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The Effects of Cisplatin on Gastric Motility

in the Rat

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Major professor

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THE EFFECTS OF CISPLATIN ON GASTRIC MOTILITY IN THE RAT

By

James Dominic San Antonio

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ABSTRACT

THE EFFECTS OF CISPLATIN ON GASTRIC MOTILITY IN THE RAT

By

James Dominic San Antonio

Cisplatin (cis-diamminedichloroplatinum II) was injected intraperitoneally (i.p.) into rats at 7 mg/Kg to study the effects of the drug on gastric motility. In vitro, fundal strips from drug-treated rats contracted normally in Tyrodes solution, but were hypercontractile to acetylcholine and serotonin in calcium-free Tyrodes solution. Histochemical acetylcholinesterase localization studied by light microscopy revealed normal enzyme levels and distribution in drug-treated gastric muscle. ^{3}H-Choline was injected i.p. into rats and its uptake into the gastric tissues was studied at 5 minutes, 3 and 12 hour intervals by scintillation counting and autoradiography. Results show an increased ^{3}H-choline uptake in drug-treated stomachs. Based on the results from muscle contractility and acetylcholinesterase experiments, it is proposed that cisplatin causes a non-deviation type supersensitive response in gastric muscle.

To my Parents

ACKNOWLEDGEMENTS

Special thanks go to Dr. Aggarwal for serving as my research advisor, and faithful racquetball companion. I also thank Dr. R. Pax and Dr. C. C. Chou for their valuable assistance throughout this project. Appreciation also goes to Mary Lynn Bajt, for her help with running some of the experiments, and for her advice concerning the preparation of this manuscript.

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INTRODUCTION

Cisplatin (cis-diamminedichloroplatinum II) is a potent anti-cancer drug used in clinics since 1972, primarily for the treatment of testicular and ovarian tumors (29). The drug is effective against a broad spectrum of tumors when used alone (29), but has proven to be more effective in combination chemotherapy (7). Though the biological activity of cisplatin is poorly understood (29), it is thought to form intra and inter-strand DNA crosslinks (38), thereby interfering with DNA replication in cells. The drug may also interfere with tumor cell division through disruption of microfilamental organization (1).

Cisplatin is not free from its toxic side effects which include kidney damage, nausea and vomiting, deafness, peripheral neuropathy (10), hypomagnesemia (29) and hypocalcemia (28,30). The most severe dose limiting side effects are kidney toxicity, and nausea and vomiting (29). Kidney toxicity can be reduced to tolerable levels by slow infusion of the drug (9), hydration, and diuresis of the patient (9,28). The nausea and vomiting is only slightly decreased if antiemetic drugs like metaclopromide and dropreridol are administered (29). Because of the severity

of the nausea and vomiting, cisplatin treatment has to be discontinued for some patients (37). It is therefore imperative that these toxic side effects be controlled. The present study is an effort to better understand the cisplatin-induced nausea and vomiting, using the rat as a model system.

When rats are injected with therapeutic dose levels of cisplatin and are sacrificed three days later, the stomach is extremely swollen and filled with food (27). The appearance of the stomach suggests that there has been an inhibition of peristalsis, which is the contractile activity providing the force for food propulsion and gastric emptying (20). Gastric peristalsis results from endogenous rhythmic contractions of smooth muscle, with amplitude and frequency modified by acetylcholine release from postganglionic parasympathetic neurons (20). The acetycholine release is controlled by stimulation from the vagus nerve, intramural reflex arcs, and hormone action (20). Peristaltic inhibition due to cisplatin could therefore be exerted by direct action on the smooth muscle, or by affecting the nervous or endocrine modulation of muscle activity.

Aggarwal et. al. (3) have shown that stomach smooth muscle strips from cisplatin-treated rats in vitro exhibit hypercontractility to acetylcholine (ACh), indicating that the muscle is supersensitive to this agent. It is not known whether the observed supersensitivity to ACh is of the deviation or the non-deviation type. The deviation type

response in cholinergic systems is only elicited by ACh, and this altered state occurs primarily through inhibition of acetylcholinesterase activity (35,36). Contrary to deviation-type supersensitivity, the non-deviation response can be elicited by a wide array of pharmacologically unrelated drugs, and is common to smooth muscle which has been deprived of normal activity through blockage of neuromuscular interactions (36).

