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Effect of Doxorubicin on Catecholamine Release in the Isolated Perfused Rat Adrenal Gland

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The present study was undertaken to investigate the effect of doxorubicin (DX) on secretion of catecholamines (CA) evoked by ACh, high K^+, DMPP and McN-A-343 from the isolated perfused rat adrenal gland and to establish the mechanism of its action. DX (10^{-7}~10^{-6} M) perfused into an adrenal vein for 60 min produced relatively dose- and time-dependent inhibition of CA secretory responses evoked by ACh (5.32×10^{-3} M), DMPP (10^{-4} M) and McN-A-343 (10^{-4} M). However, lower dose of DX did not affect CA secretion by high K^+ (5.6×10^{-2} M), but its higher doses depressed time-dependently CA secretion evoked by high K^+. DX itself did also fail to affect basal CA output. In adrenal glands loaded with DX (3×10^{-7} M), CA secretory responses evoked by Bay-K-8644, an activator of L-type Ca^{2+} channels and cyclopiazonic acid, an inhibitor of cytoplasmic Ca^{2+}-ATPase were time-dependently inhibited. Furthermore, daunorubicin (3×10^{-7} M), given into the adrenal gland for 60 min, attenuated CA secretory responses evoked by ACh, high K^+, DMPP and McN-A-343. Taken together, these results suggest that DX causes relatively dose- and time-dependent inhibition of CA secretory responses evoked by stimulation of cholinergic (both nicotinic and muscarinic) receptors from the isolated perfused rat adrenal gland. However, lower dose of DX did not affect CA secretion by high K^+, and higher doses of DX reduced time-dependently CA secretion of high K^+. It is thought that these effects of DX may be mediated by inhibiting both influx of extracellular calcium into the rat adrenomedullary chromaffin cells and intracelluar calcium release from the cytoplasmic store. Also, there was no difference in the mode of action between DX and daunorubicin in rat adrenomedullary CA secretion.

Key Words: Doxorubicin, Daunorubicin, Catecholamine secretion, Adrenal gland

INTRODUCTION

Doxorubicin (DX, adriamycin) is an anthracycline that is a highly effective chemotherapeutic agent used largely in the treatment of solid tumors (Singal & Iliskovic, 1998; Feldman et al, 2000; Slamon et al, 2001). In cardiac cells, DX is metabolized to the corresponding semiquinone free radical by flavin reductase (Boveris, 1977; Bachur et al, 1977). This or a related species induces apoptosis in cardiomyocytes and is prevented by free radical-scavengers (Della-Torre, 1996) or by chelating iron (Sparano, 1988).

Bounias and his coworkers (1997) have shown that catecholamines (CA) including epinephrine, norepinephrine and dopamine, and DOPA enhance the generation of hydroxyl radicals by chemotherapeutic antibiotics (DX, farmorubicin and mitomycin C). It has also been found that in closed-chest pure-bred beagles infused with DX into coronary artery, the plasma norepinephrine concentration as well as plasma natriuretic peptide levels were greatly increased (Toyoda et al, 1998). Some previous studies also suggested that CA could play a role in DX-induced cardiotoxicity. Increased circulating and intracardiac CA levels have been reported in experimental animals treated with DX or daunorubicin, a closely related anthracycline (Bristow et al, 1979, Bristow et al, 1981; Soldani et al, 1981). Moreover, at a lower concentration (3×10^{-6} M), DX facilitated CA secretion induced by acetylcholine and 51 mM K^+ from the bovine adrenal medulla. However, a higher concentration of DX resulted in a significant and irreversible inhibition of spontaneous CA of secretion as well as that by acetylcholine or high K^+ (Pinto et al, 1987), suggesting that these increased blood CA levels could probably be originated from adrenal medulla.

In contrast to these results, Robison & Giri (1987) reported that plasma CA and myocardial guanylate cyclase activity at 14 weeks after treatment with DX were unchanged. In acute and chronic studies in rabbits treated with DX, myocardial CA levels were also unchanged

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ABBREVIATIONS: DX, doxorubicin; CA, catecholamines; ACh, acetylcholine; DMPP, 1.1-dimethyl-4-phenyl piperazinium iodide; Bay-K-8644, methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate; McN-A-343, (3-(m-chloro-phenyl-carbamoyl-oxy)-2-butynyl trimethyl ammonium chloride.

(Jackson et al, 1984). Chen & his coworkers (1987) found that in the rat isolated Langendorff perfused heart, CA did not seem to have a major role in the development of the early cardiotoxicity induced by equimolar concentrations of some anthracycline compounds. On the other hand, it has been shown that chronic adriamycin treatment rather inhibits neuronal exocytotic release of CA at the cardiac sympathetic nerve terminals of the rabbits (Kawada et al, 2000). Thus, there is much controversy about CA release evoked by DX, especially in relation to DX-induced cardiotoxicity. Therefore, the present study was undertaken to investigate the effect of DX on secretion of CA in the isolated perfused model of the rat adrenal glands and to elucidate its action mechanism.

