The Inhibitory Effect of the Toxic Fraction from Sea Urchin (Toxopneustes pileolus) Venom on \(^{45}\text{Ca}^{2+}\) Uptake in Crude Synaptosome Fraction from Chick Brain

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The effects of toxic peaks (P-I, P-II and P-III eluted from Sephadex G-200 column) from the sea urchin Toxopneustes pileolus on time-dependent \(^{45}\text{Ca}^{2+}\) uptake in chick P2 fraction (crude synaptosome fraction) were studied under physiological ionic conditions. Time-dependent \(^{45}\text{Ca}^{2+}\) uptake was inhibited by P-II and P-III, but not by P-I. P-II had the greatest inhibitory effect. The inhibitory effect of P-II was not due to the inhibition of \(^{45}\text{Ca}^{2+}\) binding on P2 fraction, because P-II did not affect \(^{45}\text{Ca}^{2+}\) binding in osmotically-shocked P2 fraction. P-II did not affect KCl-stimulated \(^{45}\text{Ca}^{2+}\) uptake in P2 fraction, \((\text{Ca}^{2+}-\text{Mg}^{2+})\)-ATPase activity in the synaptic plasma membrane (SPM) fraction, or (Na\(^+-\text{K}^{+}\))-ATPase and Mg\(^{2+}\)-ATPase activities in osmotically-shocked P2 fraction. In contrast, the Na\(^+/\text{Ca}^{2+}\) exchanger blocker 2,4-dichlorobenzamil (DCB; 100 \(\mu\text{M}\)), with a poor specificity, inhibited not only time-dependent \(^{45}\text{Ca}^{2+}\) uptake but also KCl-stimulated \(^{45}\text{Ca}^{2+}\) uptake, \((\text{Ca}^{2+}-\text{Mg}^{2+})\)-ATPase, and (Na\(^+-\text{K}^{+}\))-ATPase. Involvement of Na\(^+/\text{Ca}^{2+}\) exchanger in the time-dependent \(^{45}\text{Ca}^{2+}\) uptake was ruled out, since it was not inhibited by replacement of Na\(^+\) with Li\(^+\) in reaction medium. These results suggested that the inhibition by P-II on time-dependent \(^{45}\text{Ca}^{2+}\) uptake appeared to be more specific than the commercially available Na\(^+/\text{Ca}^{2+}\) exchanger blocker DCB, although the mechanism is not clear yet.

Key words: sea urchin; P2 fraction; \(^{45}\text{Ca}^{2+}\) uptake; calcium ion channel; \((\text{Ca}^{2+}-\text{Mg}^{2+})\)-ATPase; Na\(^+/\text{Ca}^{2+}\) exchanger

Toxic substances extracted from the globiferous pedicellariae of Toxopneustes pileolus, Toxopneustes pileolus, have many pharmacological effects, such as muscle contraction or relaxation, cardioactivity, sedation, reduction of body temperature and regulation of catecholamine or histamine release.\(^{1-6}\) In a comprehensive review of previous research, we found that these effects inevitably correlated to changes in intracellular Ca\(^{2+}\) concentrations. It suggests that the pharmacological effects of toxic substances from sea urchin are possibly to be involved in some systems of Ca\(^{2+}\) mobilization, such as the Ca\(^{2+}\) channel, Ca\(^{2+}\) pump or Na\(^+\)/Ca\(^{2+}\) exchanger, etc. In addition, the influence of toxic substances on the central nerve system attracted our attention.

In this study, Ca\(^{2+}\) uptake in crude synaptosome fraction (P2 fraction) was examined in the presence of toxic peaks (P-I, P-II and P-III), which were partially purified from the crude venom of sea urchin (Toxopneustes pileolus) by Sephadex G-200 gel filtration. The effects of P-II on the \(^{45}\text{Ca}\) uptake were also evaluated with inhibitors of Ca\(^{2+}\) channel, Ca\(^{2+}\) pump and Na\(^+\)/Ca\(^{2+}\) exchanger.

