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# Influence of Strychnine on Catecholamine Release Evoked by Activation of Cholinergic Receptors from the Perfused Rat Adrenal Gland

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The present study was attempted to investigate the effect of strychnine on catecholamine (CA) secretion evoked by ACh, high $K^+$, DMPP and McN-A-343 from the isolated perfused rat adrenal gland. The perfusion of strychnine ($10^{-4}$ M) into an adrenal vein for 20 min produced great inhibition in CA secretory responses evoked by ACh ($5.32\times10^{-3}$ M), DMPP ($10^{-4}$ M for 2 min) and McN-A-343 ($10^{-4}$ M for 2 min), but did not alter CA secretion by high $K^+$ ($5.6\times10^{-2}$ M). Strychnine itself did also fail to affect basal catecholamine output. Furthermore, in adrenal glands preloaded simultaneously with strychnine ($10^{-4}$ M) and glycine (an agonist of glycinergic receptor, $10^{-4}$ M), CA secretory responses evoked by ACh, DMPP and McN-A-343 were considerably recovered to some extent when compared with those evoked by treatment with strychnine only. However, CA secretion by high $K^+$ ($5.6\times10^{-2}$ M) was not affected. Taken together, these results demonstrate that strychnine inhibits greatly the CA secretory responses evoked by stimulation of cholinergic (both nicotinic and muscarinic) receptors, but does not affect that by membrane depolarization. It is suggested that strychnine-sensitive glycinergic receptors are localized in rat adrenal medullary chromaffin cells.

Key Words: Strychnine, Adrenal gland, Cholinergic stimulation, Catecholamine secretion

## INTRODUCTION

Strychnine, the convulsive alkaloid, has been established as a specific antagonist of the postsynaptic receptor for the inhibitxory neurotransmitter, glycine, in the central nervous system (Curtis et al, 1971; Young & Snyder, 1973; Fostholm & Rotter, 1985). The strychnine recognition site appears to be located on the 48 KDa subunit of the glycine receptor (Graham et al, 1983; Pfeiffer et al, 1984), which has significant structural and amino acid sequence homology with the nicotinic acetylcholine receptor polypeptide family (Grenningloh et al, 1987). It has been shown that bovine adrenal medullary chromaffin cells possess a high affinity [$^3$H]-strychnine binding site (Yadid et al, 1989; 1993). It is also found that glycine and milacemide, a glycine prodrug, can evoke catecholamine (CA) release from slices of adrenal medullary tissue and from freshly isolated chromaffin cells (Yadid et al, 1991; 1992). Dar & Zinder (1995) have also reported that strychnine inhibits CA release stimulated by acetylcholine or nicotine in a primary culture of bovine adrenal medullary chromaffin cells through acting on a regulatory site of the nicotine-cholinergic receptors, which is genetically similar to the strychnine-binding 48 KD subunit of the glycine receptors. Moreover, recently, Yadid & his colleagues (1998) have suggested that strychnine and glycine interact with the agonist-binding site of the nicotinic acetylcholine receptors in bovine adrenomedullary chromaffin cells, thus exerting a pharmacological effect that may have a modulatory role

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on the acetylcholine receptors.

However, Kuijpers & his coworkers (1994) have shown that strychnine inhibits the nicotinic stimulation of CA secretion and the increase in intracellular $Ca^{2+}$ concentration from bovine adrenal medullary chromaffin cells. This inhibitory action of strychnine is reversible and is selective for nicotinic stimulation with no effect observed on secretion elicited by a high external $K^+$ concentration, histamine, or angiotensin II. Glycine does not affect CA release nor the inhibitory effect of strychnine on this CA release. This result demonstrates that strychnine exerts a pharmacological effect independent of the glycine receptor. Thus, there is some controversy in relationship between strychnine and glycine receptor in CA secretion from the adrenal medulla.

In the present study, it was attempted to investigate the effect of strychnine on CA release evoked by cholinergic stimulation and membrane depolarization from the isolated perfused rat adrenal gland and to establish the relationship between strychnine and glycine receptors.