METHODS

Experimental procedure

Male Sprague-Dawley rats, weighing 180 to 250 grams, were anesthetized intraperitoneally with thiopental sodium (40 mg/kg). 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 retractor. 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. Before ligating vessels and cannulations, heparin (400 IU/ml) was injected into vena cava to prevent blood coagulation. 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. The adrenal gland, along with ligated blood vessels and the cannula, was then carefully removed from the animal and placed on a platform of a leucite chamber. The chamber was continuously circulated with water heated at $37 \pm 1°C$.

Perfusion of adrenal gland

The adrenal glands were perfused by means of a peristaltic pump (WIZ Co.) at a rate of 0.3 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 pH of the solution was maintained at 7.4 ~ 7.5. The solution contained disodium EDTA (10 μg/ml) and ascorbic acid (100 μg/ml) to prevent oxidation of CAs.

Drug administration

The perfusions of DMPP (100 μM) and McN-A-343 (100 μM) for 2 minutes, and Bay-K-8644 (10 μM) and cyclopiazonic acid (10 μM) for 4 minutes were made into perfusion stream, respectively. A single injection of ACh (5.32 mM) and KCl (56 mM) in a volume of 0.05 ml was injected 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, McN-A-343, Bay-K-8644 and cyclopiazonic acid returned to pre-injection level in about 4 min, but the responses to DMPP in 8 min.

Collection of perfusate

Prior to stimulation with various secretagogues, perfusate was routinely collected for 4 min to determine spontaneous secretion of CA (background sample). Immediately after the collection of the background sample, the perfusates were continuously collected 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 that secreted from the stimulated sample to obtain the net secretion value of CA, which is shown in all of the figures.

To study the effects of DX on the spontaneous and evoked secretion, the adrenal gland was perfused with Krebs solution containing DX for 60 min immediately after the perfusate was collected for a certain minute (background sample). And the medium was then 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 fluorospectrophotometrically (Kontron Co. Italy) measured directly by the fluorometric method of Anton & Sayre (1962) without intermediate purification on alumina for the reasons described earlier (Wakade, 1981).

A volume of 0.2 ml perfusate was used for the reaction. The CA content in the glands perfusate stimulated by secretogagues in the present work was high enough to obtain several folds greater readings than that of control samples (unstimulated). The sample blanks were also the 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 utilizing 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 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

Doxorubicin hydrochloride, 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), and daunorubicin hydrochloride were purchased from Sigma Chemical Co., U.S.A. Cyclopiazonic acid and (3-(m-

chloro-phenyl-carbamoyl-oxy)-2-butynyl trimethyl ammonium chloride [McN-A-343] were purchased from RBI Co., U.S.A. Drugs were dissolved in distilled water (stock) and added to the normal Krebs solution as required except Bay-K-8644, which was 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 doxorubicin (DX) on CA secretion in the perfused rat adrenal glands evoked by ACh, high K^+, DMPP and McN-A-343

After the initial perfusion with oxygenated Krebs-bicarbonate solution for 1 hr, basal CA release from the isolated perfused rat adrenal glands amounted to 21 ± 2.3 ng/2 min (n=8). It has earlier been shown that, at a lower concentration (3×10^{-6} M), DX facilitated CA secretion from the bovine adrenal medulla induced by acetylcholine and 51 mM K^+. However, a higher concentration of DX resulted in a significant and irreversible inhibition of the spontaneous secretion of CA as well as that by acetylcholine or high K^+ (Pinto et al, 1987). Therefore, it was decided initially to examine the effects of DX on CA secretion from perfused rat adrenal glands evoked by cholinergic receptor stimulation as well as membrane depolarization. Secretagogues were given at 15 to 20 min-intervals, and DX was introduced for 60 min after obtaining the control secretory response of each secretagogue. In the present study, it was found that DX itself did not affect basal CA output (data not shown).

When ACh (5.32×10^{-3} M) in 0.05 ml volume was injected into the perfusion stream, the amount of CA secreted was 295 ± 20 ng for 4 min. However, pretreatment of 10 adrenal glands with DX in the range of $10^{-7} \sim 10^{-6}$ M for 20 min significantly inhibited ACh-stimulated CA secretion to ~53% of the control response in a concentration-dependent manner (Fig. 1). Also, it has earlier been found that depolarizing agent such as KCl stimulates sharply CA secretion, however, in the present work, excess K^+ (5.6×10^{-2} M)-stimulated CA secretion in the presence of lower concentration of DX was not affected. But at higher concentration it was significantly inhibited to ~71% of the corresponding control secretion (132 ± 9 ng for 0~4 min) from 6 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 (507 ± 44 ng for 0~8 min). As shown in Fig. 3, DMPP-stimulated CA secretion after pretreatment with DX was greatly reduced to 64% of the control secretion in 8 rat adrenal glands. As illustrated in Fig. 4, McN-A-343 (10^{-4} M), which is a selective muscarinic M1-agonist (Hammer and Giachetti, 1982), perfused into an adrenal gland for 4 min increased CA secretion (107 ± 9 ng for 0~4 min) from 8 glands. However, McN-A-343-stimulated CA secretion in the presence of pinacidil was markedly inhibited to ~61% of the corresponding control secretion.