MATERIALS AND METHODS

Preparation of the Toxic Fraction For preparation of toxic fractions, thirty-six specimens of T. pileolus (8—11 cm in diameter) were collected along the coast of Tokushima Prefecture, Shikoku Island, Japan, in July 1995 and December 1996. The crude venom was extracted from the large globiferous pedicellariae of T. pileolus and fractionated by chromatography on gel filtration as reported previously.\(^{7}\) Briefly, the extracted venom (11.5 mg protein) was applied to a Sephadex G-200 column (2.6×60 cm) equilibrated with 0.15 M NaCl solution, and eluted with the same solution at a flow rate of 15 ml/h. Fractions of 10 ml each were collected.

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trifuged at 131,000 × g for 1 h. The intermediate phase between 0.6 and 0.8 m sucrose was collected, diluted with distilled water, and centrifuged at 117,000 × g for 30 min. The pellet was resuspended with distilled water, and the solution was used for the assay of (Ca²⁺−Mg²⁺)-ATPase.

All preparative experiments were carried out at 0–4 °C. Protein content of P2, osmotically shocked P2 or SPM fraction was measured by the Lowry method. BSA was used as the standard.

**Assay for ⁴⁵Ca⁺⁺ Uptake** High KCl-stimulated ⁴⁵Ca⁺⁺ uptake in P₂ fraction was assayed by the method previously reported. Time-dependent ⁴⁵Ca⁺⁺ uptake under physiological ionic solution (PIS; in mM: 120 NaCl, 5 KCl, 1 MgCl₂, 30 HEPES, 10 d-glucose, 1.5 Na₂HPO₄–NaH₂PO₄, pH 7.4) was assayed according to the same method as above, except that the P-I, P-II or P-III was added to the reaction medium after pre-incubation (9 min) of P₂ fraction in PIS, then adding ⁴⁵CaCl₂ (1–2 kBq/μl medium) 1 min later. After incubation for various times (1–15 min), a 400 μl aliquot of the reaction solution was filtered through a nitrocellulose filter. After washing the filter, the radioactivity on the filter (in 5 ml Clear Sol-I) was counted with a Liquid Scintillation Counter (Packard, 2050CA, Tri-CARB).

**Assay of ATPase Activity** The activities of (Na⁺−K⁺)-ATPase and (Ca²⁺−Mg²⁺)-ATPase were assayed by a modification of the previous methods. ATPase activities are presented as nmol of Pi/hr/mg of protein. (Na⁺−K⁺)-ATPase activity was calculated from the difference between (Na⁺−K⁺−Mg²⁺)-ATPase activity and Mg²⁺-ATPase activity. (Na⁺−K⁺−Mg²⁺)-ATPase activity was assayed in a 180 μl reaction medium containing 40 mM imidazole–HCl (pH 7.5 at 37 °C), 0.01% BSA, 130 mM NaCl, 20 mM KCl, 3 mM Mg (CH₃COO)₂, 40 μM Tris–HCl (pH 7.5 at 37 °C), osmotically shocked P₂ fraction (11–16 μg of protein/tube) and 3 mM ATP. The reaction was started by adding 20 μl of starting solution containing Mg(CH₃COO)₂, Tris–HCl and ATP to the reaction medium. After incubation of 20 min at 37 °C, the reaction was stopped by adding 20 μl 5% trichloroacetic acid (TCA). The reaction mixture was then centrifuged at 5000 × g for 10 min. 160 μl aliquots of supernatant were transferred to a micro-tube, and inorganic phosphorus was assayed by the method reported. Mg²⁺-ATPase activity was determined by the same method as for (Na⁺−K⁺)-ATPase activity, except that 1 mM ouabain was contained in the reaction medium.

(Ca²⁺−Mg²⁺)-ATPase activity was basically assayed by the same method as for (Na⁺−K⁺)-ATPase activity, except that the reaction medium contained 40 mM imidazole–HCl (pH 7.5 at 37 °C), 0.5 mM ouabain, 0.01% BSA, 100 mM KCl, 100 μM ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 0–150 μM CaCl₂, 3 mM Mg (CH₃COO)₂, 40 μM Tris–HCl (pH 7.5 at 37 °C), SPM (about 20 μg of protein/tube) and 3 mM ATP. (Ca²⁺−Mg²⁺)-ATPase activity was calculated as the difference between the activities in the presence and absence of calcium.

