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• BASIC RESEARCH •

The pre-synaptic blocker toosendanin does not inhibit secretion in exocrine cells

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Abstract

AIM: Toosendanin is a pre-synaptic blocker at the neuromuscular junction and its inhibitory effect is divided into an initial facilitative/stimulatory phase followed by a prolonged inhibitory phase. The present study investigated whether the subsequent inhibitory phase was due to exhaustion of the secretory machinery as a result of extensive stimulation during the initial facilitative phase. More specifically, this paper examined whether toosendanin could directly inhibit the secretory machinery in exocrine cells.

METHODS: Rat pancreatic acinar cells were isolated by collagenase digestion. Secretion was assessed by measuring the amount of amylase released into the extracellular medium as a percentage of the total present in the cells before stimulation. Cholecystokinin (CCK)-induced increases in intracellular calcium in single cells were measured with fura-2 microfluorometry.

RESULTS: Effects of toosendanin on CCK-induced amylase secretion and calcium oscillations were investigated. Toosendanin of 87-870 μM had no effect on 10 pM-100 nM CCK-stimulated amylase secretion, nor did 8.7-870 μM toosendanin inhibit 5 pM CCK-induced calcium oscillations. In contrast, 10 nM CCK_1 receptor antagonist FK 480 completely blocked 5 pM CCK-induced calcium oscillations.

CONCLUSION: The pre-synaptic "blocker" toosendanin is a selective activator of the voltage-dependent calcium channels, but does not interfere with the secretory machinery itself.

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INTRODUCTION

Toosendanin is a tetracyclic triterpenoid isolated from the seeds and barks of *Melia toosendan Seib. et Zucc* and *Melia azedarach L.* It has been used as an anthelmintic for many centuries, and has also been found to have pesticidal effects[1-5], and have anti-botulismic and other effects in whole animals[6, 7]. Work on nerve-muscle and other preparations (neuromuscular junction)[8-14] has established that toosendanin is a potent, long-lasting pre-synaptic inhibitor. The neuromuscular blocking effect of toosendanin is divided into two phases: an early stimulatory phase that is due to direct activation of voltage-dependent calcium channels[8, 12-18], and a delayed inhibition[9, 14]. However, it is not known whether or not the delayed blockade of the neuromuscular transmission is due to direct interference with the secretory machinery involved in neurotransmitter release.

Whether toosendanin has any direct effect on the secretory machinery can only be examined in secretory cells with no voltage-dependent calcium channels. If indeed the delayed blocking effect of toosendanin is due to direct inhibition of the secretory machinery, only will an inhibitory phase be observed in secretory cells which lack voltage-dependent calcium channels. The pancreatic acinar cell is an ideal model in which to address this question. The molecular mechanisms of secretion in pancreatic acinar cells are well elucidated[19-24], and these non-excitable cells have no voltage-dependent calcium channels[25, 26]. Therefore, this study examined if toosendanin has any inhibitory effect on secretion induced by a physiological secretagogue, cholecystokinin (CCK), in freshly isolated rat pancreatic acinar cells.

MATERIALS AND METHODS

Materials

CCK octapeptide, α-amylase and amylose azure were purchased from Sigma (St. Louis, MO, USA), Cell-Tak was purchased from Collaborative Biomedicals (Bedford, MA, USA). Toosendanin was a gift from Professor He LI (Department of Chemistry, Beijing Normal University). Collagenase P was bought from Boehringer Mannheim (Mannheim, Germany). Fura-2 AM was purchased from Molecular Probes (Eugene, OR, USA). (s)-N-[1-(2-fluorophenyl)-3,4,6,7-tetrahydro-4-oxo-pyrrolo[3,2,1-jk][1,4] benzodiazepin-3yl]-1H-indole-2-carboximide (FK480) was donated by Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan). Toosendanin and FK480 were both dissolved in DMSO as stock solutions before dilution to final concentration.

Isolation of rat pancreatic acini

Pancreatic acini were isolated from male Sprague-Dawley rats with body weight ranged from 170 g to 250 g according to the method reported previously[23, 27, 28].

Measurement of amylase secretion

Isolated acini were aliquoted into 2 ml portions and stimulated at 37 ℃ in a shaking water bath (50 cycle · min^{-1}) for 30 min. The amylase secreted into the buffer was assayed according to the procedures reported previously[29- 32] and expressed as percentage of the total present in the acini before stimulation.

