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Influence of Staurosporine on Catecholamine Release Evoked by Cholinergic Stimulation and Membrane Depolarization from the Rat Adrenal Gland

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The present study was attempted to examine the effect of staurosporine (STS) on secretion of catecholamines (CA) evoked by cholinergic stimulation and membrane depolarization from the isolated perfused rat adrenal gland and to establish its mechanism of action. The perfusion of STS ($3\times10^{-7}\sim3\times10^{-8}$ M) into an adrenal vein for 20 min produced a dose-dependent inhibition in CA secretion evoked by ACh (5.32×10^{-3} M), high K^+ (5.6×10^{-2} M), DMPP (10^{-4} M for 2 min), McN-A-343 (10^{-4} M for 2 min), cyclopiazonic acid (10^{-5} M for 4 min) and Bay-K-8644 (10^{-5} M for 4 min). Also, in the presence of tamoxifen (2×10^{-6} M), which is known to be a protein kinase inhibitor, CA secretory responses evoked by ACh, high K^+, DMPP, McN-A-343, Bay-K-8644 and cyclopiazonic acid were also significantly depressed. However, in adrenal glands preloaded with STS (10^{-7} M) under the presence of phorbol-12, 13-dibutyrate (10^{-7} M), a specific activator of protein kinases (for 20 min), the inhibitory effect of STS on CA secretory responses evoked by ACh, high K^+, DMPP, McN-A-343, Bay-K-8644 and cyclopiazonic acid was greatly recovered to the extent of the control release as compared to those in the presence of STS only. These results demonstrate that STS causes the marked inhibition of CA secretion evoked by stimulation of cholinergic (both nicotinic and muscarinic) receptors as well as by membrane depolarization, indicating strongly that this effect may be mediated by inhibiting influx of extracellular calcium and release in intracellular calcium in the rat adrenomedullary chromaffin cells through preventing activation of protein kinases. Furthermore, these findings also suggest that these STS-sensitive protein kinases play a modulatory role partly in regulating the rat adrenomedullary CA secretion.

Key Words: Staurosporine, Catecholamine secretion, Protein kinase, Adrenal gland

INTRODUCTION

It has been known that staurosporine (STS), an alkaliod with antifungal activity produced by *Streptomyces staurosporeus* (Omura et al, 1977), is widely used to study the roles of protein kinases in cellular function. Although STS was thought initially to be a specific inhibitor of protein kinase C (Tamaoki et al, 1986), it inhibits many protein kinases, presumably by binding to the ATP binding site of these enzymes (Nakadate et al, 1988; Ruegg & Burgess, 1989). It also affects intracellular Ca^{2+} mobilization (Himpens et al, 1993; Wong et al, 1992) and Ca^{2+} homeostasis (Cheng et al, 1994).

Maurer & McKay (1992a; 1994) have reported that STS induces a time-dependent reduction in catecholamine (CA) secretion from cultured bovine adre-

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nal chromaffin cells. In permeabilized chromaffin cells, higher concentrations of free Ca^{2+} (2~20 μM) appear to overcome the inhibitory effect of STS on Ca^{2+}-stimulated CA release (Maurer & McKay, 1994). These results indicate that the drug treatment interferes with distal steps of the exocytotic process. Moreover, Maurer & his coworkers (1995) have shown that STS treatment reduces basal and A23187-stimulated CA release from the cultured bovine chromaffin cells, but does not inhibit Ca^{2+} influx. Some previous studies have shown that acute treatment with STS has little or no effect on CA release stimulated by depolarizing concentrations of KCl (TerBush & Holz, 1990; Bunn & Boyd, 1992) while STS does reduce KCl-stimulated CA secretion. However, this effect is time-dependent and occurs only after a relatively prolonged pretreatment with the alkaloid (Maurer & McKay, 1992a; 1992b). Moreover, Isosaki & his coworkers (1991) have found that protein kinase C plays a modulatory role in CA secretion from the digitonin-permeabilized bovine adrenal medullary cells rather than being essential for initiating CA secretion.

