \mu\text{g/L})\) on ATP-evoked \(\text{Ca}^{2+}\) response \((U-73122 \text{ with PNS vs control }, p>0.05, n=20, \text{ in Fig.4B and C})\).
Fig. 4. Effects of U-73122 and H89 on the reduction of PNS. (A) Preincubation with H89 (10 μM, 10 min) didn’t abolish the inhibitory effects of PNS (n = 20). (B) Preincubation with U-73122 (10 μM, 10 min) prevent the inhibitory effects of PNS (30μg/L) on ATP-evoked Ca\(^{2+}\) response in Sertoli cells (n=20). (C) Summary data revealed that preincubation with U-73122, but not H89 influenced the ATP-evoked [Ca\(^{2+}\)], responses when co-applied with PNS. *P < 0.05 compared to control.

4. Discussion
In the present study, we show that PNS markedly decreased ATP-evoked [Ca\(^{2+}\)], response in cultured Sertoli cells, A-317491 further facilitated inhibitory effects of PNS. In addition, PNS failed to prevent the effect of caffeine on [Ca\(^{2+}\)], but it significantly reduced the elevation of [Ca\(^{2+}\)], induced by Mch. Meanwhile, H89 didn’t influence the [Ca\(^{2+}\)], level in application with PNS while U-73122 reversed the reduction of [Ca\(^{2+}\)], level in application of PNS.

Sertoli cells are important in maintaining a gradient of ions, small molecules, and proteins between blood and tubular fluid and hence providing a suitable microenvironment for spermatogenesis [9]. The fluid creates a unique environment for the developing germ cells [10]. To maintain a normal spermatogenesis, it is important to protect Sertoli cells from injury. In the present study, our results demonstrated that PNS prevent the Ca\(^{2+}\)-elevation induced by ATP. More importantly, A-317491, an inhibitor of P2X receptors, further facilitated inhibitory effects of PNS. All of these data strongly show that PNS may reduce calcium influx through inhibiting P2X receptor.

When extracellular ATP stimulates basic ion influx via P2X receptor on the Sertoli cell, cAMP and Ca\(^{2+}\), the second messengers, will play their important roles in a series of physiological activity. It is well documented that triggering P2X receptor can further activate CICR from the endoplasmic reticulum Ca\(^{2+}\) store [11]. Caffeine is known as a specific agent that acts on the ryanodine receptor and directly evokes intracellular Ca\(^{2+}\) release from the ryanodine-sensitizing Ca\(^{2+}\) store in Ca\(^{2+}\)-free solution [12]. We found in the absence of extracellular Ca\(^{2+}\), pretreatment with PNS didn’t influence the caffeine-induced [Ca\(^{2+}\)], level. However, pretreatment with PNS did inhibit the Mch (an agonist for IP\(_{3}\) receptor)-evoked [Ca\(^{2+}\)], released. These results thus further show that the sensitization of IP\(_{3}\) receptor may contribute to the inhibitory effects of PNS.

Membrane-associated P2X receptor is thought to be linked to activation of cyclic AMP/PKA and phospholipase C (PLC)/IP\(_{3}\) signaling cascades. In the present study, we discovered that U73122 (PLC inhibitor), not H89 (membrane-permeant PKA inhibitor), successfully reversed the inhibitory effect of PNS on ATP-evoked [Ca\(^{2+}\)], level in Sertoli cells, suggesting that the inhibitory effect of PNS is dependent on the activation of PLC.

5. Conclusion
In summary, PNS decrease the ATP-triggered intracellular Ca\(^{2+}\) release from endoplasmic reticulum Ca\(^{2+}\) stores by P2X receptor in Sertoli cells. This inhibitory effect may be resulted from the activation of PLC signaling cascades and subsequent PLC-mediated phosphorylation of IP\(_{3}\) receptors.

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Conflict of interest
The authors state no conflict of interest.

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