In the present studies, attempts were made to characterize cisplatin-induced smooth muscle supersensitivity using muscle bath experiments and an acetylcholinesterase localization test. The effects of cisplatin on the acetylcholine metabolism of enteric nerves was studied by measuring ^{3}H-choline uptake and its metabolism in gastric tissues.

MATERIALS AND METHODS

In each experiment inbred male Wistar rats (Charles River Breeding Lab, Willington, MA) ranging from 2 to 6 months of age were used, all being from 2 or 3 sibling groups, and all of approximately equal age and weight (250-500 g). Rats were weighed daily for the duration of the experiment, starting a day before injections. Animals were assigned to control or experimental groups so that each group had the same number of rats and a roughly equal weight distribution. Cisplatin (Johnson Matthey Research Laboratories, U.K.) was dissolved in normal saline by gently heating the solution, and animals were injected intraperitoneally (i.p.) with a single dose of 7 mg/Kg. Control animals received comparable saline injections. Animals were given laboratory rat chow and water ad lib., except when specified, and when possible each cage housed both the control and drug-treated rats in equal numbers. On day 3 after injection animals were sacrificed by decapitation or cervical dislocation.

Muscle Bath Experiments

Rats were starved, but given water ad lib. starting one day before injection and until 3 days after, when they were sacrificed. The starvation was necessary because it

decreased the stomach bloating which occurs in cisplatin-treated rats which are fed ad lib. After sacrificing, stomachs were removed and placed in warm Tyrodes solution. A smooth muscle strip from each stomach was prepared according the the method of Vane (33), as follows. The fundic and pyloric regions of the stomach were separated by cutting along the dividing line between the regions with a scissors. The fundus of the stomach was cut along the lesser curvature, opened and rinsed, then cuts spaced 0.5 cm apart were made parallel to the longitudinal muscle layer. A 5-6 cm strip was obtained by unfolding the fundic preparation, and from this a strip 3 cm long was cut. One end of the strip was tied with surgical thread to a glass rod tissue holder, the other end was tied with a thread to which a small metal hook was attached. The tissue and the glass holder were placed into the muscle bath which contained 15 ml of Tyrodes solution at 37°C into which a 95% O_2 and 5% CO_2 mixture was continually bubbled. The free end of the muscle strip was connected to a myograph transducer by the threaded hook, and muscle contractions were recorded isotonically on a multichannel physiograph (A&M Instruments, Houston, TX).

The muscle strip was allowed to equilibrate for 10 min under a resting tension of 6 g; during this period no readings were made. After 10 minutes the experiments were begun by washing the bath. This wash, and all subsequent ones consisted of 3 quick rinses with Tyrodes followed by a

bath refill. In all experiments the following procedures were the same. During the periods when spontaneous contractility of the muscle was recorded, the bath was washed every 10 minutes. At the time when drugs were to be added, the bath was washed, and since the muscle usually contracted at this time it was allowed to reach baseline on the physiograph. Twenty-five seconds after reaching baseline 1 ml of Tyrodes containing acetylcholine or serotonin was added to the bath with a pre-calibrated pipette. The muscle response was recorded for 1.5 min, then the bath was rinsed. Subsequent drug additions were done the same way. Dose response curves to acetylcholine and serotonin were obtained for all strips tested, and the maximal response was arbitrarily chosen to be 38 mg contractile force. Drugs were mixed in the same Tyrodes used in the muscle bath (normal or calcium-free) the day of the experiments, and were kept in an ice bucket during use. Data expressing muscle response to drugs was statistically analyzed by use of the Mann-Whitney-U test. Spontaneous contractility data was analyzed by use of the Students t-test.

Experiments in Physiological Tyrodes

Tyrodes was prepared according to Weinstock and Weiss (34), and consisted of (mM); NaCl (136.8), KCl (2.7), $CaCl_2$ (1.8), $MgCl_2$ (1.6), NaH_2PO_4 (0.4), $NaHCO_3$ (11.9), and glucose (5.6). Spontaneous contractility in muscle strips was recorded for 20 min, then acetylcholine (ACh) was added,

starting at muscle bath concentrations of 10^{-8}M and increasing concentrations 10X with each addition, until a dose response curve was obtained. Serotonin (5-HT) was then added, starting at muscle bath concentrations of $3.6x10^{-8}$M and adding further doses as with ACh until a dose response curve was obtained.