Measurement of intracellular calcium $[Ca^{2+}]_i$

Ten microlitre of 1mM Fura-2 AM was added to1 ml of isolated acini (final concentration 10 μM) and the mixture was incubated in a shaking water bath at 37 ℃ and 50 cycle · min^{-1} for 40 min.

Fura-2-loaded acini were attached to the cover-slip of a Sykus-Moore chamber and perfused on the stage of an Olympus fluorescence miscroscope (IX70) attached to a microfluorometric calcium measurement system (M40, Photon Technology International, NJ, USA). Calcium increases were expressed as fluorescence ratios measured at 510 nm (F340/F380)[23, 27, 33-35].

Standard buffer used in this work was composed of (all in mM) NaCl 118, KCl 4.7, $MgCl_2$ 1.16, $CaCl_2$ 2.5, NaH_2PO_4 1.16, glucose 5.6, bovine serum albumin 2 mg • ml^{-1}, soybean trypsin inhibitor 0.1 mg • ml^{-1}, N-(2-hydroxyethyl) piperazine-N' -(2-ethanesulfonic acid) (HEPES) 10, MEM amino acid mixture (GIBCOBRL, Grand Island, NY, USA) 2 % and glutamine 2. The buffer was adjusted for pH to 7.4 with 4 mM NaOH and oxygenated with O_2 for 30 min before use.

RESULTS

Effects of toosendanin on CCK-induced amylase secretion

CCK stimulated amylase secretion from the freshly isolated rat pancreatic acini in a concentration-dependent manner (Figure 1). The maximum stimulation was achieved at CCK concentration of 100 pM, with a percentage secretion of 33± 1.6 (*n*=6). This bell-shaped dose response curve is consistent with previous report[36].

In separate experiments, acini were first incubated with toosendanin at 870 μM or 87 μM for 10 min before stimulation with CCK for a further 30 min in the continued presence of toosendanin (Figure 2). When toosendanin was added at 87 μM, the maximum stimulating CCK concentration shifted from 100 pM (18.2±1.6, *n*= 6) to 1 nM (19.7±1.2, *n*=6). With the addition of toosendanin at 870 μM, the maximum CCK concentration also shifted from 100 pM (18.2±1.6, *n*=6) to 1 nM (19.2±1.9, *n*=4). The slight rightward shift indicates a mild but statistically insignificance inhibition (Student' s *t* test, *P*>0.05). In control experiments, neither 0.01 % solvent DMSO nor toosendanin at each concentration used had any effect on amylase secretion (data not shown).

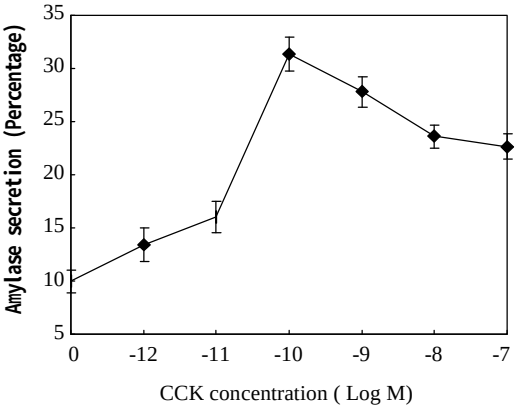
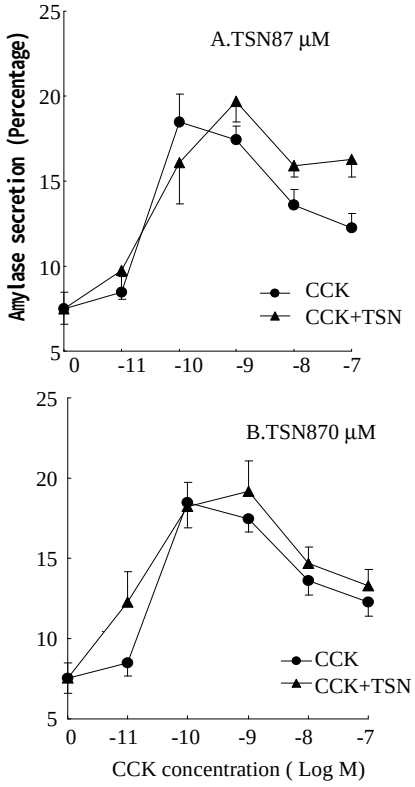


Figure 1 Concentration dependence of cholecystokinin (CCK)-stimulated amylase secretion. Note that maximum secretion was achieved at 100 pM of CCK. *n*=6