**Statistics** All statistical analyses proceeded in one factor ANOVA, Fisher’s Protected LSD.

**Materials** ω-Conotoxin GVIA (ω-CTX GVIA) and FMRF-amide (FMRFa) were purchased from the Peptide Institute Inc. (Japan); ATP disodium salt trihydrate, ammonium molybdate, 1-amino-2-naphthol-4-sulfonic acid, d-glucose, imidazole, 2',4'-dichlorobenzamid-HCl, magnesium acetate tetrahydrate, ouabain octahydrate, sodium bisulfite, sodium sulfite and trichloroacetic acid were from Wako Pure Chemical Ind. Ltd. (Japan); BSA, EGTA and Trizma Base (Tris) were obtained from Sigma Chemical Co. Clear Sol-I and sucrose were from Nakalai Tesque Inc. (Japan); ⁴⁵CaCl₂ was from Du Pont de Nemours & Co., Inc. and Serva Blue G (C.I. 42655) was from Serva (Heidelberg, Germany).

**RESULTS**

The effects of P-I, P-II and P-III, partially purified from crude venom of sea urchin (T. pilosus), on time-dependent ⁴⁵Ca⁺⁺ uptake in chick P₂ fraction were investigated. P-II and P-III almost completely inhibited the ⁴⁵Ca⁺⁺ uptake in a dose-dependent manner (Fig. 1B and C), while P-I even at a higher concentration (7.8 μg of protein/ml) did not (Fig. 1A). The inhibitory effect of P-II was greater than that of P-III. From Fig. 1, we can estimate that the IC₅₀ values for P-II and P-III were about 0.03 and 0.57 μg of protein/ml, respectively. Furthermore, we investigated effects of P-II on ⁴⁵Ca⁺⁺ binding to the osmotically-shocked P₂ fraction. As shown in Fig. 1D, ⁴⁵Ca⁺⁺ binding was not affected by P-II (2.8 μg of protein/ml). This result suggests that the inhibitory effect of P-II
on time-dependent $^{45}$Ca$^{2+}$ uptake was not due to the inhibition of $^{45}$Ca$^{2+}$ binding.

It is possible that time-dependent $^{45}$Ca$^{2+}$ uptake related to the Na$^{+}$/Ca$^{2+}$ exchanger, Ca$^{2+}$ channel, etc. Truly, dichlorobenzamid (DCB; an inhibitor of Na$^{+}$/Ca$^{2+}$ exchanger) significantly inhibited time-dependent $^{45}$Ca$^{2+}$ uptake in the P$_2$ fraction at 100 $\mu$M (Fig. 2A). However, 120 mM Li$^+$ (in place of 120 mM Na$^+$ in the PIS) did not inhibit the $^{45}$Ca$^{2+}$ uptake (Fig. 2B), suggesting little role to the Na$^{+}$/Ca$^{2+}$ exchanger in time-dependent $^{45}$Ca$^{2+}$ uptake. Moreover, 100 $\mu$M FMRFa (another inhibitor of Na$^{+}$/Ca$^{2+}$ exchange)$^{15}$ and 1 $\mu$M $\alpha$-CTX GVIA (an inhibitor of N-type Ca$^{2+}$ channel)$^{16}$ showed no effect on time-dependent $^{45}$Ca$^{2+}$ uptake (Fig. 2C), even though $\alpha$-CTX GVIA (1 $\mu$M) was reported to inhibit KCl-stimulated $^{45}$Ca$^{2+}$ uptake in chick P$_2$ fraction.$^{17}$ As shown in Fig. 2D, P-II (0.03—2.8 $\mu$g of protein/ml) did not inhibit KCl-induced $^{45}$Ca$^{2+}$ uptake, while DCB (1—100 $\mu$M) selectively inhibited it. From these results, no exact evidence was obtained to prove that the effect of P-II on the time-dependent $^{45}$Ca$^{2+}$ uptake was due to the inhibition of Na$^{+}$/Ca$^{2+}$ exchanger. In addition, we confirmed that P-II does not affect voltage-dependent Ca$^{2+}$ channels (i.e. N-type Ca$^{2+}$ channel) in the chick P$_2$ fraction.