## METHODS

*Experimental procedure*

Male Sprague-Dawley rats, weighing 180 to 300 grams, were anesthetized with thiopental sodium (40 mg/kg) intraperitoneally. The adrenal gland was isolated by the methods described previously (Wakade, 1981). The abdomen was opened by a mid-line incision, and the left adrenal gland and surrounding area were exposed by placing three-hook retractors. The stomach, intestine and portion of the liver were not removed, but pushed over to the right side and covered by saline-soaked gauge pads and urine in bladder was removed in order to obtain enough working space for tying blood vessels and cannulations.

A cannula, used for perfusion of the adrenal gland, was inserted into the distal end of the renal vein after all branches of adrenal vein (if any), vena cava and aorta were ligated. Heparin (400 IU/ml) was injected into vena cava to prevent blood coagulation before ligating vessels and cannulations. A small slit was made into the adrenal cortex just opposite to entrance of adrenal vein. Perfusion of the gland was started, making sure that no leakage was present, and the perfusion fluid escaped only from the slit made in adrenal cortex. Then the adrenal gland, along with ligated blood vessels and the cannula, was carefully removed from the animal and placed on a platform of a leucite chamber. The chamber was continuously circulated with water heated at $37 \pm 1^{\circ}C$.

*Perfusion of adrenal gland*

The adrenal glands were perfused by means of a peristaltic pump (ISCO Co., Lincoln, NB, USA) at a rate of 0.31 ml/min. The perfusion was carried out with Krebs-bicarbonate solution of following composition (mM): NaCl, 118.4; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.18; $NaHCO_3$, 25; $KH_2PO_4$, 1.2; glucose, 11.7. The solution was constantly bubbled with 95% $O_2$+5% $CO_2$ and the final pH of the solution was maintained at 7.4~7.5. The solution contained disodium EDTA (10 $\mu$g/ml) and ascorbic acid (100 $\mu$g/ml) to prevent oxidation of catecholamine.

*Drug administration*

The perfusions of DMPP (100 $\mu$M) and McN-A-343 (100 $\mu$M) for 2 minutes, and a single injection of ACh (5.32 mM) and KCl (56 mM) in a volume of 0.05 ml were made into perfusion stream via a three way stopcock, respectively. In the preliminary experiments it was found that upon administration of the above drugs, secretory responses to ACh, KCl and McN-A-343 returned to preinjection level in about 4 min, but the responses to DMPP in 8 min. All secretagoues were administered at 15-min intervals for 60 min, but DMPP at 20-min intervals.

*Collection of perfusate*

As a rule, prior to stimulation with various secretagogues, perfusate was collected for 4 min to determine the spontaneous secretion of CA (background sample). Immediately after the collection of the background sample, collection of the perfusate was continued in another tube as soon as the perfusion medium containing the stimulatory agent reached the adrenal gland. Stimulated samples were collected for 4 to 8 min. The amounts secreted in the background sample have been subtracted from those secreted from the stimulated sample to obtain the net secretion value of CA, which is shown in all of the figures. In order to study the effect of strychnine on the spontaneous and evoked secretion, the adrenal gland was perfused

with Krebs solution containing strychnine for 60 min. Then the perfusate was collected for the background sample, and then the medium was changed to the one containing the stimulating agent and the perfusates were collected for the same period as that for the background sample. Generally, the adrenal gland's perfusate was collected in chilled tubes.

*Measurement of catecholamines*

CA content of perfusate was measured directly by the fluorometric method of Anton & Sayre (1962) without the intermediate purification alumina for the reasons described earlier (Wakade, 1981) using fluorospectrophotometer (Kontron Co., Italy). A volume of 0.2 ml of the perfusate was used for the reaction. The content of CA in the perfusate was expressed in terms of norepinephrine (base) equivalents.

*Statistical analysis*

The statistical significance between groups was determined by the Student's t-test. A P-value of less than 0.05 was considered to be significant unless specifically noted in the text. Values given in the text refer to means and the standard errors of the mean (S.E.M.). The statistical analysis of the experimental results was made by computer program described by Tallarida & Murray (1987).