In porcine carotid medial smooth muscle, STS also attenuated contractions and phosphorylation of myosin light chain induced by phorbol-12,13-dibutyrate, histamine and KCl (Singer, 1990). Oxytocin- and endothelin-induced contractions were also reduced by STS (Karibe et al, 1990; Ohlstein et al, 1984; Danthuluri & Brock, 1990). It has been also shown that STS is a relatively specific inhibitor of protein kinase C in intact porcine arteries at lower concentrations (Kageyama et al, 1991). However, at higher concentrations it may have actions unrelated to its inhibitory effect of protein kinase C, which includes the inhibition of calcium influx into smooth muscle cells through the voltage-dependent Ca^{2+} channel (Kageyama et al, 1991). There is little report so far whether STS influences CA release from the perfused adrenal model. In the present study, therefore, it was attempted to investigate the effect of STS on CA secretion from the isolated perfused rat adrenal gland and to establish the mechanism of action.

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 midline 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 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 ISCO pump (WIZ Co.) at a rate of 0.33 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±0.05. The solution contained disodium EDTA (10 μg/ml) and ascorbic acid (100 μg/ml) to prevent oxidation of CA.

Drug administration

The perfusions of DMPP (10^{-4} M for 1min) and McN-A-343 (10^{-4} M for 4 min) and/or a single injection of ACh (5.32×10^{-3} M) and KCl (5.6×10^{-2} M) in a volume of 0.05 ml were made into perfusion stream via a three way stopcock, respectively. Bay-K-8644 (10^{-5} M) and cyclopiazonic acid (10^{-5} M) were

also perfused for 4 min, respectively.

In the preliminary experiments, it was found that upon administration of the above drugs, secretory responses to ACh, KCl, McN-A-343, Bay-K-8644 and cyclopiazonic acid returned to preinjection level in about 4 min, but the responses to DMPP in 8 min.

Collection of perfusate

As a rule, prior to stimulation with various secretagogues, the 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 perfusates was continued in another tube as soon as the perfusion medium containing the stimulatory agent reached the adrenal gland. Stimulated sample's perfusate was collected for 4 to 8 min. The amounts secreted in the background sample have been subtracted from that secrected from the stimulated sample to obtain the net secretion value of CA, which is shown in all of the figures.

To study effects of STS on the spontaneous and evoked secretion, the adrenal gland was perfused with Krebs solution containing STS for 20 min, then the perfusate was collected for a certain period (background sample). 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 (Anton & Sayre, 1962) without the intermediate purification with 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 CA content in the perfusate of stimulated glands by secretagogues used in the present work was high enough to obtain readings several folds greater than the reading of control samples (unstimulated). The sample blanks were also lowest for perfusates of stimulated and non-stimulated samples. 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 represent statistically significant changes 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 and Murray (1987).

Drugs and their sources

The following drugs were used

Acetylcholine chloride, 1.1-dimethyl-4-phenyl piperazinium iodide (DMPP), norepinephrine bitartrate, methyl-1, 4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate (BAY-K-8644) (Sigma Chemical Co., U.S.A.), phorbol-12,13-dibutyrate, tamoxifen, cyclopiazonic acd, 3-(m-cholrophenyl-carbamoyl-oxy)-2butynyl trimethyl ammonium chloride [McN-A-343] (RBI, U.S.A.). Drugs were dissolved in distilled water (stock) and added to the normal Krebs solution as required except phorbol-12, 13-dibutyrate, tamoxifen and Bay-K-8644, which were dissolved in 99.5% ethanol and diluted appropriately (final concentration of alcohol was less than 0.1%). Concentrations of all drugs used are expressed in terms of molar base.

RESULTS

Effect of STS on CA secretion evoked by ACh, excess K^+, DMPP, McN-A-343, Bay-K-8644 and cyclopiazonic acid 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 ± 2.8 ng for 2 min (n=9). The pretreatment with STS in cultured bovine adrenal chromaffin cells has reduced basal and calcium ionophore-induced CA secretion, but not inhibited the activated Ca^{2+} homeostasis (Maurer et al, 1995). Therefore, it was decided initially to examine the effects of STS on cholinergic receptor stimulation- as well as membrane depolarization-mediated CA secretion from perfused rat adrenal glands. Secretagogues were given at 20 to 30 min-

