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Inhibition by ouabain of palytoxin-induced catecholamine secretion and calcium influx into cultured bovine adrenal chromaffin cells

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Abstract—The effect of ouabain on palytoxin (PTX)-induced catecholamine secretion from cultured bovine adrenal chromaffin cells was examined in relation to its effect on calcium (Ca^{2+}) influx into the cells. Ouabain showed concentration-dependent inhibition of catecholamine secretion induced by PTX. Ouabain also inhibited $[^{45}Ca]^{2+}$ influx induced by PTX, this inhibition being parallel with that of catecholamine secretion. The inhibitory effects of ouabain on PTX-induced catecholamine secretion and $[^{45}Ca]^{2+}$ influx were both overcome by increasing the concentrations of PTX, indicating that ouabain inhibited the actions of PTX in a competitive manner. These results suggest that the ouabain-sensitive (or -binding) site on the cell membrane might be the target site of action of PTX, which causes an increase in Ca^{2+} permeability and initiation of catecholamine secretion.

Key words: palytoxin; ouabain; catecholamine secretion; Ca^{2+} influx; adrenal chromaffin cells

PTX*, a marine toxin, isolated from the zoanthid Palythoa species [1], has been shown to cause Na^+-dependent, TTX-insensitive depolarization of many excitable tissues and increase in Ca^{2+} influx into cells, leading to contraction of smooth and cardiac muscles [2–8] and the release of norepinephrine from nerve terminals [9–11] and PC12 cells [12]. Recently, we demonstrated that PTX at relatively low concentrations (10^{-10}–10^{-6} M) also causes marked catecholamine secretion from cultured bovine adrenal chromaffin cells, mediated by activation of TTX-insensitive Na^+-dependent Ca^{2+} influx [13–15]. PTX has also been shown to stimulate the efflux of K^+ from erythrocytes [16–18], Hela cells and adrenal medullary cells [16]. The PTX-induced contraction of muscle and efflux of K^+ from cells are reported to be inhibited by ouabain [9, 16, 17]. These results seem significant for clarifying the mode of action of PTX on the cell membrane, in relation to the ouabain-sensitive (or -binding) site, probably Na^+-K^+ ATPase [4]. Therefore, in the present study, we examined the effect of ouabain on PTX-induced catecholamine secretion and $[^{45}Ca]^{2+}$ influx of cultured bovine adrenal chromaffin cells to elucidate whether it also affects the secretory response of these cells induced by PTX.

Materials and Methods

Fresh bovine adrenal glands were obtained from a local slaughterhouse and placed on ice. Isolated adrenal chromaffin cells were prepared by sequential digestion of adrenal medullary slices with collagenase [19]. As described previously, the cells were maintained as monolayers for 3 days in 24-well plates (5×10^5 cells/well) to measure the catecholamine secretion and $[^{45}Ca]^{2+}$ influx [20]. The cells were washed once with 1 mL of BSS [135 mM NaCl, 5.6 mM KCl, 1.2 mM $MgSO_4$, 2.2 mM $CaCl_2$, 10 mM glucose and 20 mM *N*-2-hydroxy-ethylpiperazine-*N'*-2-ethane-sulfonic acid (HEPES)/NaOH, pH 7.4], and then incubated at 37° for 10 min in 250 μL of BSS. After incubation, the medium was withdrawn and the cells were lysed by adding 250 μL of 10% acetic acid and then freeze–thawing. Catecholamine in the medium and the cell lysate was assayed fluorometrically [21]. For measurement of $[^{45}Ca]^{2+}$ influx into the cells, 3.0 μCi of $[^{45}Ca]Cl_2$ was added to the incubation medium in the presence or absence of PTX. After 10 min incubation, the medium was discarded, and the cells were washed four times with 1 mL volumes of ice-cold, Ca^{2+}-free BSS. Then the intracellular $[^{45}Ca]^{2+}$ was extracted with 1% Triton X-100 and measured in a liquid scintillation counter. PTX was kindly donated by Dr I. Muramatsu (Department of Pharmacology, Fukui Medical School, Japan).