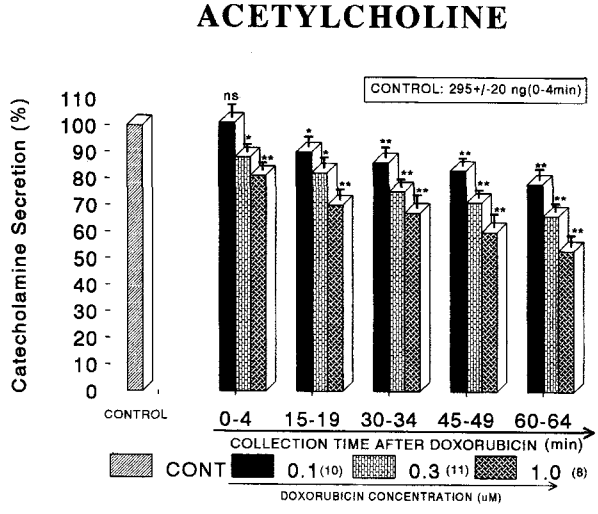

Fig. 1. Dose-dependent effect of doxorubicin on the secretory responses of catecholamines (CA) evoked by acetylcholine (ACh) from the isolated perfused rat adrenal glands. CA secretion by a single injection of ACh (5.32×10^{-3} M) in a volume of 0.05 ml was evoked at 15 min intervals after preloading with 0.1, 0.3 and 1.0 μM of doxorubicin for 60 min as indicated at an arrow mark, respectively. Numbers in the parenthesis indicate number of rat adrenal glands. Vertical bars on the columns represent the standard error of the mean (S.E.M.). Ordinate: the amounts of CA secreted from the adrenal gland (% of control, 295 ± 20 ng for 4 min). Abscissa: collection time of perfusate (min). Statistical difference was obtained by comparing the corresponding control (CONT) with each concentration-pretreated group of doxorubicin. ACh-induced perfusate was collected for 4 minutes. *: $P<0.05$, **: $P<0.01$. ns: not statistically significant.

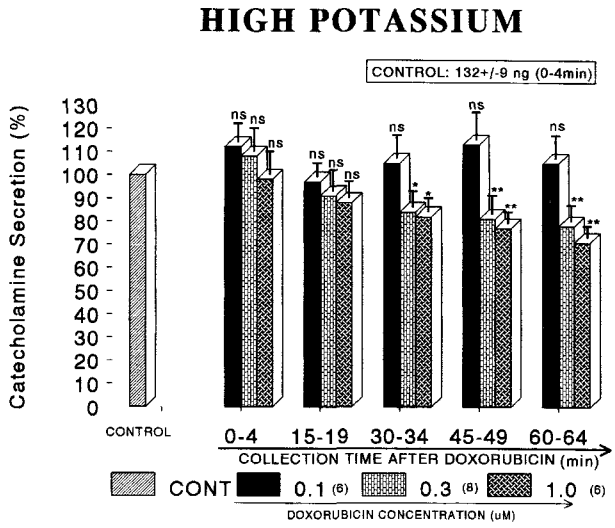

Fig. 2. Dose-dependent effect of doxorubicin on the secretory responses of catecholamines (CA) evoked by high K^+ from the isolated perfused rat adrenal glands. CA secretion by a single injection of K^+ (56 mM) was injected in a volume of 0.005 ml at 15 min intervals after preloading with 0.1, 0.3, and 1.0 μM of doxorubicin for 60 min, respectively. Statistical difference was obtained by comparing the corresponding control (CONT, 132 ± 9 ng for 4 min) with each concentration-pretreated group of doxorubicin. K^+-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. *: $P<0.05$, **: $P<0.01$. ns: not statistically significant.