Experiments in Calcium-free Tyrodes

Tyrodes was prepared according to Weinstock and Weiss (34), except that $CaCl_2$ was omitted and .1 mM Na_2-EDTA (ethylenediaminetetracetate) was added according to Hudgins and Weiss (21). Spontaneous contractility was recorded for 70 min, then ACh and 5-HT were added to the bath, at starting concentrations of 10^{-9}M and $3.6x10^{-9}$M, respectively.

Muscle Bath Description

The glass muscle bath (Made by the Chemistry Dept., Mich. State U.) consisted of an outer and inner chamber. The inner chamber had a volume of 17 ml, and had 3 outlets, one through which the bath was connected to a Tyrodes reservoir held above, another to the sink for drainage, and the third to an O_2/CO_2 (95:5) tank. The tank outlet contained a fritted glass filter which served to decrease the size of the O_2/CO_2 bubbles as they entered the chamber. The outer chamber enveloped the inner one and was not continuous with it. A pump was used to constantly circulate water at 37°C from a water bath through the outer chamber.

Acetylcholinesterase Localization

After sacrificing the rats, stomachs were removed and stomach strips were obtained by cutting mediolongitudinally along the full length of the greater curvature, beginning at the tip of the forestomach and ending 0.5 cm from the pyloric sphincter. The final dimensions of the strips were 2x5 cm. The pyloric sphincter area including tissue 0.5 cm on both sides of the sphincter was also obtained. The sphincter tissue and the stomach strip were placed on a microtome stub, covered with cryostat tissue embedding compound (Miles Laboratories, Ill.), and were frozen by immersion in liquid nitrogen for 15 sec or by supercooling in a cryostat. Tissue sections (10 um) were cut in the freezing microtome, and were collected on glass coverslips and placed at 4°C until their use several hours later. Sections were treated according to the Karnovsky method (23) for acetylcholinesterase localization. All solutions were kept at 4°C and tissue sections remained on coverslips and were transferred into coplin jars with forceps throughout the experiment. All sections were fixed for 3 min in 4% formaldehyde solution with 0.075 M phosphate buffer and 0.44 M sucrose at a pH of 6.0. Control sections were placed in identical media except they contained physostigmine sulfate (10^{-4}M), or tetramonoisopropylpyrophosphortetramide (iso-OMPA)($3x10^{-5}$M)(5), depending on whether acetylcholinesterase or pseudocholinesterases were to be inhibited. Tissues were then transferred into 10 ml freshly

mixed incubation media consisting of the following components, mixed in the order listed: 2mM acetylthiocholine Iodide, 0.065 M Tris Maleate buffer (pH 6.0), 5 mM sodium citrate, 3 mM copper sulfate, 1 ml distilled water, 0.5 mM potassium ferricyanide, and 0.44 M sucrose. Some control tissues were placed in incubation media containing all components except the substrate, acetylthiocholine Iodide. After 45 min sections were removed, washed in 0.1 M Tris Maleate buffer with .44 M sucrose (pH 6.0), then mounted in glycerin jelly on standard microscope slides. Reaction product was considered to be any reddish brown precipitate, and reaction intensity was assigned numbers ranging from 0 (no reaction product) to 4 (most intense reaction). All slides were scored blindly, and all conclusions are based on results obtained from 9 cisplatin-treated and 9 control animals.

^{3}H-Choline Uptake Experiments

Three days after drug-treatment rats were injected with choline chloride (Methyl-^{3}H)(New England Nuclear, Boston, MA) i.p. at doses of 6 uC/Kg (for choline uptake and autoradiography studies) or 60 uC/Kg (only for autoradiography studies). Animals were sacrificed at 5 minutes, 3 and 12 hour intervals. Stomachs were removed, trimmed of fat and nerve and opened with a scissors by cutting mediolongitudinally and then washing in normal saline (4°C) for 2 min by gently agitating the tissue while

immersed. The stomach was divided into three parts by cutting along the line separating the cardiac from the pyloric regions, and by cutting 0.5 cm on both sides of the pyloric sphincter. Separate stomach regions were placed in individual scintillation vials and stored (4°C). Stomachs were thawed, and cleaned of remaining food and hair with a forceps. Tissues were weighed, then cut into pieces about 1 mm^3 and placed back into the vials. Tissues were digested in the vials by adding 2.5 ml protosol (New England Nuclear, Boston, MA) or sometimes 2 N or 10 N NaOH, at 55°C for 48 hrs with intermediate agitation. After digestion 20 ul of concentrated HCl was added to each sample, samples were divided into several vials until all sample volumes equaled 2.5 ml, and 15 ml scintillation cocktail was added. Cocktail was prepared according to Beckman specifications, and consisted of 5 g PPO (2,5-Diphenyloxazole) and 100 g Napthalene to 1 L Dioxane. Sample radioactivity was measured by use of a Beckman scintillation counter. All data was statistically analyzed by use of the Students t-test.