Results and Discussion

As shown in Fig. 1A, ouabain inhibited 10^{-8} M PTX-induced catecholamine secretion from cultured bovine adrenal chromaffin cells in a concentration-dependent manner. A significant inhibitory action of ouabain on the secretory response to PTX was observed at concentrations of above 3×10^{-6} M, and inhibition was maximal at 10^{-4} M. These concentrations of ouabain were inhibitory on Na^+-K^+ ATPase in adrenal chromaffin cell membrane [22]. Ouabain (10^{-5}–10^{-4} M) itself caused secretion of only 2–4% of the total cellular catecholamine.

Next, we examined the effect of ouabain on the PTX-induced increase in $[^{45}Ca]^{2+}$ influx into the cells, to elucidate whether the inhibitory effect of ouabain on PTX-induced catecholamine secretion was due to its inhibition of Ca^{2+} influx induced by PTX. As shown in Fig. 1B, ouabain concentration dependently inhibited $[^{45}Ca]^{2+}$ influx induced by PTX (10^{-8} M). This inhibitory effect on $[^{45}Ca]^{2+}$ influx was similar to that on catecholamine secretion induced by PTX. Thus the inhibitory effect of ouabain on PTX-induced catecholamine secretion was attributable to its inhibitory effect on Ca^{2+} influx induced by PTX, which is necessary to cause catecholamine secretion from the cells [15].

The characteristics of the inhibitory effects of ouabain on PTX-induced catecholamine secretion and $[^{45}Ca]^{2+}$ influx were also examined. Figure 2A shows the dose–response curve for the PTX-induced catecholamine secretion in the presence and absence of ouabain (10^{-5} M). PTX-induced catecholamine secretion was observed at concentrations of more than 10^{-10} M and was maximum at 10^{-6} M, where approximately 60% of the total cellular catecholamine was secreted. The inhibitory effect of ouabain was remarkable at lower concentrations of PTX and was overcome by increasing the concentration of PTX. As shown in Fig. 2B, PTX-induced $[^{45}Ca]^{2+}$ influx was also observed at concentrations of more than 10^{-10} M and the concentration–response curve was similar to that for PTX-induced catecholamine secretion. Ouabain caused significant inhibition of the $[^{45}Ca]^{2+}$ influx induced by lower concentrations of PTX, and this inhibition by ouabain was

* Abbreviations: PTX, palytoxin; TTX, tetrodotoxin; BSS, balanced salt solution.

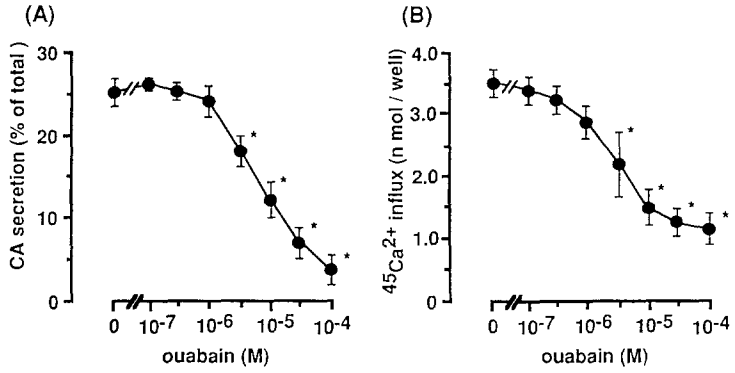

Fig. 1. Effects of ouabain on catecholamine secretion (A) and $[^{45}Ca]^{2+}$ influx (B) induced by PTX. Cultured bovine adrenal chromaffin cells were incubated with PTX (10^{-8} M) at 37° for 10 min in the presence of various concentrations of ouabain. (A) Catecholamine (CA) secretion is expressed as a percentage of the total cellular content. Since ouabain itself at the concentrations used (10^{-5}–10^{-4} M) caused secretion of about 2–4% of the total cellular catecholamine, the presented values were calculated by subtracting the values with ouabain only from the observed values. (B) For measurement of $[^{45}Ca]^{2+}$ influx into the cell, 3.0 μCi of $[^{45}Ca]Cl_2$ was added to the incubation medium. The influx of $[^{45}Ca]^{2+}$ into the cells is shown in nmol/well. Points and bars are means ± SEM for three to four experiments. *$P < 0.005$ vs control value.