We can not exclude the possibility that the inhibitory effect of P-II on time-dependent $^{45}$Ca$^{2+}$ uptake may be due to the influence of various kinds of ATPases in the P$_2$ fraction. Therefore, we investigated the effects of P-II on (Ca$^{2+}$-Mg$^{2+}$)-ATPase in SPM, and on Mg$^{2+}$-ATPase or (Na$^{+}$/K$^+$)-ATPase in the osmotically-shocked P$_2$ fraction. P-II did not affect these ATPase activities, while DCB (100 $\mu$M) inhibited all of these ATPase activities with no selectivity (Fig. 3A, B and C). In addition (Fig. 3D and E), the time-dependent $^{45}$Ca$^{2+}$ uptake was not influenced by ouabain [(Na$^{+}$/K$^+$)-ATPase inhibitor] or ruthenium red [selectively inhibited (Ca$^{2+}$/ Mg$^{2+}$)-ATPase in SPM fraction, data not shown].

These findings indicated that the P-II inhibited time-dependent $^{45}$Ca$^{2+}$ uptake, and the inhibition was different from that of DCB which showed inhibitory effects on not only time-dependent $^{45}$Ca$^{2+}$ uptake but also KCl-stimulated $^{45}$Ca$^{2+}$ uptake and various ATPase activities.

DISCUSSION

In this study, P-II and P-III partially purified from sea urchin venom, inhibited time-dependent $^{45}$Ca$^{2+}$ uptake in the
P₂ fraction from chick brain (Fig. 1B and C). The estimated IC₅₀ (0.03 μg protein/ml) of P-II was nineteen times lower than the IC₅₀ (0.57 μg protein/ml) of P-III. Because the peak of P-III partially overlapped with the peak of P-II when eluted from the Sephadex G-200 column,²⁰ the inhibitory effect of P-III may arise from a little of P-II which was mixed in P-III. Therefore, we used P-II [at least 3 bands appeared on (sodium dodecyl sulfate)-polyacrylamide gel electrophoresis; molecular weight 6500—46000] for further investigations.

Based on all of the results, we can draw the following conclusions regarding the mechanism by which P-II inhibits time-dependent ⁴⁵Ca²⁺ uptake under PⅢ conditions: (1) The time-dependent ⁴⁵Ca²⁺ uptake in the P₂ fraction was different from the ⁴⁵Ca²⁺ binding to membrane of the P₂ fraction, because only the former could be inhibited by P-II (Fig. 1B and D). (2) It was unlikely that the inhibitory effect of P-II was the result of direct binding between ⁴⁵Ca²⁺ and P-II. If such binding occurs during time-dependent ⁴⁵Ca²⁺ uptake, the concentration of free Ca²⁺ in the reaction medium will decrease, so that Ca²⁺ binding, KCl-stimulated ⁴⁵Ca²⁺ uptake and (Ca²⁺—Mg²⁺)-ATPase activity in the P₂ fraction will also be affected. However, our results show that these activities are not affected by P-II (Fig. 1D, 2D and 3C). Moreover, the high concentration of Ca²⁺ (1 mM) and the very low concentration of P-II (0.28 μg protein/ml) in the reaction medium appeared to deny the possibility that the inhibitory effect of P-II was due to the binding of Ca²⁺ to P-II, although the lack of binding between P-II and Ca²⁺ remains to be directly confirmed. (3) For the same reason as in (2), the inhibitory effect of P-II is unlikely to be due to an increase in the intracellular Ca²⁺ concentration as a result of Ca²⁺ release from intracellular Ca²⁺ pools(s) induced by P-II.

