Effect of 2,4,6-Trinitrophenol on Catecholamine Secretion from Perfused Bovine Adrenal Glands

Kyozo YAMANAKA*, Shizuo YAMADA and Eiichi HAYASHI
Department of Pharmacology, Shizuoka College of Pharmaceutical Sciences, Shizuoka 422, Japan
*Department of Pharmacology, Fukui Medical School, Matsuoka, Fukui 910-11, Japan
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Abstract—2,4,6-Trinitrophenol (PA) evoked a prolonged catecholamine (CA) secretion from perfused bovine adrenal glands. The PA-evoked CA secretion was concentration-dependent, required the presence of extracellular calcium and resulted from a direct action of PA on the chromaffin cells. Furthermore, PA reduced Mg$^{2+}$-ATPase activities in the plasma membrane-rich microsome and granule-rich fraction from the adrenal medulla. These results indicate that PA evokes CA secretion through the actions on both the chromaffin cell membranes and granule membranes.

2,4,6-Trinitrophenol (picric acid: PA) is well known to stimulate the release of acetylcholine from pre- and post-ganglionic cholinergic nerve endings (1-3). Recently, Sorimachi and Yamagami (4) found that PA stimulates the release of catecholamines (CA) from perfused rabbit adrenal glands and suggested a direct action of PA on the adrenal chromaffin cells. This evidence indicates that PA stimulates the releasing process at different types of secretory cells. However, the underlying mechanism of the PA action still remains unclear. The present study was an attempt to further clarify the action of PA on the adrenal medulla.

Fresh bovine adrenal glands obtained at the local slaughter house were perfused retrogradely with Locke’s solution (pH 7.2, 22-25°C) equilibrated with 5% CO$_2$ in O$_2$, as described previously (5, 6). The flow rate was maintained at 4 ml/min and the perfusate was collected at 3-min intervals. The amount of CA was estimated fluorometrically (7). In the experiments with the ATPase assay, adrenal medullary microsomes and granule-rich fraction were obtained by the centrifugation technique described in our recent report (8). Briefly, the adrenal medulla was homogenized in 0.3 M sucrose, and the homogenate was centrifuged to separate cell debris and nuclei (800×g for 10 min), granules and mitochondria (27,000×g for 20 min), and microsomes (105,000×g for 60 min), consecutively. Plasma membrane-rich microsomes and granule-rich fraction were then obtained by using discontinuous sucrose density gradients. ATPase activities were assayed by measuring inorganic phosphate liberated during incubation of plasma membrane-rich microsomes and granule-rich fraction for 15 min at 37°C. The incubation medium contained 33 mM Tris/HCl (pH 7.0), divalent cations (2 mM CaCl$_2$ for the Ca$^{2+}$-ATPase assay or 2 mM MgCl$_2$ for the Mg$^{2+}$-ATPase assay) and ATP (sodium salt, 4 mM) in a final volume of 3 ml. Blank experiments were carried out without divalent cations. Inorganic phosphate and protein were measured by the methods of Fiske and Subbarow (9) and Lowry et al. (10), respectively. Statistical evaluation was performed by using Student’s $t$-test. PA was used as the sodium salt (Wako), and other drugs used in this study were purchased from commercial sources.