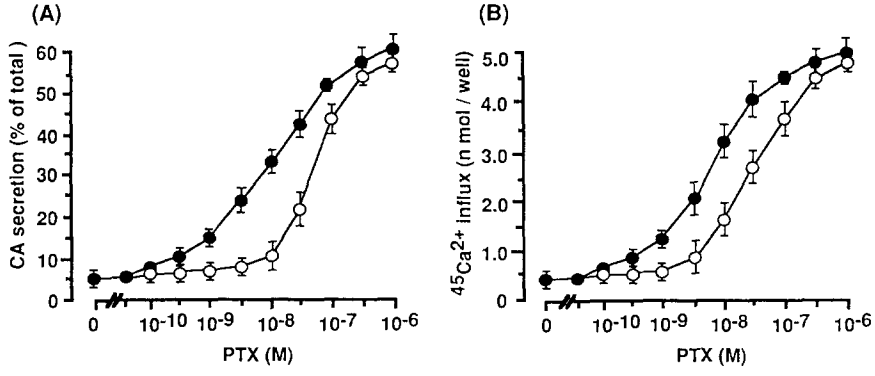

Fig. 2. Concentration–response curves for PTX-induced catecholamine secretion (A) and $[^{45}Ca]^{2+}$ influx (B) in the presence and absence of ouabain. Cultured bovine adrenal chromaffin cells were incubated with various concentrations of PTX at 37° for 10 min in the presence (○) or absence (●) of 10^{-5} M ouabain. (A) Catecholamine secretion is expressed as a percentage of the total cellular content. Since ouabain itself at 10^{-5} M induced secretion of about 3% of the catecholamine, the presented values were calculated by subtracting the values with ouabain alone from the observed values. (B) For measurement of $[^{45}Ca]^{2+}$ influx into the cells, $[^{45}Ca]Cl_2$ was added to the incubation medium. The influx of $[^{45}Ca]^{2+}$ into the cells is shown in nmol/well. Points and bars are means ± SEM for three to four experiments.

also overcome by increasing the concentration of PTX. These results suggest that the inhibitory effect of ouabain on PTX-induced catecholamine and $[^{45}Ca]^{2+}$ influx is competitive, and that the ouabain-sensitive (or -binding site), probably Na^+-K^+ ATPase, might be the target site of PTX on the chromaffin cell membrane. This possibility is consistent with previous reports that ouabain inhibits PTX-induced muscle contraction and efflux of K^+ from cells through its interaction with Na^+-K^+ ATPase [4, 9, 16, 17].

Although PTX at higher concentrations (10^{-7} M) was shown to inhibit Na^+-K^+ ATPase activity in erythrocyte membrane [17], PTX at the concentrations used here did not affect Na^+-K^+ ATPase, because, unlike ouabain [22], it did not inhibit, but slightly increased, ^{86}Rb influx into the cells (data not shown). The inhibition by ouabain of the actions of PTX seems unlikely to be due to inhibition of Na^+-K^+ ATPase, because PTX-induced catecholamine secretion was not affected in K^+-free medium, in which Na^+-K^+ ATPase activity is diminished.

It is generally thought that ouabain inhibits Na^+-K^+ ATPase and causes accumulation of Na^+ in the cells, thereby increasing the influx of Ca^{2+} through acceleration of the Na^+–Ca^{2+} exchange reaction, and thus leading to the contraction of muscles and release of neurotransmitters. Previously, PTX-induced noradrenaline release from sympathetic nerve terminals was also reported to be potentiated by ouabain [9]. However, in the present study, we found that PTX-induced Ca^{2+} influx and catecholamine secretion in cultured bovine adrenal chromaffin cells were

competitively inhibited by ouabain. Thus we suggest that the action site of PTX on the cell membrane might be the ouabain-sensitive (or -binding) site, probably Na^+-K^+ ATPase, as already suggested [4, 17]. The mechanism by which PTX acts on the ouabain-sensitive (or -binding) site on the membrane, resulting in an increase in Ca^{2+} influx and catecholamine secretion is under our investigation.

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