*Drugs and their sources*

*The following drugs were used*: stychnine nitrate, acetylcholine chloride, 1.1-dimethyl-4-phenyl piperazinium iodide (DMPP), norepinephrine bitartrate, glycine (Sigma Chemical Co., USA), (3-(m-cholro-phenyl-carbamoyl-oxy)-2-butynyl trimethyl ammonium chloride [McN-A-343] (RBI, USA). Drugs were dissolved in distilled water (stock) and added to the normal Krebs solution as required. Concentrations of all drugs used are expressed in terms of molar base.

## RESULTS

*Effect of strychnine on CA secretion evoked by ACh, high $K^+$, DMPP and McN-A-343 from the perfused rat adrenal glands*

After the initial perfusion with oxygenated Krebs-bicarbonate solution for 1 hr, basal CA release from the isolated perfused rat adrenal glands amounted to $22.5 \pm 2.0$ ng/2 min (n=8). It is shown that strychnine inhibits CA release stimulated by acetylcholine or nicotine in a primary culture of bovine adrenal medullary chromaffin cells through acting on a regulatory site of the nicotine-cholinergic receptors (Dar & Zinder, 1995). Thus, it was of interest to examine the effects of strychnine itself on CA secretion from perfused rat adrenal glands. However, in the present study, strychnine alone did not produce any effect on basal CA output from perfused rat adrenal glands (data not shown). Therefore, it was decided to investigate the effects of strychnine on cholinergic receptor stimulation-as well as membrane depolarization-mediated CA secretion. Secretagogues were given at 15 to 20 min-intervals. Strychnine was present 15~20 min before initiation of stimulation.

When ACh ($5.32 \times 10^{-3}$ M) in a volume of 0.05 ml was injected into the perfusion stream, the a-

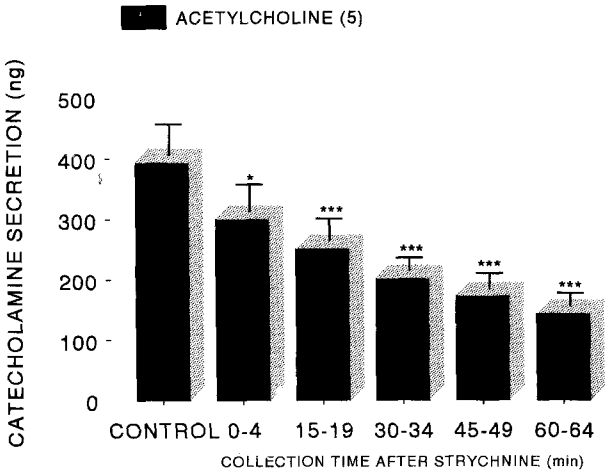


**Fig. 1.** Effects of strychnine on secretory responses of catecholamines (CA) evoked by acetylcholine from the isolated perfused rat adrenal glands. CA secretion by a single injection of ACh ($5.32 \times 10^{-3}$ M) was induced before (CONTROL) and after preloading with $10^{-4}$ M of strychnine for 60 min, respectively. Number in the parenthesis indicates number of experimental rat adrenal glands. Vertical bars represent the standard error of the mean (S.E.M.). Ordinate: the amounts of CA secreted from the adrenal gland (ng). Abscissa: Time of perfusate collection. Statistical difference was obtained by comparing the "CONTROL" with each period (for 4 min) after the initiation of strychnine perfusion. Perfusates were collected for 4 minutes at 15-min intervals, respectively. *: $P<0.05$, ***: $P<0.01$.

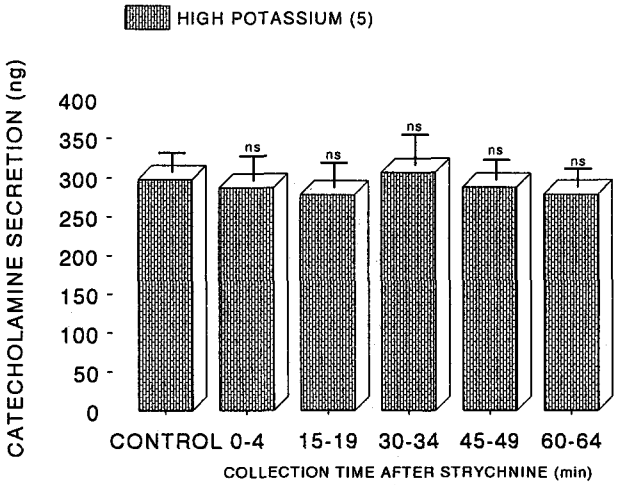


**Fig. 2.** Effects of strychnine on secretory responses of catecholamines (CA) evoked by high potassium from the isolated perfused rat adrenal glands. CA secretion by a single injection of excess $K^+$ ($5.6\times10^{-2}$ M) was induced before (CONTROL) and after preloading with $10^{-4}$ M of strychnine for 60 min, respectively. Perfusates were collected for 4 minutes at 15-min intervals, respectively. ns: Statistically nonsignificant.