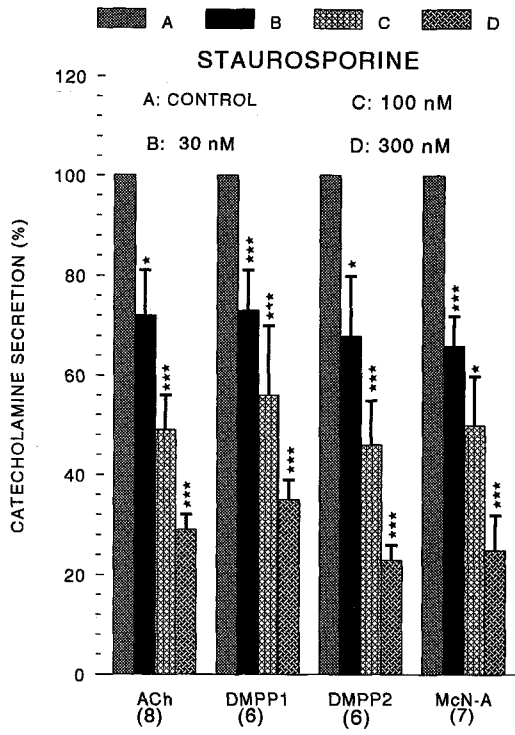

Fig. 1. Dose-dependent effects of staurosporine on secretory responses of catecholamines (CA) evoked by Ach, DMPP and McN-A343 from the isolated perfused rat adrenal glands. CA secretion evoked by ACh, DMPP (10^{-4} M) and McN-A-343 (10^{-4} M) was induced before (A) and after preloading with 3×10^{-8} M (B), 10^{-7} M (C), 3×10^{-7} M (D) of staurosporine for 20 min, respectively. Numbers in the parenthesis indicate 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 (% of control). Abscissa: secretagogues. Statistical difference was obtained by comparing the corresponding control (A) with each concentration of staurosporine-pretreated group (B, C, and D). DMPP-induced perfusates were collected twice successively for 4 minutes, respectively (DMPP-1; 0~4 min, DMPP-2; 4~8 min) but ACh- or McN-A-343-induced perfusate only for 4 minutes. ACh: acetylcholine, McN-A: McN-A-343. *: $P<0.05$, ***: $P<0.01$

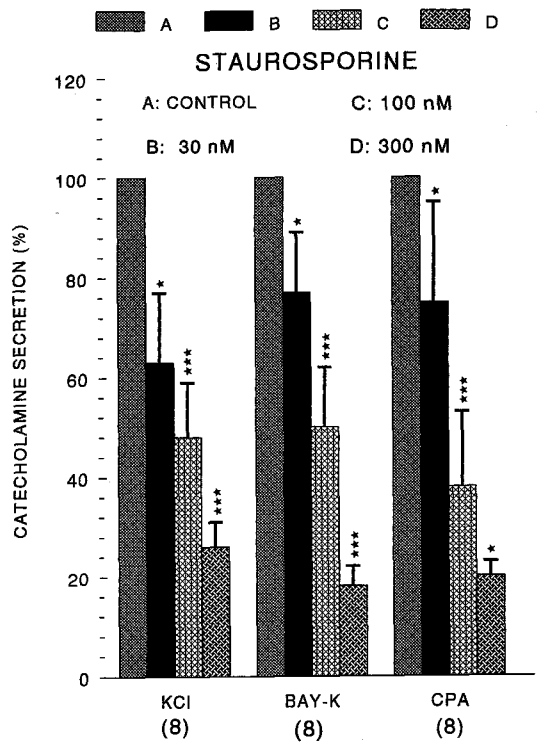

Fig. 2. Dose-dependent effects of staurosporine on CA secretory responses evoked by excess K^+, Bay-K-8644 and cyclopiazonic acid from the rat adrenal glands. Bay-K-8644 (10^{-5} M) and cyclopiazonic acid (10^{-5} M) were perfused into an adrenal vein for 4 min and excess K^+ (5.6×10^{-2} M) was injected before (A) and after preloading with 3×10^{-8} M (B), 10^{-7} M (C), 3×10^{-7} M (D) of staurosporine for 20 min, respectively. The perfusate was collected for 4 minutes, respectively. Other legends are as in Fig. 1. Bay-K: Bay-K-8644, CPA: Cyclopiazonic acid. *: $P<0.05$, ***: $P<0.01$

intervals. STS was present 20 min before stimulation with secretagogue. In the present study, it was found that STS itself did not produce any effect on basal CA.

When ACh (5.32×10^{-3} M) in a volume of 0.05 ml was injected into the perfusion stream, the amounts of CA secreted was 969 ± 234 ng for 4 min. However, the pretreatment with STS inhibited significantly ACh-stimulated CA secretion in a concentration-dependent manner in the range of $3\times10^{-8}\sim3\times10^{-7}$ M for 20 min from 8 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^+ (5.6×10^{-2} M)-stimulated CA secretion after the pretreatment with STS was markedly inhibited as compared with its corresponding control secretion (100%) from 8 glands (Fig. 2).