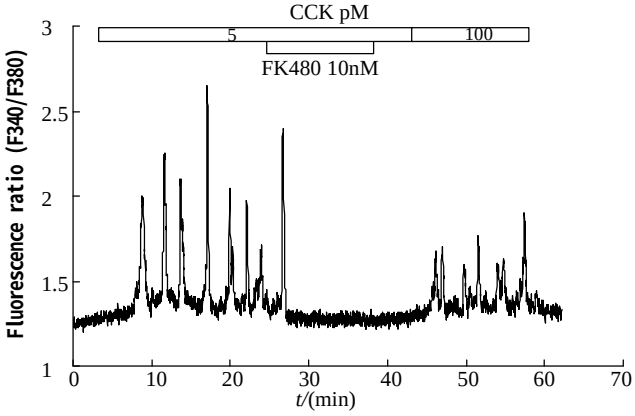
Effects of toosendanin on CCK-induced $[Ca^{2+}]_i$ oscillations

CCK of 5 pM induced regular calcium oscillations in perfused rat pancreatic acinar cells (Figure 3), which is consistent with previous reports[27, 28]. Addition of toosendanin at 8.7 μM to the CCK-stimulated acinar cells for 10-20 min had no apparent effect on CCK-induced calcium oscillations (Figure 3A, *n*=4). Even when toosendanin was increased to 87 μM, there was still no obvious inhibition observed (Figure 3B, *n*=7). At 870 μM, toosendanin induced a very mild inhibition: a single spike appeared missing in the trace shown (Figure 3C, *n*=8).

For comparison, FK480, an antagonist for CCK_1 receptors[37-39], produced immediate and complete inhibition of 5 pM CCK-induced calcium oscillations (Figure 4, *n*=5). FK480 at 10 nM abolished 5 pM CCK-induced calcium oscillations immediately upon addition, reducing the calcium to pre-stimulation level (Figure 4). After washout of FK480, calcium oscillations did not re-appear immediately, indicating that FK480 might bind to the acinar cells very tightly. It was possible, however, to re-introduce calcium oscillations when CCK was increased to 100 pM. At a lower FK480 concentration of 1 nM, a much longer time was needed before complete abolition of 5 pM CCK-induced Ca^{2+} oscillations was observed (data not shown).



igure 2 Effect of toosendanin (TSN) on CCK-stimulated amy-ıse secretion. TSN was added to the pancreatic acini 10 min efore stimulation with CCK which was maintained for an-ther 30 min. A. TSN 87 μM, *n*=6. B. TSN 870 μM, *n*=4. Note ıat, for clarity, the same CCK dose-response curve (*n*=6) was



the horizontal bars. *n*=5.