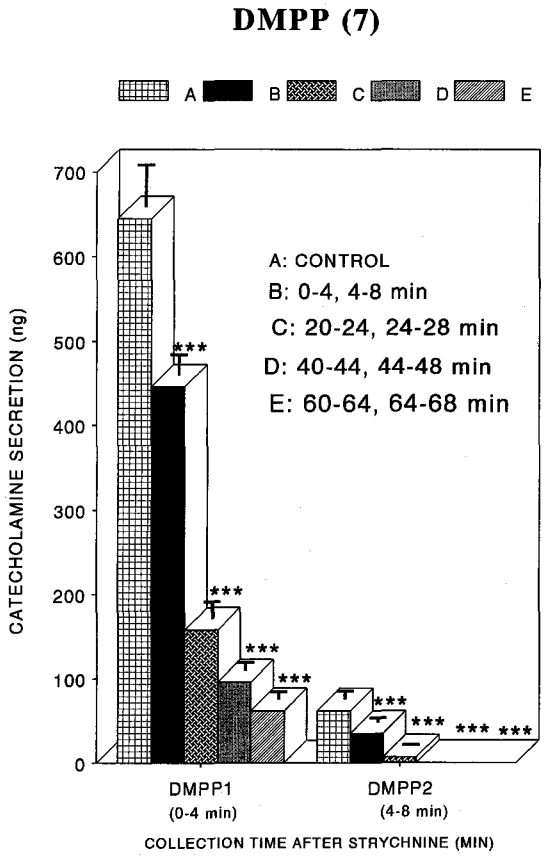


**Fig. 3.** Effects of strychnine on secretory responses of catecholamines (CA) evoked by DMPP from the isolated perfused rat adrenal glands. CA secretion by perfusion of DMPP ($10^{-4}$ M) for 2 min was induced before (A, CONTROL) and after (B, C, D and E) preloading with $10^{-4}$ M of strychnine for 60 min, respectively. DMPP-induced perfusates were collected consecutively twice at 4-min intervals for 8 minutes every 20 min, respectively (DMPP-1; 0~4 min, DMPP-2; 4~8 min). Other legends are the same as in Fig. 2. ***: $P<0.01$.

mounts of CA secreted was 394±52 ng for 4 min. However, the pretreatment with 100 M strychnine for 60 min inhibited significantly ACh-stimulated CA secretion in a time-dependent manner from 5 adrenal glands, as shown in Fig. 1. Also, it has been found that depolarizing agent like KCl stimulates sharply CA secretion. In the present work, excess $K^+$ ($5.6\times10^{-2}$ M)-stimulated CA secretion after the pretreatment with strychnine was not affected as compared with its corresponding control secretion (298±25 ng for 0~4 min) from 5 glands (Fig. 2). When perfused through the rat adrenal gland, DMPP ($10^{-4}$ M for 2 min), which is a selective nicotinic receptor agonist in autonomic sympathetic ganglia, evoked a sharp and rapid increase in CA secretion. However, as shown in Fig. 3, DMPP-stimulated CA secretion after pretreatment with 100 $\mu$M strychnine was relatively time-dependently reduced as compared with its corresponding control secretion (645±50 ng for 0~4 min; 62±10 ng for 4~8 min) in 7 rat adrenal glands. McN-A-343 ($10^{-4}$ M), which is a selective muscarinic M1-agonist (Hammer & Giachetti, 1982), perfused into an adrenal gland for 2 min caused an increased CA secretion (96±19 ng for 0~4 min) from 7 glands. However, McN-A-343-stimulated CA secretion was perfectly blocked at 45~49 min after loading with 100 $\mu$M strychnine as compared to the corresponding control secretion as depicted in Fig. 4.