When perfused through the rat adrenal gland, DMPP (10^{-4} M for 1 min), which is a selective nicotinic receptor agonist in autonomic sympathetic ganglia, evoked a sharp and rapid increase in CA secretion. As shown in Fig. 1, DMPP-stimulated CA secretion for both periods (0~4 min and 4~8 min) after pretreatment with STS was reduced greatly in 6 rat adrenal glands. As illustrated in Fig. 1, McN-A-

343 (10^{-4} M), which is a selective muscarinic M1-agonist (Hammer & Giachetti, 1982), perfused into an adrenal gland for 4 min caused an increased CA secretion from 7 glands. However, McN-A-343-stimulated CA secretion in the presence of STS was markedly inhibited as compared to the corresponding control secretion.

Since Bay-K-8644 is known to be a calcium channel activator which enhances basal Ca^{2+} uptake (Garcia et al, 1984) and CA release (Lim et al, 1992), it was of interest to determine the effects of STS on Bay-K-8644-stimulated CA secretion from the isolated perfused rat adrenal glands. Fig. 2 illustrates the inhibitory effect of STS on Bay-K-8644- and cyclopiazonic acid-evoked CA secretory responses. Bay-K-8644 (10^{-5} M)-stimulated CA secretion under the presence of STS was strikingly depressed as compared to the corresponding control release.

Cyclopiazonic acid, a mycotoxin from *Aspergillus* and *Penicillium*, has been described as a highly selective inhibitor of Ca^{2+}-ATPase in skeletal muscle sarcoplasmic reticulum (Goeger & Riley, 1989; Seidler et al, 1989). The inhibitory action of STS on cyclopiazonic acid-evoked CA secretory response was observed as shown in Fig. 2. Under the effect of STS in 8 rat adrenal glands, cyclopiazonic acid (10^{-5} M)-evoked CA secretion was reduced as compared to the control response.

Effect of tamoxifen on CA secretion evoked by ACh, excess K^+, DMPP, McN-A-343, Bay-K-8644 and cyclopiazonic acid from the perfused rat adrenal glands

In the previous experimental results as shown in Fig. 1~2, it was found that STS showed a dose-dependent inhibition in CA secretory responses. Since it has been known that tamoxifen inhibits Ca^{2+}- and phospholipid-dependent protein kinase C (O'Brian et al, 1985; 1986; Su et al, 1985), therefore, it is of great interest to examine the effect of tamoxifen on CA secretion evoked by various secretagogues.

CA release evoked by ACh (5.32×10^{-3} M) and excess K^+ (5.6×10^{-2} M) after preloading with tamoxifen (2×10^{-6} M) for 20 min amounted to $47\pm8\%$ ($P<0.01$, n=12) and $42\pm9\%$ ($P<0.01$, n=6), respectively as compared to each corresponding control secretion (100%) as shown in Fig. 3 and 4. DMPP (10^{-4} M)- and McN-A-343 (10^{-4} M)-stimulated CA releases after preloading with tamoxifen were also

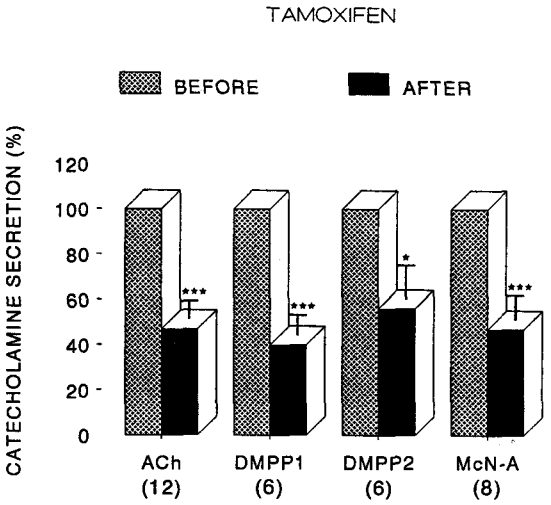

Fig. 3. Effect of tamoxifen on CA release evoked by ACh, DMPP- and McN-A-343. CA secretion evoked by ACh (5.32×10^{-3} M), Perfusion of DMPP (10^{-4} M) and McN-A-343 (10^{-4} M) for 2 min was induced before and after preloading with tamoxifen (2×10^{-6} M) for 20 min, respectively. Other legends are as in Fig. 1. *: $P<0.05$, ***: $P<0.01$

significantly reduced as shown in Fig. 3.