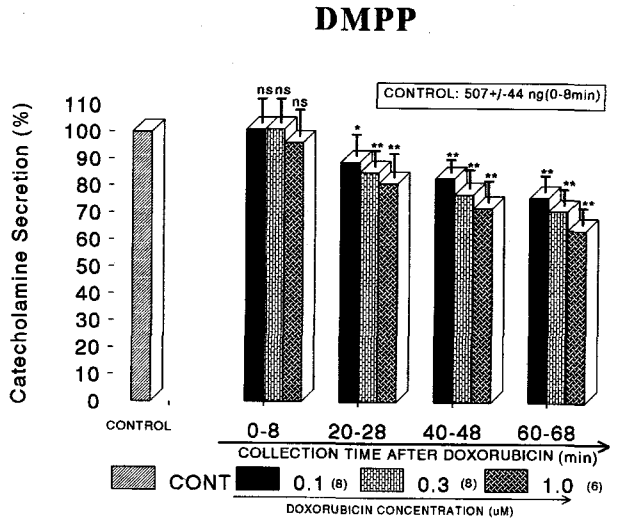

Fig. 3. Dose-dependent effect of doxorubicin on the secretory responses of catecholamines (CA) evoked by DMPP from the isolated perfused rat adrenal glands. CA secretion by a single injection of DPPP (10^{-4} M) was infused for 2 min at 20 min intervals after preloading with 0.1, 0.3 and 1.0 μM of doxorubicin for 60 min, respectively. Statistical difference was obtained by comparing the corresponding control (CONT, 507±44 ng for 8 min) with each concentration-pretreated group of doxorubicin. DMPP-induced perfusate was collected for 8 minutes. Other legends are the same as in Fig. 2. *: $P<0.05$,**: $P<0.01$.ns: not statistically significant.

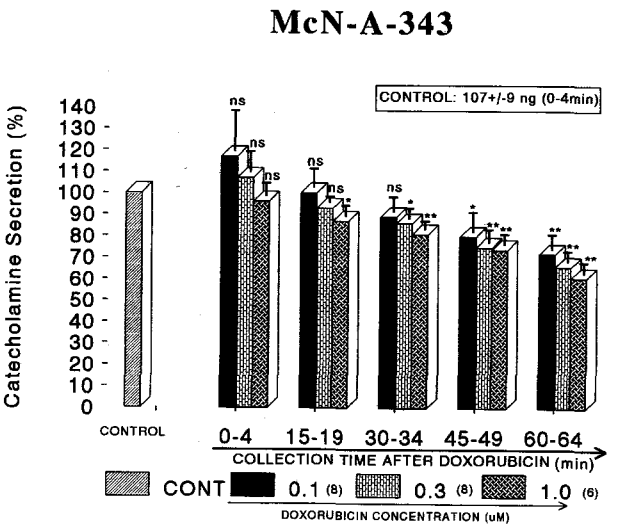

Fig. 4. Dose-dependent effect of doxorubicin on the secretory responses of catecholamines (CA) evoked by McN-A-343 from the isolated perfused rat adrenal glands. CA secretion by a single injection of McN-A-343 (10^{-4} M) was infused for 4 min at 15 min intervals after preloading with 0.1, 0.3 and 1.0 μM of doxorubicin for 60 min, respectively. Statistical difference was obtained by comparing the corresponding control (CONT, 107±9 ng for 4 min) with each concentration-pretreated group of doxorubicin. McN-A-343-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. *: $P<0.05$, **: $P<0.01$. ns: not statistically significant.

Effect of doxorubicin (DX) on CA secretion in the perfused rat adrenal glands evoked by Bay-K-8644 and cyclopiazonic acid

It has been found that Bay-K-8644 is a calcium channel activator that causes positive inotropy and vasoconstriction in isolated tissues and intact animals (Schramm et al, 1982; Wada et al, 1985), and enhances basal Ca^{2+} uptake (Garcia et al, 1984) and CA release (Lim et al, 1992). Therefore, it was of interest to examine the effects of DX on Bay-K-8644-evoked CA secretion from the isolated perfused rat adrenal glands. Fig. 5 shows the inhibitory effect of 3×10^{-7}

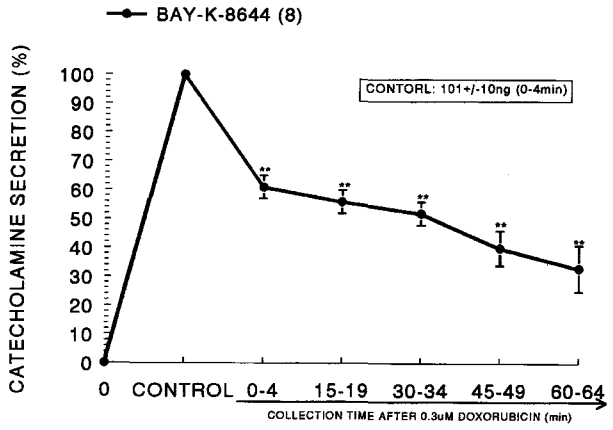

Fig. 5. Effects of doxorubicin on CA release evoked by Bay-K-8644 from the rat adrenal glands. Bay-K-8644 (10^{-5} M) was perfused into an adrenal vein for 4 min at 15 min intervals after preloading with doxorubicin (0.3 μM) for 60 min, respectively. Statistical difference was obtained by comparing the corresponding control (CONT, 101±10 ng for 4 min) with each period after pretreatment with doxorubicin. Other legends are the same as in Fig. 2. **: $P<0.01$.