*The effects of strychnine plus glycine on CA release evoked ACh, high $K^+$, DMPP and McN-A-343 from the perfused rat adrenal glands*

It has been found that strychnine and glycine interact with the agonist-binding site of the nicotinic acetylcholine receptors in bovine adrenomedullary chromaffin cells, thus exerting a pharmacological effect that may have a modulatory role on the acetylcholine receptors (Yadid et al, 1998). Therefore, it was tried to determine the effect of strychnine in the presence

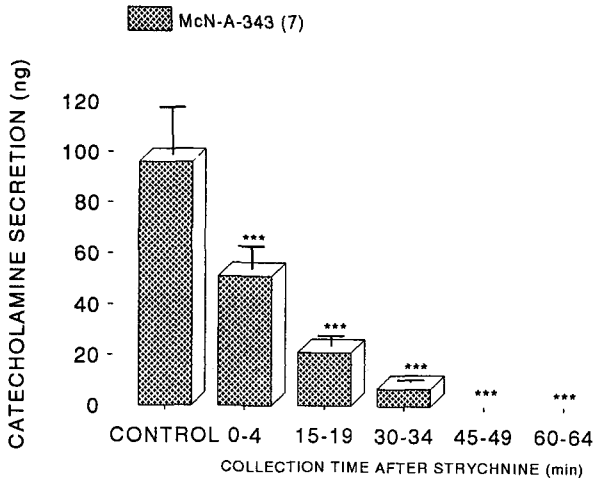


**Fig. 4.** Effects of strychnine on secretory responses of catecholamines (CA) evoked by McN-A343 from the isolated perfused rat adrenal glands. CA secretion by perfusion of McN-A-343 ($10^{-4}$ M) for 2 min was induced before (CONTROL) and after preloading with $10^{-4}$ M of strychnine for 60 min, respectively. McN-A-343-induced perfusate was collected for 4 minutes at 15 min-interval. Other legends are the same as in Fig. 2. ***: $P<0.01$.

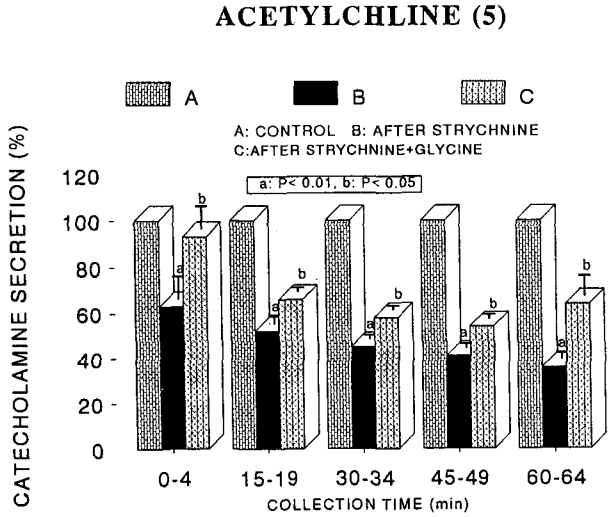


**Fig. 5.** Effects of strychnine plus glycine on CA release evoked by acetylcholine from the isolated perfused rat adrenal glands. CA secretion by a single injection of ACh ($5.32\sim10^{-3}$ M) was induced before (A) and after preloading with $10^{-4}$ M strychnine only (B) or $10^{-4}$ M strychnine + $10^{-4}$ M glycine (C) for 60 min, respectively. Ordinate: the amounts of CA secreted from the adrenal gland (% of control release). Abscissa: Collection time of perfusate (min). Statistical difference was obtained by comparing "A" with strychnine-treatment only (B), and by comparing strychnine-treatment only (B) with strychnine + glycine (C), respectively.