As shown in Fig. 4, in the presence of tamoxifen (2×10^{-6} M), the CA secretory response by cyclopiazonic acid (10^{-5} M) given into the adrenal gland was reduced to $65\pm15\%$ ($p<0.05$) as compared to the corresponding control response (100%) from 8 experiments. Also, Bay-K-8644-stimulated CA secretion under the effect of tamoxifen was strikingly depressed as compared to the corresponding control release; thus, the release was reduced to $58\pm20\%$ ($P<0.05$, n=8) of the control secretion (Fig. 4).

Effects of staurosporine plus phorbol-12, 13-dibutyrate on CA release evoked by ACh, excess K^+, DMPP, McN-A-343, Bay-K-8644 and cyclopiazonic acid from the perfused rat adrenal glands

Since phorbol esters have been found to facilitate CA release evoked by various stimuli, such as nicotine, excess potassium and calcium ionophore, in bovine chromaffin cells (Brocklehurst et al, 1985; Pocotte et al, 1985; Brocklehurst & Pollard, 1986) and in anesthetized dog (Suzuki et al, 1994), we tried to determine the effect of STS in the presence of phorbol-12,13-dibutyrate on CA secretion evoked by various secretagogues from the isolated rat adrenal glands. When given into an adrenal vein in a volume

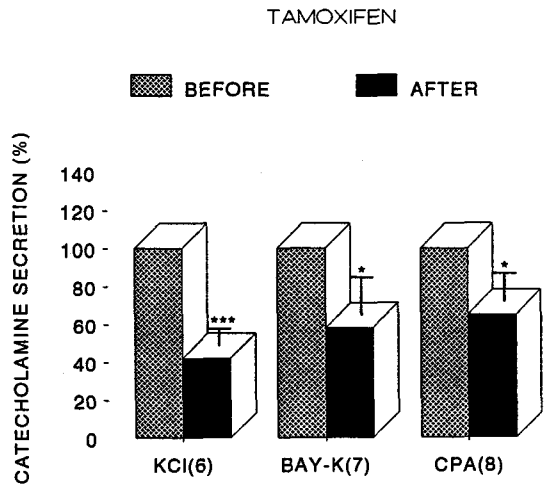

Fig. 4. Influence of tamoxifen on CA release evoked by excess K^+, Bay-K-8644 and cyclopiazonic acid. Bay-K-8644 (10^{-5} M) and cyclopiazonic acid (10^{-5} M) were perfused into an adrenal vein for 4 min and Excess K^+ (5.6×10^{-2} M) was injected "BEFORE" and "AFTER" preloading with of tamoxifen (2×10^{-6} M) for 20 min, respectively. Other legends are as in Fig. 1. CPA: cyclopiazonic acid. *: $P<0.05$, ***: $P<0.01$

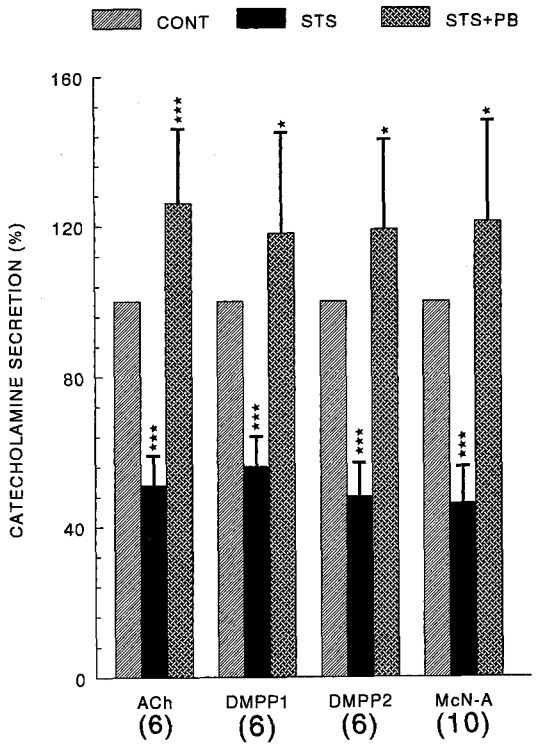

Fig. 5. Effect of staurosporine plus phorbol-12, 13-dibutyrate on CA release evoked by ACh, DMPP and McN-A-343. A single injection of ACh (5.32×10^{-3} M) and perfusion of DMPP (10^{-4} M) and McN-A (10^{-4} M) for 2 min were induced before and after preloading with 10^{-7} M STS only or 10^{-7} M STS plus 10^{-7} M PB for 20 min, respectively. Statistical difference was obtained by comparing CONT with STS-treatment only, and by comparing STS-treatment only with STS+PB, respectively. Other legends are as in Fig. 1. CONT: control. STS: staurosporine, PB: Phorbol-12, 13-dibutyrate, McN-A: McN-A-343. *: $P<0.05$, ***: $P<0.01$.