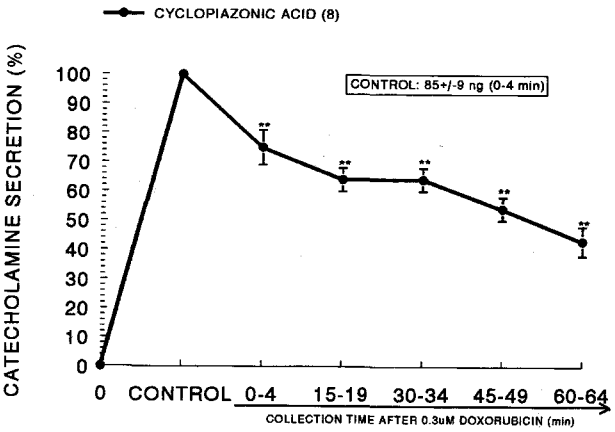

Fig. 6. Effects of doxorubicin on CA release evoked by cyclopiazonic acid from the rat adrenal glands. Cyclopiazonic acid (10^{-5} M) were perfused into an adrenal vein for 4 min at 15 min intervals after preloading with doxorubicin (0.3 μM) for 60 min, respectively. Statistical difference was obtained by comparing the corresponding control (CONT, 85±9 ng for 4 min) with each period after pretreatment with doxorubicin. Other legends are the same as in Fig. 2. **: $P<0.01$.

M DX on Bay-K-8644-evoked CA secretory responses. In the absence of DX, Bay-K-8644 (10^{-5} M) given into the perfusion stream evoked CA secretion of 117±12 ng (0~4 min) from 8 rat adrenal glands. However, in the presence of 3×10^{-7} M DX, Bay-K-8644-stimulated CA secretion was inhibited to ~33% of 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 (Georger & Riley, 1989; Seidler et al, 1989), and is an extremely valuable pharmacological tool for investigating intracellular Ca^{2+} mobilization and ionic current regulated by intracellular calcium (Suzuki et al, 1992). As shown in Fig. 6, in the presence of 3×10^{-7} M DX from 8 adrenal glands, cyclopiazonic acid (10^{-5} M)-evoked CA secretion was depressed to ~43% of the control response (93±10 ng, 0~4 min).

Effect of daunorubicin on CA secretion in the perfused rat adrenal glands evoked by ACh, excess K^+, DMPP and McN-A-343

As shown earlier in Fig. 1~4, DX was found to inhibit dose-dependently CA secretory responses evoked by cholinergic secretagogues and membrane depolarization. Since it has been known that daunorubicin is also one of the

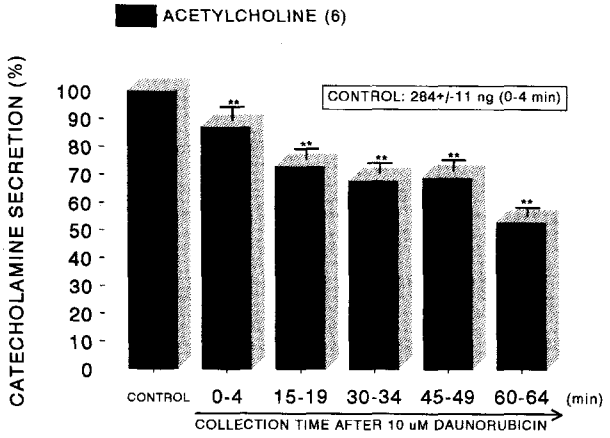

Fig. 7. Effects of daunorubicin on the secretory responses of catecholamines (CA) evoked by acetylcholine (ACh) from the isolated perfused rat adrenal glands. CA secretion by a single injection of ACh (5.32×10^{-3} M) was induced before (CONTROL) and after preloading with 10 μM daunorubicin for 60 min, respectively. Perfusates were collected for 4 minutes at 15 min-intervals, respectively. Other legends are the same as in Fig. 2. **: $P<0.01$.

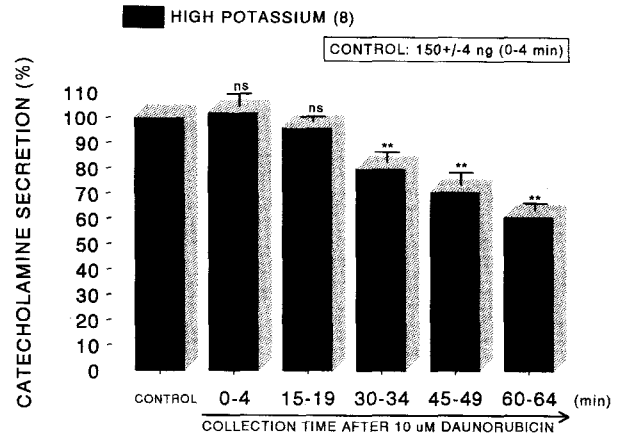

Fig. 8. Effects of daunorubicin on the secretory responses of catecholamines (CA) evoked by high potassium from the isolated perfused rat adrenal glands. CA secretion by a single injection of high K^+ (5.6×10^{-2} M) was induced before (CONTROL) and after preloading with 10 μM daunorubicin for 60 min, respectively. Perfusates were collected for 4 minutes at 15 min-intervals, respectively. Other legends are the same as in Fig. 2. **: $P<0.01$, ns: not statistically significant.