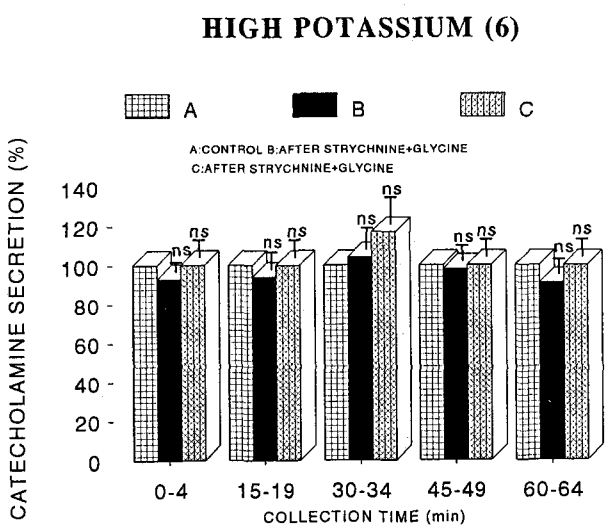


**Fig. 6.** Effects of strychnine plus glycine on CA release evoked by high $K^+$ from the isolated perfused rat adrenal glands. CA secretion by a single injection of high $K^+$ ($5.6\times10^{-2}$ M) was induced before (A) and after preloading with $10^{-4}$ M of strychnine only (B) or $10^{-4}$ M strychnine + $10^{-4}$ M glycine (C) for 60 min, respectively. Other legends are the same as in Fig. 5. ns: Statistically nonsignificant.

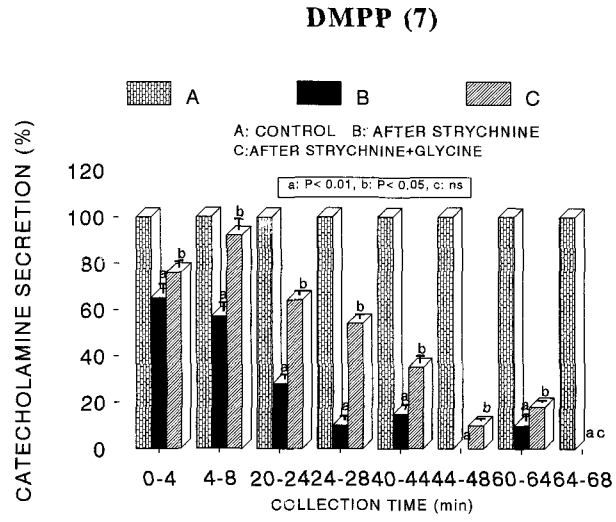


**Fig. 7.** Effects of strychnine plus glycine on CA release evoked by DMPP from the isolated perfused rat adrenal glands. CA secretion by a perfusion of DMPP ($10^{-4}$ M) for 2 min was induced before (A) and after preloading with $10^{-4}$ M strychnine only (B) or $10^{-4}$ M strychnine + $10^{-4}$ M glycine (C) for 60 min, respectively. DMPP-induced prefusats were collected consecutively twice at 4-minintervals for 8 min every 20 min. Other legends are the same as in Fig. 5.

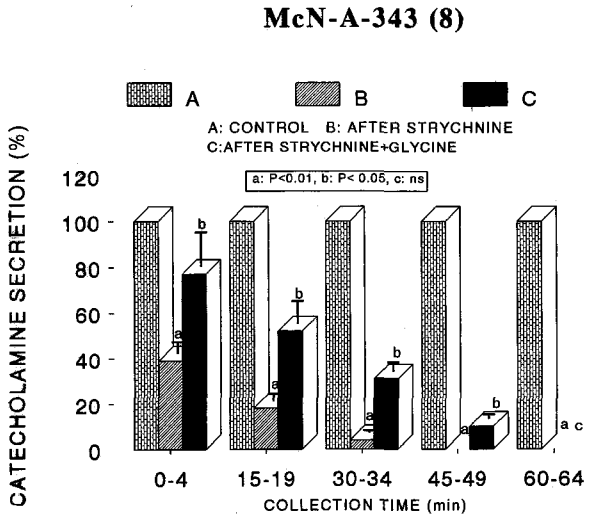


**Fig. 8.** Effects of strychnine plus glycine on CA release McN-A-343 from the isolated perfused rat adrenal glands. CA secretion by a perfusion of McN-A-343 ($10^{-4}$ M) for 2 min was induced before (A) and after preloading with $10^{-4}$ M strychnine only (B) or $10^{-4}$ M strychnine + $10^{-4}$ M glycine (C) for 60 min, respectively. Other legends are the same as in Fig. 5.

of glycine on CA secretion evoked by ACh, high $K^+$, DMPP and McN-A-343 from the isolated rat adrenal glands.