of 0.05 ml, ACh (5.32×10^{-3} M)- and excess K^+ (5.6×10^{-2} M)-induced CA releases in the presence of STS (10^{-7} M) along with phorbol-12,13-dibutyrate (10^{-7} M) were considerably recovered to 126±20% ($P<0.01$, n=6) and 81±13% ($P<0.05$, n=10) of their control secretion (100%), respectively as compared to their secretory responses of 51±8% and 44±9% of their controls in the presence of STS (10^{-7} M) only (Fig. 5 & 6). As depicted in Fig. 5, CA secretions under the existence of STS plus phorbol-12,13-dibutyrate were also greatly recovered to 118±27% (0~4 min, $P<0.05$, n=6) and 119±24% (4~8 min, $P<0.05$, n=6) in response to DMPP and 121±27% (0~4 min, $P<0.05$, n=10) in response to McN-A-343 of their corresponding control responses, respectively as compared to the secretory responses of 56±8% (0~4 min) and 48±9% (4~8 min) to DMPP, and 46±10% (0~4 min) to McN-A-343 of the control in the presence of STS (10^{-7} M) only.

CPA (10^{-5} M)- and Bay-K-8644 (10^{-5} M)-induced CA secretory responses after preloading with Krebs solution containing STS (10^{-7} M) along with phorbol-12,13-dibutyrate (10^{-7} M) for 20 min amounted to 104±16% ($P<0.01$, n=6) and 113±19% ($P<0.05$, n=6) of each corresponding control (100%), respectively as compared to the secretory responses of 35±7% and 40±12% of the controls in the presence of STS only as shown in Fig. 6.

DISCUSSION

These experimental results demonstrate that STS inhibits CA secretion evoked by cholinergic stimulation and membrane depolarization from the isolated perfused rat adrenal gland in a dose-dependent manner. This inhibitory effect of STS may be mediated by the direct inhibition of extracellular Ca^{2+} influx and intracellular Ca^{2+} release in the rat adrenomedullary chromaffin cells, which seems to be associated

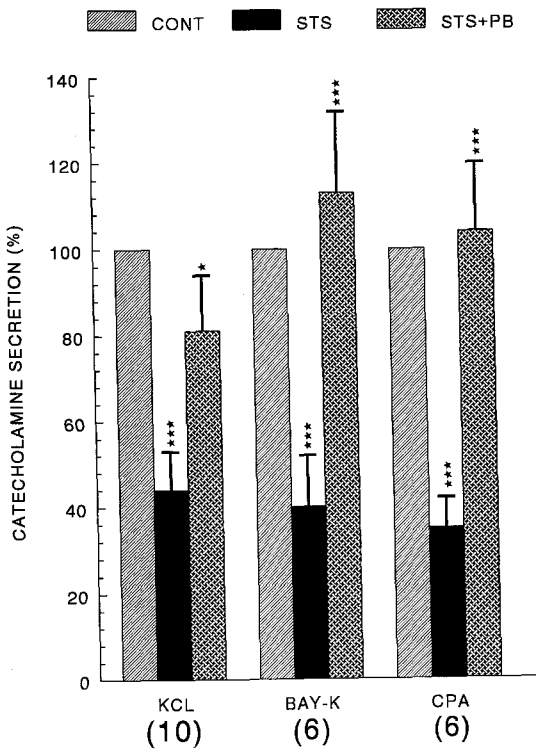

Fig. 6. Effect of staurosporine plus phorbol-12, 13-dibutyrate on CA release evoked by excess K^+, Bay-K-8644 and cyclopiazonic acid. Excess K^+ (5.6×10^{-2} M), Bay-K-8644 (10^{-5} M) and cyclopiazonic acid (10^{-5} M) were induced before and after preloading with 10^{-7} M STS only or 10^{-7} M STS plus 10^{-7} M PB for 20 min, respectively. Other legends are the same as in Fig. 1. CONT: control. STS: staurosporine, PB: Phorbol-12, 13-dibutyrate, BAY-K; Bay-K-8644, CPA; cyclopiazonic acid. *: $P<0.05$, ***: $P<0.01$.

at least with inhibition of protein kinases.