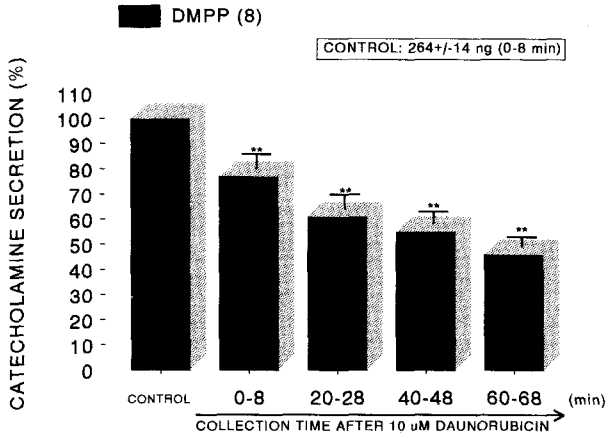

Fig. 9. Effects of daunorubicin on the secretory responses of catecholamines (CA) evoked by DMPP from the isolated perfused rat adrenal glands. CA secretion by perfusion of DMPP (10^{-4} M) for 2 min was induced before (CONTROL) and after preloading with 10 μM daunorubicin for 60 min, respectively. DMPP-induced perfusates were collected for 8 minutes at 20 min intervals, respectively. Other legends are the same as in Fig. 2. **: $P<0.01$.

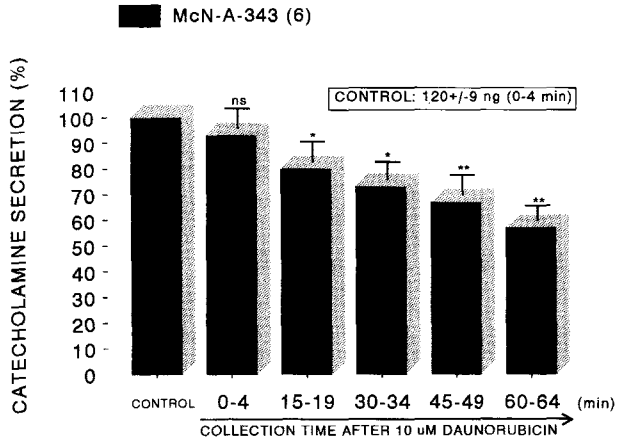

Fig. 10. Effects of daunorubicin on the secretory responses of catecholamines (CA) evoked by McN-A343 from the isolated perfused rat adrenal glands. CA secretion by perfusion of McN-A343 (10^{-4} M) for 2 min was induced before and after preloading with 10 μM daunorubicin for 60 min, respectively. DMPP-induced perfusates were collected for 4 minutes at 15 min intervals, respectively. Other legends are the same as in Fig. 2. *: $P<0.05$, **: $P<0.01$, ns: not statistically significant.

anthracycline antibiotics derived from the fungus *Streptococcus peucetius var. caesius* (Hardman et al, 2001), it was of interest to examine the effect of daunorubicin on CA secretion evoked by cholinergic secretagogues and membrane depolarization.

CA release evoked by ACh (5.32×10^{-3} M) after preloading with daunorubicin (10^{-5} M) was time-dependently reduced to ~53% of the corresponding control secretion (284±11 ng, 0~4 min) (Fig. 7). Also, in the presence of daunorubicin (10^{-5} M), excess K^+ (5.6×10^{-2} M) amounted to ~61% of the corresponding control secretion (150±4 ng, 0~4 min) (Fig. 8). As shown in Fig. 9, DMPP (10^{-4} M)-evoked CA release following the pretreatment with daunorubicin (10^{-5} M) was inhibited to ~46% of the control secretion (364±14 ng, 0~8 min). McN-A-343 (10^{-5} M)-stimulated CA releases after preloading with daunorubicin (10^{-5} M) were also time-dependently reduced to ~57% of the corresponding control release (Fig. 10).

DISCUSSION

The present experimental results strongly demonstrate that DX, an anthracyclinic cytotoxic drug, causes relatively dose- and time-dependent inhibition of CA secretory responses from the isolated perfused rat adrenal gland evoked by stimulation of cholinergic (both nicotinic and muscarinic) receptors. However, low dose of DX did not affect CA secretion by high K^+, and high doses of DX reduced time-dependently CA secretion of high K^+. It is suggested that these effects of DX may be mediated by both inhibiting influx of extracellular calcium into the rat adrenomedullary chromaffin cells and release of intracelluar calcium from the cytoplasmic store.

In support of this idea, Pinto & his coworkers (1987) showed that a higher concentration of DX (10^{-4} M) resulted in a significant and irreversible inhibition of spontaneous secretion of CA, as well as that caused by ACh or high K^+ from the bovine adrenal medulla. High concentration of DX did not modify the CA release induced by NaCl deprivation. These results suggested that DX effects could be mediated at the plasma membrane of the chromaffin cells. The present results that DX greatly inhibited the CA secretory responses evoked by DMPP as well as by ACh suggest that this inhibitory effect of DX may be mediated by the direct blockade of neuronal nicotinic receptors in the perfused rat adrenal medulla.