In the existence of strychnine ($10^{-4}$ M) plus glycine ($10^{-4}$ M) from 4 rat adrenal glands ACh ($5.32 \times 10^{-3}$ M)-induced CA secretory responses were recovered to 93~54% of the corresponding control response (100%). However, they were inhibited significantly to 63~36% of the control secretory response (100%) in the presence of strychnine ($10^{-4}$ M) only (Fig. 5). However, as shown in Fig. 6, in the presence of strychnine and glycine, high potassium ($5.6 \times 10$ M)-evoked Ca secretory responses were scarcely affected as compared to those cases of strychnine only-treated group. There was no difference between strychnine-treated group and control group from 6 experiments. As depicted in Fig. 7, under the existence of strychnine ($10^{-4}$ M) plus glycine ($10^{-4}$ M) from 7 rat adrenal glands, DMPP ($10^{-4}$ M)-induced CA secretory responses for first 4 min at each perfusion period were recovered considerably to 76~18% of the control secretory response (100%). Whereas they were depressed greatly to 65~0% of the control secretory response (100%) by the treatment with strychnine only. Also, DMPP ($10^{-4}$ M)-induced CA secretory responses for second 4 min at each perfusion period were recovered to 92~10% of the control release (100%) in the presence of strychnine ($10^{-4}$ M) plus glycine ($10^{-4}$ M) from 7 rat adrenal glands. However, they were also reduced to 57~0% of the control secretory response (100%) in the presence of strychnine only, except the case of last period (64~68 min) as shown in Fig. 7. In 8 rat adrenal glands, McN-A-343 ($10^{-4}$ M)-induced CA secretory responses for each perfusion period were inhibited to 39~4% of the control secretory response (100%) in the presence of strychnine only. However, in the existence of strychnine ($10^{-4}$ M) plus glycine ($10^{-4}$ M) they were recovered to 77~10% of the control secretion, except the case of the last period (60~64 min) as shown in Fig. 8.

## DISCUSSION

In the present study, the experimental results demonstrate that, strychnine inhibits CA secretory responses evoked by stimulation of cholinergic (nicotinic and muscarinic) receptors, but not by direct membrane depolarization from the isolated perfused rat adrenal glands. This inhibitory action of strychnine was considerably counteracted by the simultaneous administration of glycine. This finding suggests strongly that strychnine-sensitive glycinergic receptors may be located on the rat adrenomedullary chromaffin cells.

In support of this idea, Dar & Zinder (1995) have found that strychnine inhibits CA release evoked by ACh or nicotine from a primary culture of bovine adrenal medullary cells. Strychnine might be acting on a regulatory site of the nicotinic receptor, which is genetically similar to the strychnine-binding 48 KD submit of the glycine receptor.

Modulatoy effects of strychnine on cholinergic transmission have been reported, although it is best knows as a powerful convulsant, which acts directly on the central nervous system (Beers & Reich, 1970). There have been a growing body of evidence suggesting that the strychnine-binding 48 KD submit of the glycine receptor resembles that found in the nicotinic receptor (Grenninghloh et al, 1987), and the $GABA_A$ receptor polypeptides (Schoefield et al, 1987). Strychnine, as a potent antagonist of the glycine receptor, may thus bind to one of the nicotinic cholinergic receptor polypeptides, most probably to the subunit that shows the highest similarity to the glycine receptor. The two receptors share structural

and functional characteristics and are considered to evolve by duplication of a common ancestral gene (Noda et al, 1983; Boulter et al, 1986)

It is possible that strychnine acts by regulation of the cholinergic receptor itself, since secretion of CA evoked by secretagogue acting beyond the point of receptor activation, such as depolarization via high potassium, was not inhibited. In the present results, it has been shown that strychnine effect is both rapid and lasting, tending to confirm that strychnine action is most probably parallel to that of the neurotransmitter on the nicotinic cholinergic receptor. Supportive evidence to this result is provided by data demonstrating that strychnine effect was competitive with ACh (Dar & Zinder, 1995). Moreover, Yadid & his coworkers (1998) have reported that strychnine and glycine interact with the agonist-binding site of the nicotinic ACh receptor in the bovine adrenomedullary chromaffin cells, thus exerting a pharmacological effect that may have a modulatory role on the ACh receptors.