The present data seem to be similar to the previous results that STS inhibits CA release stimulated by 10 μM nicotine, depolarizing concentration of potassium (56 mM), and 2 mM $BaCl_2$ in cultured bovine adrenal chromaffin cells (Maurer & McKay, 1992a, b). In the present study, the finding that STS reduced KCl, DMPP, McN-A-343 and ACh-evoked CA release in a concentration-dependent fashion, suggests strongly that the same phenomenon underlies these effects. Because CA secretion evoked by DMPP, McA-A-343 and high KCl as well as by ACh was inhibited greatly by the pretreatment of STS, stimulus-secretion coupling appears to be uncoupled at some steps distal to cell depolarization. It is also known that STS reduces BaCl2-evoked CA release in cultured bovine adrenal chromaffin cells (Morita et al, 1990; Maurer et al, 1995). Maurer et al (1995) have suggested that regardless of the exact mechanism by which Ba^{2+} stimulates CA secretion, STS must interfere with pathway at some step at, or distal to Ba^{2+} entry (at or after the opening of the voltage-sensitive Ca^{2+} channels). However, Morita & his colleagues (1990) have found that the stimulatory action of Ba^{2+} on CA biosynthesis in the bovine chromaffin cells is mediated by the activation of protein kinase C because Ba^{2+}-induced CA formation is inhibited significantly by the protein kinase C inhibitor, polymyxin B. In view of these findings, in the present investigation, STS-induced inhibitory effect on CA secretions evoked by cholinergic stimulation and high K^+ was considerably recovered to the control level of each corresponding secretagogue when both STS and phorbol-12, 13-dibutyrate were perfused simultaneously into the adrenal gland. This result suggests that STS suppresses the CA secretory responses through inhibition of protein kinase C. Furthermore, in support of this possibility, several in vitro studies in bovine adrenal chromaffin cells demonstrated that phorbol esters (protein kinase activators) stimulate CA secretion (Brocklehurst et al, 1985; Pocotte et al, 1985; Brocklehurst & Pollard, 1986; Suzuki et al, 1994), and the stimulatory effects are abolished by protein kinase inhibitors (Tamaoki et al, 1986; Isosaki et al, 1991; Suzuki et al, 1994). Thus, the results of the present study obtained under in vitro condition are consistent with the in vivo observations showing that inhibition of protein kinase C causes inhibitory responses on stimulus (secretagogue)-induced CA secretion.

The main purpose of the present study was to investigate possible involvement of protein kinases in the action of STS on CA release from the perfused adrenal gland in responses to cholinergic (nicotinic and muscarinic) stimulation and membrane depolarization. For this purpose, two inhibitors, STS and tamoxifen were used in the present experiments. Perfusion of STS into the rat adrenal gland had no effect on basal CA release, whereas this drug attenuated greatly CA releasing effects evoked by DMPP and McN-A-343 as well as high potassium and ACh in a concentration-dependent fashion. These results suggest that protein kinase is activated by cholinergic stimulation and raise the possibility that activated protein kinase C may play a facilitatory role in CA release evoked by various secretagogues. The assumption that protein kinase C is activated by nico-

tinic stimulation is supported by results of several studies dealing with the effects of nicotinic agonists on exocytotic events such as translocation of protein kinase C to membrane (as an index of protein kinase activation), calcium influx, protein phosphorylation, and CA secretion in bovine chromaffin cells (Pocotte et al, 1985; TerBush & Holz, 1986; Terbush et al, 1988). Suzuki & his coworkers (1994) have shown that protein kinase C activated by nicotinic stimulation during splanchnic nerve stimulation probably modulates CA release not only by affecting a nicotinic receptor-dependent release pathway but by stimulating release through a nicotinic receptor-independent pathway. Moreover, in the present study, the finding that tamoxifen-treatment also depressed CA secretory responses evoked by DMPP, McN-A-343 and ACh as well as high K^+ supports the inhibitory effect of STS. Tamoxifen has been also known to inhibit Ca^{2+} and phospholipid-dependent protein kinase (O'Brian et al, 1985; 1986; Su et al, 1985; Issandou et al, 1990).