P-II had no effect on voltage-dependent calcium channels (VDCC) in the P₂ fraction (Fig. 2D). In other words, time-dependent ⁴⁵Ca²⁺ uptake did not relate to VDCC in the chick P₂ fraction. Besides calcium channels, it remained to be clarified whether or not a Na⁺/Ca²⁺ exchanger is responsible for the time-dependent ⁴⁵Ca²⁺ uptake. Because Na⁺/Ca²⁺ exchange in synaptosomes from rat brain has been confirmed as an important mechanism for Ca²⁺ influx. To clarify this point, we tried to block the Na⁺/Ca²⁺ exchanger in a time-dependent ⁴⁵Ca²⁺ uptake experiment. Generally, three kinds of blockers for Na⁺/Ca²⁺ exchanger have been reported. (1) Aminophylline analogues (i.e. DCB) can completely block the Na⁺/Ca²⁺ exchanger, but these analogues are not selective. Our present results with DCB were comparable to those in previous work that DCB was not selective for the Na⁺/Ca²⁺ exchanger. (2) XIP-like peptides (exchanger inhibitory peptide, the amino acid sequence is similar to a calmodulin binding domain) can inhibit not only Na⁺/Ca²⁺ exchangers which possess a putative calmodulin binding domain in their intracellular side loop, but also other calmodulin-dependent enzymes. Moreover, to inhibit the Na⁺/Ca²⁺ exchanger, XIP-like peptides must bind to the intracellular side of the Na⁺/Ca²⁺ exchanger. Therefore, XIP-like peptides would not be good selective blockers of Na⁺/Ca²⁺ exchanger in our experiment. (3) The mollusc peptide FMRFa and related peptides with naloxone-like activity can modulate the function of the Na⁺/Ca²⁺ exchanger. In this study, FMRFa (100 μM) did not inhibit time-dependent ⁴⁵Ca²⁺ uptake (Fig. 2C), although we can not exclude the possibility that FMRFa did not arrive to its binding site located on the intracellular side of the Na⁺/Ca²⁺ exchanger. Furthermore, Na⁺/Ca²⁺ exchange should be inhibited when Na⁺ (in a physiological ion condition) is replaced by Li⁺ in the time-dependent ⁴⁵Ca²⁺ uptake because Na⁺ is indispensably for Na⁺/Ca²⁺ exchange. As in Fig. 2B, no effect of Li⁺ (instead of Na⁺) was found in the time-dependent ⁴⁵Ca²⁺ uptake. According to above results, we can not regard the Na⁺/Ca²⁺ exchanger as a target of P-II.

We also can not exclude the possibility that the inhibitory effect of P-II on time-dependent ⁴⁵Ca²⁺ uptake was due to a change in (Ca²⁺—Mg²⁺)-ATPase or (Na⁺—K⁺)-ATPase activity on the plasma membranes. Therefore, the effects of P-II on these ATPase activities were examined (Fig. 3A, B and C). Relatively high activities of (Na⁺—K⁺)-ATPase on Mg²⁺-ATPase were measured in the osmotically-shocked P₂ fraction, but the activity of (Ca²⁺—Mg²⁺)-ATPase in the osmotically-shocked P₂ fraction was too low to detect. The SPM fraction was prepared with the modified method described in this paper. SPM had higher (Ca²⁺—Mg²⁺)-ATPase activity than the other fractions prepared by the sucrose density gradient centrifugation of the osmotically-treated synaptosomal fraction (data not shown). As a result, P-II did not significantly inhibit these ATPase activities. Therefore the inhibitory effect of P-II did not result from its effect on Ca²⁺—Mg²⁺ and/or (Na⁺—K⁺)-ATPase activity. Moreover, it has been reported that Mg²⁺-ATPase played an important role in modulating the turnover of neurotransmitters such as γ-amino butyric acid (GABA) and glutamate. In this study, P-II had no significant effect on Mg²⁺-ATPase activity as shown in Fig. 3B, suggesting that the inhibition of P-II on time-dependent ⁴⁵Ca²⁺ uptake is not related to a GABA or glutamate receptor (or channel). The above results were confirmed by the fact that the time-dependent ⁴⁵Ca²⁺ uptake could not be inhibited by ouabain (Fig. 3D) or ruthenium red (Fig. 3E).

On nerve cells, no direct pharmacological effects caused by partially purified toxic substances from sea urchin venom was reported until now, though the toxic substances of sea urchin can induce some reactions in the central nervous system of animals or humans. Because time-dependent ⁴⁵Ca²⁺ uptake occurs under normal physiological ion conditions, it remains to be investigated whether or not P-II affects the voltage-independent Ca²⁺ channels or spontaneous fluctuations of intracellular Ca²⁺ concentration in some nerve cells. This kind of research will help us to understand better the mechanisms of Ca²⁺ mobilization in plasma membranes. However, the results from studies using a crude fraction like P-II have to be discussed with reservations, because the phenomena observed might reflect only the apparent effects due to the multiple components in P-II.

In conclusion, both P-II and DCB could inhibit time-dependent ⁴⁵Ca²⁺ uptake in the chick P₂ fraction, but the inhibitory mechanisms might be different. Full purification and characterization of the active component(s) from the venom of sea urchin (T. pileolus) is a promising work for further clarification of the mechanisms of Ca²⁺ mobilization (i.e. time-dependent ⁴⁵Ca²⁺ uptake) in nerve cells.
REFERENCES