Moreover, it has also been demonstrated that 4hr treatment with colchicine, vinblastine, tubulozole, podophyllotoxin and taxol has similar inhibitory effects on the nicotinic ACh receptor (nAChR)-mediated adrenal CA release from the bovine adrenal chromaffin cells in culture (Gu et al, 1994). However, it had no effects on secretion stimulated by other secretagogues that operate by non-nAChR mechanisms, thus demonstrating the selectivity of the antimitotic drugs to nAChR-stimulated release. Therefore, the site of action of these drugs appears to be at a step before depolarization and the opening of the voltage sensitive ion channels. This would suggest that all of antimitotic drugs act at the level of the adrenal nAChR. McKay & Schneider (1984) reported similar conclusion with taxol and vinblastine. Based on these findings, the present results that daunorubicin also inhibited the CA secretory response evoked by nicotinic stimulation with DMPP as well as ACh suggest that this inhibitory effect of DX may be mediated through the direct blockade of neuronal nicotinic receptors in the perfused rat adrenal medulla. Daunorubicin is also known to be an anthracyclinic antibiotic, just like DX (Hardman et al, 2001). Also, Lopez & McKay (1997) demonstrated that vincristine, tubulozole, podophyllotoxin, and demecolcine inhibited nAChR-stimulated CA release noncompetitively and in a concentration-dependent manner from the cultured bovine adrenal chromaffin cells. These studies indicate an association of adrenal nicotinic ACh receptors with microtubules, and suggest that the mechanism, by which the antimitotic drugs interfere with the signal transduction pathway, is by inducing dissociation of nAChRs from the microtubular network (Lopez & McKay, 1997). Furhtermore, it seems that these high concentrations of DX can also produce direct cardiotoxic effects, such as an acute depression of chronotropic and inotropic activity (Villani et al, 1978; Politi et al, 1985), a nonspecific -adrenergic blocking effect (Politi et al, 1985), as well as other toxic actions, such as free radical production and membrane lipid damage (Bachur et al, 1977; Facchinetti et al, 1982).

However, on contrary to the above, DX at 3×10^{-6} M concentration facilitated the secretory response from the cultured bovine adrenal chromaffin cells induced by ACh and 56 mM K^+, but did not affect the spontaneous CA output or that evoked by NaCl deprivation (Pinto et al, 1987). Since lower concentration of the drug sensitizes the adrenal medulla to physiologic stimuli (ACh, depolarization), there is a good reason to believe that the medulla may become supersensitive to these stimuli and overreact to them in the presence of DX. DX administration can increase CA release to the circulation by a direct action on the adrenal medulla, and high blood CA concentration in turn can in part account for the atrial and ventricular tachyarrhythmias frequently observed after DX treatment (Essesse et al, 1980; Signori & Guevara, 1981). This hypothesis is supported by the partial protection against DX-induced cardiotoxicity by pretreatment with-adrenoceptor blocking drugs (Bristow et al, 1979). Moreover, DX increased both ACh- and high K^+-evoked CA release from isolated adrenal glands. Both stimuli are calcium-dependent (Pinto et al, 1983). Conversely, DX failed to modify the secretory response to NaCl deprivation, a calcium-independent mechanism (Lastowecka & Trifaro, 1974). These results suggested that the action of DX on the adrenal gland could probably be located at the plasma membrane level. In the light of this fact, the present results that DX rather inhibited CA release evoked by Ach, DMPP and high K^+ from the rat adrenal medulla are not in agreement with this finding. In the present study, DX at concentrations less than 50 nM never affected the CA secretion evoked by ACh, high K^+ and DMPP (data not shown). Therefore, there might be species difference in reactivity between animals used.

In the present study, the results that DX as well as daunorubicin inhibited CA secretion evoked by stimulation of muscarinic receptors with McN-A-343, a selective muscarinic M_1-receptor agonist, strongly suggested that DX could be involved in the regulation of overall secretory responses evoked by muscarinic stimulation. In support of this hypothesis, it has been shown that muscarinic stimulation generates a depolarizing signal which triggers the firing of action potentials, thereby resulting in the incresed CA release in the rat chromaffin cells (Akaike et al, 1990; Lim & Hwang, 1991). These observations are in agreement

with a previous report showing that Bay-K-8644 almost triples the peak secretory response to muscarine in perfused Ca^{2+} cat adrenal glands (Ladona et al, 1987; Uceda et al, 1992). In the present experiment, DX also depressed greatly CA secretion induced by Bay-K-8644, which is found to potentiate the release of CA by increasing Ca^{2+} influx through L-type Ca^{2+} channels in chromaffin cells (Garcia et al, 1984). This finding that DX inhibited CA secretion evoked by high K^+ and also by Bay-K-8644 suggest that this DX inhibits directly the voltage-dependent Ca^{2+} channels, similar to Ca^{2+} channel blockers (Cena et al, 1983), which have direct actions on voltage-dependent Ca^{2+} channels. In the bovine chromaffin cells, stimulation of nicotinic, but not muscarinic ACh receptors is known to cause CA secretion by increasing Ca^{2+} influx largely through voltage-dependent Ca^{2+} channels (Oka et al, 1979; Burgoyne, 1984). Thus, it seems that these anthracyclinic antibiotics inhibit DMPP-evoked CA secretion by inhibiting Ca^{2+} influx through voltage-dependent Ca^{2+} channels activated by nicotinic ACh receptors with DMPP.