Glycine has an important role as a neurotransmitter in many inhibitory synapses in the mammalian central nervous system and the inhibitory response to glycine can be specifically antagonized by the plant alkaloid, strychnine, at the receptor level (Young & Snyder, 1973). Thus, strychnine has been used for localization of glycine receptor in brain tissues (Bristraw et al, 1986). It has been also reported that a high affinity [$^3$H] strychnine binding site is presented on adrenal medulla chromaffin cells (Yadid et al, 1989; 1993), a finding that supports the possibility that the high affinity binding sites for glycine are in fact receptors. Furthermore, chromaffin cells are activated by glycine to secrete CA by a strychnine-sensitive and $Ca^{2+}$-dependent mechanism (Yadid et al, 1991) in accordance with a receptor-mediated process. A strychnine-sensitive glycine receptor has been also identified in hippocampal slices and has been linked to a stimulatory role in CA secretion (Raiteri et al, 1990).

The direct effect of glycine and glycinamide on CA secretion, and the demonstration that the release occurs together with the rise in cytoplasmic glycine, suggest that these metabolite are able to traverse the plasma membrane and to exert the effect within the cell. Glycine is known to be taken up into frog CNS via an active transport mechanism (Davidoff & Adair, 1976) with two distinct saturable uptake system, and has been also shown to induce CA release from the rat brain (Schmidt & Taylor, 1990).

However, these results do not exclude the possibility that the binding of glycine to specific receptors on the plasma membranes triggers an intracellular cascade of events culminating in CA secretion. It has been shown that in adrenal medulla chromaffin cells (Yadid et al, 1989). A high affinity binding site for [$^3$H]-strychnine is presented (Curtis et al, 1971). This finding supports the evidence for the existence of glycine receptor activity (Yadid et al, 1989) on these cells similar to that seen in the brain (Young & Snyder, 1974).

The inhibition of McN-A-343-evoked CA release by strychnine can be interpreted in light of findings by Shirvan et al (1991), that in chromaffin cells, the nicotinic ACh receptor is activated by the otherwise muscarinic agonist oxotremorine. Shirvan & his colleagues (1991) have argued that nicotine and oxotremorine occupy different sites on this unique cholinergic receptor. These experimental results indicate that two receptors can not be distinguished in the present study, since DMPP and McN-A-343-evoked CA release appear to be agent equally sensitive to inhibition by strychnine.

On the other hand, Kuijpers & his coworkers (1994) have reported that the absence of an effect of glycine on the inhibitory effect of strychnine suggests that strychnine exerts its effect through a direct blockade of the nicotinic ACh receptors. Several studies have shown that at high micromolar concentrations (10～300 $\mu$M), strychnine blocks a number of ion channels in nervous tissues (Shapiro, 1977a, b; Cahalan & Almers, 1979; Yamamoto, 1986), and channels in brain and cardiac muscle (Ramos, 1974; O'Neill & Bolger, 1990). The fact that the inhibitory effect of strychnine on DMPP-evoked CA secretion is considerably counteracted by simultaneous treatment with glycine, and the fact that high $K^+$-evoked CA release is not affected, however, argues against a blockade of non-receptor coupled ion channels as the mechanism for the inhibition. The chemical similarities between the nicotine and strychnine molecules (Beers & Reich, 1970), as well as the homology of the well-characterized strychnine-binding subunits between the inhibitory glycine receptor and nicotinic ACh receptor polypeptides (Grenningloh et al, 1987) are in accordance with the results that strychnine can inhibit CA secretion evoked by stimulation of the nicotinic ACh receptors.

In conclusion, the results of the present study demonstrate that strychnine specifically inhibits DMPP

and McN-A-343-evoked CA release from the isolated perfused rat adrenal medulla, most likely by the blockade of both nicotinic and muscarinic ACh receptors through the interaction with glycinergic receptors.

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