Figure 1 shows a typical example of adrenal CA secretion evoked by prolonged...
perfusion of PA (3 mM) for 60 min. The PA-evoked CA secretion increased during the first 15 min and reached 10 times prestimulation level as the amount of CA released for a 3-min period. The evoked CA secretion was then followed by a progressive decline, but the level of CA secretion still remained above the prestimulation level 60 min later. Interestingly, occasional but short-lived increases in CA secretion were observed during the course of decline of the PA-evoked secretory response. A similar pattern of the evoked CA secretion was obtained in an additional three experiments with 3 mM of PA, but short-lived increases in CA secretion varied in frequency and the amount of CA released. The PA-evoked CA secretion for the first 15 min was concentration dependent: 74.9±11.5 μg/15 min, 158.0±33.7 μg/15 min and 265.6±23.6 μg/15 min (n=4-6) at concentrations of 0.3, 1 and 3 mM, respectively. The CA secretion evoked by PA (1 mM) was markedly inhibited and enhanced by removal of calcium (with 1 mM EDTA present) and raising the level of calcium in the Locke’s solution, respectively, whereas it was unaffected by hexamethonium (1 mM), hemicholinium-3 (30 μM), morphine (90 μM) and tetrodotoxin (0.63 μM) (data not shown). These results indicate that PA evokes a prolonged CA secretion from the adrenal medulla, and they support the idea by Sorimachi and Yamagami (4) that PA-evoked CA secretion may be due to an increase in calcium entry into the chromaffin cells through a direct action of PA on the chromaffin cells.

Fig. 1. A typical example of PA-evoked catecholamine secretion from perfused bovine adrenal gland. The arrow indicates the beginning of perfusion of PA (3 mM) for 60 min and the abscissa, the time after beginning perfusion of the gland. Each white column shows an amount of catecholamine secretion evoked by PA for a 3-min period and the black bar, the level of spontaneous catecholamine secretion.

Several agents that produce a prolonged adrenal CA secretion have been demonstrated to produce a significant decrease in the activity of Mg2+-ATPase in adrenal medullary microsomes (8). Accordingly, we examined the effect of PA on the ATPase activities to further clarify the PA action on the adrenal medulla. As shown in Table 1, PA (1 mM) reduced Mg2+-ATPase activity in the plasma membrane-rich microsomes by about 20% (significant at P<0.05), without affecting Ca2+-ATPase activity. Similarly, PA reduced...
Mg$^{2+}$-ATPase activity located in the granule-rich fraction by about 50% (significant at P<0.05). Mg$^{2+}$-ATPases have been suggested to involve the efflux and uptake of intracellular calcium in the plasma membrane and granules, to maintain a low resting level of intracellular calcium by these subcellular organelles. Accordingly, the significant inhibition of Mg$^{2+}$-ATPases by PA may sustain the increased cytosolic free calcium to cause a prolonged CA secretion. In our preliminary experiments, we found that PA markedly inhibits the calcium uptake by chromaffin granules stimulated by ATP plus magnesium (K. Yamanaka et al., unpublished observation).

Conclusively, the present study indicates that there are two sites for the action of PA in chromaffin cells, that is, plasma membranes and granule membranes, and suggests that the CA secretion evoked by PA may be initiated by an influx of calcium into the chromaffin cells and prolonged by the actions on both plasma membranes and granule membranes, such as an inhibition of Mg$^{2+}$-ATPases. At present, the mechanism of short-lived increases in CA secretion evoked by prolonged perfusion of PA is unclear. Recently, PA has been suggested to behave like a weak calcium ionophore in human erythrocytes (12), whereas in the chromaffin cells, an activation of calcium channels by PA was supposed (4). The underlying mechanism of calcium entry induced by PA still remains an open question.

### Table 1. Effect of PA on the activities of Mg$^{2+}$-ATPase and Ca$^{2+}$-ATPase in the plasma membrane-rich microsomes and granule-rich fraction from the adrenal medulla

|                  | PA (1 mM) | Mg$^{2+}$-ATPase | Ca$^{2+}$-ATPase |
|------------------|-----------|------------------|------------------|
| Microsome        |           |                  |                  |
| -                | 8.9±0.5   | 21.5±3.3         |                  |
| +                | 7.3±0.4*  | 20.4±2.9         |                  |
| Granule          |           |                  |                  |
| -                | 8.2±0.4   | ND               |                  |
| +                | 4.1±1.5*  | ND               |                  |

ATPase activities were expressed as µmoles Pi/hr/mg protein, and each value represents the mean value±S.E. of 4 separate experiments. *: Significantly different from the control value at P<0.05. ND: not determined.

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