In the present study, it has also been shown that DX inhibited the increase of CA secretion evoked by cyclopiazonic acid. Cyclopiazonic acid is known to be a highly selective inhibitor of Ca^{2+}-ATPase in skeletal muscle sarcoplasmic reticulum (Georger & Riley, 1989; Seidler et al, 1989) and a valuable pharmacological tool for investigating intracellular Ca^{2+} mobilization and ionic currents regulated by intracellular Ca^{2+} (Suzuki et al, 1992). Therefore, it is felt that the inhibitory effect of DX on CA secretion evoked by cholinergic stimulation as well as by membrane-depolarization may be associated with the mobilization of intracellular Ca^{2+} in the adrenomedullary chromaffin cells. This suggests that DX inhibits he release of Ca^{2+} from the intracellular pools induced by stimulation of muscarinic ACh receptors, which is partly responsible for the secretion of CA. It has been shown that Ca^{2+}-uptake into intracellular storage sites susceptible to caffeine (Ilno, 1989) is almost completely abolished by treatment with cyclopiazonic acid during the proceeding Ca^{2+} load (Suzuki et al, 1992). This is consistent with the findings obtained in skinned smooth muscle fibers of the longitudinal layer of the guinea-pig ileum, where Ca^{2+}-uptake was also inhibited by cyclopiazonic acid (Uyama et al, 1992). Suzuki & his coworkers (1992) have shown that cyclopiazonic acid easily penetrates into the cytoplasm through the plasma membrane and reduces Ca^{2+}-ATPase activity in sarcoplasmic/endoplasmic reticulum, resulting in increase of the subsequent Ca^{2+} release from those storage sites and thereby increase of Ca^{2+}-dependent K^+-current. Moreover, in bovine adrenal chromaffin cells, stimulation of muscarinic ACh receptors is also proposed to activate phosphoinositide metabolism, resulting in the formation of inositol 1,4,5-trisphosphate, which induces Ca^{2+} mobilization from the intracellular pools (Cheek et al, 1989; Challis et al, 1991). However, it is uncertain in the present study whether the inhibitory effect of the DX on Ca^{2+} movement from intracellular pools was due to their direct effect on the PI response or not.

Uceda & his coworkers (1992) have reported that intracellular Ca^{2+}-dependent K^+ channels, probably of the small-conductance type (SK), seem to be involved in the modulation of muscarinic-evoked CA release responses in cat adrenal chromaffin cells. However, in the present study, the fact that McN-A-343-evoked CA secretion was depressed by pretreatment with DX appeared to be consistent with these previous results. Furthermore, it has also been found that, in the absence of extracellular Ca^{2+}, methacholine still evoked a transient Ca^{2+} rise that declined quickly to basal levels, suggesting the release of Ca^{2+} from an intracellular pool likely associated with the smooth endoplasmic reticulum in cat chromaffin cells (Uceda et al, 1994). In line with this observation are facts that muscarinic stimulation of bovine chromaffin cells increases the formation of inositol trisphosphate (Forsberg et al, 1986) and that inositol trisphosphate mobilizes Ca^{2+} in permeabilized cells (Fohr et al, 1991). A similar rise of intracellular Ca^{2+} by muscarinic stimulation, even in the absence of extracellular Ca^{2+}, has been demonstrated in bovine chromaffin cell suspensions (Kao & Schneider, 1985; 1986; Kim & Westhead, 1989) and in cat chromaffin cells (Sorimachi et al, 1992). Furthermore, previous evidence has shown that high concentrations of anthracyclines can also reduce myocardial calcium availability through different mechanisms, such as Ca^{2+} chelation (Hofling & Bolte, 1981), reduction of plasma membrane permeability for this cation or inhibition of transmembrane Ca^{2+} movements (Villani et al, 1978; Villani et al, 1986). Thus, it is suggested that similar mechanisms could also play a role in the depression of adrenomedullary CA release at high DX concentraions.

In conclusion, the above described results suggest that DX causes relatively dose- and time-dependent inhibition of CA secretory responses evoked by stimulation of cholinergic (both nicotinic and muscarinic) receptors from the isolated perfused rat adrenal gland. However, lower dose of DX do not affect CA secretion by high K^+, and higher dose reduces time-dependently CA secretion of high K^+. It is suggested that these DX effects may be mediated by inhibiting influx of extracellular calcium into the rat adrenomedullary chromaffin cells and intracelluar calcium release from the cytoplasmic store. There seems to be no difference in the mode of action between doxorubicin and daunorubicin in rat adrenomedullary CA secretion.

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