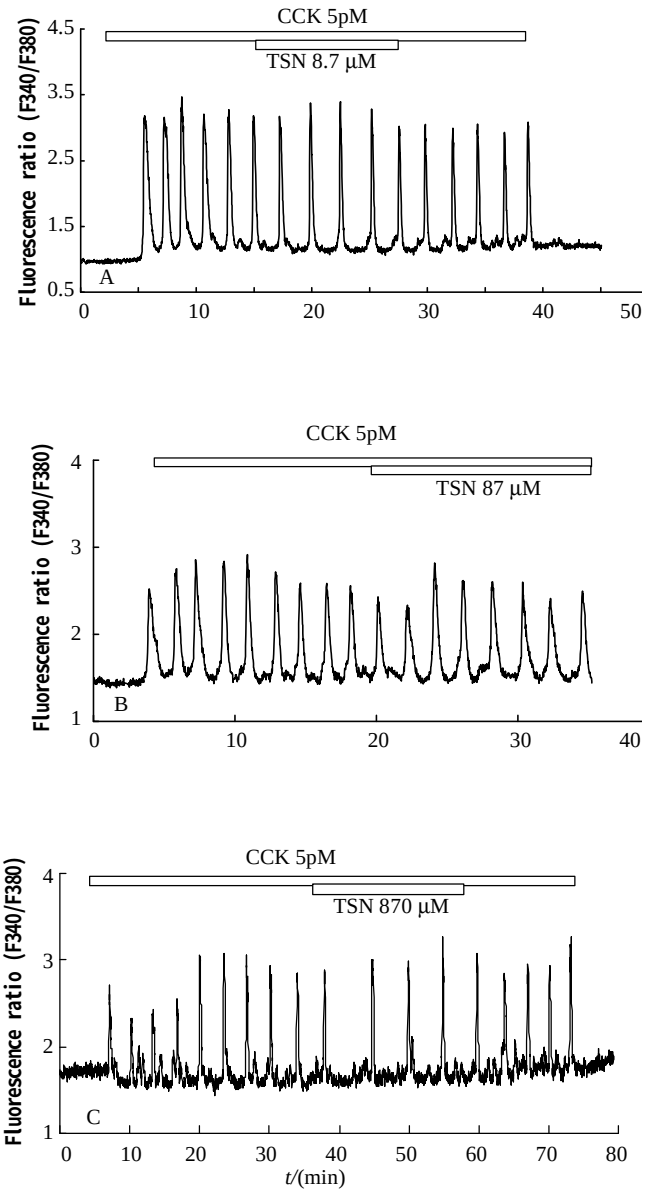


Figure 3 Effect of toosendanin on 5 pM CCK-induced calcium oscillations. CCK and TSN were added as indicated by the horizontal bars. A. TSN 8.7 μM, *n*=4. B. TSN 87 μM, *n*=7. C. TSN 870 μM, *n*=8.

DISCUSSION

The present work demonstrated that toosendanin, a pre-synaptic inhibitor of the neuromuscular transmission[7, 9, 13, 14], had little effect on CCK-stimulated amylase secretion. Although treatment with toosendanin at both concentrations tested (87 μM, 870 μM) resulted in a rightward shift in concentration of CCK that was needed to induce a maximum stimulation, i.e. from 100 pM in untreated cells to 1 nM in treated cells (Figure 2)[36], the toosendanin inhibition was not statistically significant for each CCK concentration (P>0.05).

Toosendanin of 8.7- 87 μM had no effect on 5 pM CCK-induced calcium oscillations. At a much higher concentration of 870 μM, a mild inhibition was observed because one spike seemed missing (Figure 4). This lack of marked inhibition of toosendanin on CCK-induced calcium oscillations is in sharp contrast with the complete blockade of CCK-induced calcium oscillations by FK480, a CCK_1 receptor antagonist [38, 40, 41]. Toosendanin at the neuromuscular junction had dual effects, a fast-onset stimulation which lasts about 40 min followed by a long-lasting inhibition[7-9, 12-14]. The fast phase has been postulated to be due to activation of voltage-dependent calcium channels[8, 12-18]. However, toosendanin had no effect on CCK-induced calcium oscillations in rat pancreatic acinar cells. This indicates that toosendanin had no effect on stores-operated calcium channels as they are the only calcium channels existing in the freshly isolated pancreatic acinar cells[20, 26] and are important for the continued presence of calcium oscillations[23, 27].

In view of the above findings, the pre-synaptic blocking effect of toosendanin should be looked at under a new light. The delayed inhibition of neuromuscular transmission could just be due to massive stimulation of synaptic vesicle fusion after activation of the voltage-dependent calcium channels at the nerve terminal, resulting in the depletion of synaptic vesicles and delayed depression. The fact that toosendanin administration in rat leads to a decrease in synaptic vesicles[42, 43] strongly supports this hypothesis. The initial strong stimulation of voltage-dependent calcium channels would afford the early stimulatory effects of toosendanin, providing an antidote to botulism[6, 18].

An activator for voltage-dependent calcium channels as toosendanin may be, a long-lasting inhibition of the neuromuscular junction[7- 9,12-14] or an extended period of synaptic vesicle depletion would require the stimulatory effect of toosendanin to be long-lasting. This stimulatory effect has indeed been found to last 40 min in nerve-muscle preparations[7-9, 12-14]. It is well known that voltage-dependent calcium channels inactivate rather quickly after opening, although with different kinetics[44-49]. Therefore, the long-lasting effect must be due to something other than constant opening of the calcium channels. It is known that intracellular calcium signals are subsequently encoded into activation of calcium/calmodulin-dependent protein kinases[50-52], and short-duration calcium signals could be transformed into long-lasting activation of calcium/calmodulin-dependent protein kinase II[53], therefore it would be interesting in the future to further investigate the possible effect of toosendanin on calcium oscillations in excitable cells, and on oscillation-associated activation of calcium/calmodulin-dependent protein kinase II. It would also be interesting to identify which types of voltage-dependent calcium channels are activated by toosendanin since a number of them are involved in neurotransmitter release[54-56]. In this conjunction, it is important to note that decreased calcium influx into motor nerve terminals has been found to recruit additional neuromuscular junctions during the synapse elimination period [57].

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