Contrary to the present results, acute pretreatment ($\leq$20 min) with relatively high concentrations ($\geq$1 M) of STS was shown to produce no significant change (Bunn & Boyd, 1992) or slight reduction (TerBush & Holz, 1990) in KCl-stimulated CA secretion from cultured bovine adrenal chromaffin cells. Some findings have been reported that protein kinase inhibitor, sphingosine inhibits Ca^{2+}-dependent secretion from chromaffin cells after prolonged (Burgoyne et al, 1988), but not acute (Bader et al, 1989) treatment. In view of these previous results using cultured bovine chromaffin cells, the present data indicate that STS-induced inhibitory action is considerably rapid. Tachikawa & his coworkers (1990) have reported that polymyxin B, a selective inhibitor of protein kinase C, completely inhibits CA secretion from cultured cell induced by ACh, high K^+ medium, 12-O-tetradecanoylpherbol-13-acetate, or Ca^{2+}-ionophore ionomycin. These findings indicate that activation of protein kinase C may participate in Ca^{2+}-dependent CA secretion from chromaffin cells.

In the present study, the result that STS as well as tamoxifen inhibited CA secretory response evoked by Bay-K-8644, a selective L-type Ca^{2+}-channel activator, suggest strongly that STS inhibits CA secretion stimulated by cholinergic secretagogues through inhibition of Ca^{2+} uptake into the rat adrenal chromaffin cells. In support of this idea, Nakanishi & his colleagues (1989) have shown that ML-9, a preferential inhibitor of myosin light-chain kinase, also inhibits CA secretion evoked by high K^+, veratridine or palytoxin. They are known to activate the voltage-dependent Ca^{2+} channel and initiate the CA secretion from the cultured bovine adrenal chromaffin cells. Therefore, similarly as in the present study, STS appears to suppress the functions of both cholinergic receptor-mediated Ca^{2+} channels and voltage-dependent Ca^{2+} channels and thereby to inhibit CA secretion. Kageyama & his coworkers (1991) have found that STS is a relatively specific inhibitor of protein kinase C in intact porcine coronary arteries at lower concentrations. At higher concentrations, it may have action unrelated to its inhibitory effect of protein kinase C, which include the inhibition of Ca^{2+} influx into smooth muscle cells through the voltage-dependent Ca^{2+} channel. In the light of these findings, the present study indicates that STS as well as tamoxifen inhibits cholinergic receptor-mediated and voltage-dependent Ca^{2+} channels and thereby also depresses CA release from the rat adrenal gland.

Besides, to study the effect of STS on the intracellular Ca^{2+}-dependent mechanism, the CA secretory response evoked by cyclopiazonic acid, which is known to be highly selective inhibitor of Ca^{2+}-ATPase and to increase intracellular free Ca^{2+} concentration, was examined in the presence of STS. In this experiment, STS inhibited significantly cyclopiazonic acid-evoked CA secretory response in a dose-dependent fashion. This result indicates that STS may inhibit the release of intracellular Ca^{2+} from its store as well as influx of extracellular Ca^{2+}. In support of this possibility, some investigators have found that STS inhibited the contractile response to KCl to the some extent as that to phorbol-12,13-dibutyrate from the rabbit aorta (Ohlstein et al, 1984) and swine carotid medial smooth muscle (Singer, 1990). They have suggested that an increase in intracellular Ca^{2+} induced by KCl leads to activation of protein kinase C, which is inhibited by STS. However it seems that there are some differences in opinion in relation to the effect of STS on mobilization of intracellular Ca^{2+}. In the porcine coronary arteries, it has been shown that STS suppresses the phorbol-12,13-dibutyrate-induced contraction even at concentrations that it did not affect the increase in intracellular Ca^{2+} induced by 90 mM KCl (Kageyama et al, 1991). Kageyama & his coworkers (1991) have shown that STS (10^{-7} M) had no effect on the caffeine-sensitive intracellular Ca^{2+} release in porcine coronary arteries,

although it slightly attenuated the caffeine-induced contraction. However, in the present study, STS as well as tamoxifen inhibited cyclopiazonic acid-evoked CA secretion. This finding suggests that STS-sensitive protein kinase is involved in intracellular Ca^{2+} mobilization in the rat adrenomedullary chromaffin cells.

In conclusin, These results, taken together, demonstrate that STS causes the marked inhibition of CA secretion evoked by cholinergic stimulation as well as by membrane depolarization. This inhibitory effect may be mediated by inhibiting influx of extracellular calcium and release of intracellular calcium in the rat adrenomedullary chromaffin cells by preventing activation of protein kinases. Furthermore, these findings suggest also that these STS-sensitive protein kinases play a modulatory role at least partly in regulating the rat adrenomedullary CA secretion.

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