Tissue Preparation for Autoradiography

Small tissue samples about 1 mm^3 were removed from the cardiac stomach during choline uptake experiments. These were fixed in 1% glutaraldehyde - 2% Potassium Pyroantimonate, with 0.05 M Cacodylate buffer for 12 hours at 4°C. Tissues were post-fixed in 1% buffered Osmium for 1

hour, dehydrated in an ascending acetone series and embedded in Araldite epoxy resin. Tissues were processed for autoradiography according to Aggarwal (2).

Light Microscope Autoradiography

Sections (0.5 um) were cut from each tissue block using a Cambridge ultramicrotome. Three sections were transferred into water droplets on each glass microscope slide, and were affixed by placing the slide on a hotplate and evaporating the water. Sections were stained with 0.5% methylene blue, by placing a drop of stain over the sections, putting the slide on a hotplate (low heat) for 10 sec, then rinsing the sections with distilled water.

Slides were coated with Kodak NTB 2 emulsion in a darkroom illuminated by a safelight with a Kodak series 2 Wratten filter. Emulsion was stored at 4°C until use, then placed in a water bath (43°C) for 45 min with gentle stirring until melted. Slides containing thick sections were rinsed in distilled water (43°C), drained, dipped into the emulsion, then were removed and dried upright on a slide rack for 45 min. Slides were then placed in black plastic slide boxes containing about 10 g of drierite wrapped in a kimwipe. Boxes were sealed with black electrical tape, and were placed into plastic bags (containing drierite) which were tied shut and placed in the freezer (4°C) for 6 weeks. The slides were developed in the darkroom illuminated as before, and all solutions were mixed fresh, filtered, and

kept in glass staining jars in a water bath (23°C). Slides were removed from the boxes in the darkroom and were placed into glass carriers. Carriers were placed into Microdol X (3 min), distilled water (0.5 min), Kodak rapid fixer (0.5 min) and 3 distilled water rinses (7 min each), then were dried and stored in slide boxes. Slides were examined with a Zeiss photomicroscope.

Electron Microscope Autoradiography

Glass microscope slides were cleaned, then dipped in a 4% solution of Parlodion in amylacetate, and were allowed to dry upright. Sections (0.1 um) were cut from each tissue block using an LKB ultramicrotome III. Four sections were transferred onto water droplets located 1 cm from the frosted end of the parlodion coated slides, and sections were affixed by allowing the water to evaporate. Sections were stained by placing a drop of uranyl acetate (saturated solution) on the sections for 1.5 hours, then rinsing with distilled water. Slides were coated with Ilford L 4 emulsion in a darkroom illumiated by a safelight with a Kodak Series 2 Wratten filter. Emulsion was stored at 4°C until use, then 5 g was weighed and added to 20 ml distilled water in a plastic container in a water bath (44°C), and was stirred gently for 30 min. The emulsion was then cooled to room temperature in an ice bath. A test slide was coated with emulsion using a pipette, and allowed to dry. It was examined with flourescent light to determine where the purple diffraction region was located, and this area was

taken to be the emulsion thickness composed of a monolayer of silver bromide crystals. The distance from where the emulsion line was applied to the monolayer region was recorded. When slides were emulsion-coated the use of a ruler insured that the monolayer region was located approximately over the sections. Slides were dried upright on a slide rack for 45 min, and were stored the same way as were the thick sections, for 7 weeks.

The slides were developed in the darkroom illuminated as before, and all solutions were mixed fresh, filtered, and kept in glass staining jars in a water bath (23°C). Slides were removed from the boxes in the darkroom and were placed into glass carriers. Carriers were placed into Microdol X (2.5 min), distilled water (0.5 min), Kodak rapid fixer (2 min), and 3 distilled water rinses (2 min each), then were allowed to dry. A diamond-tipped knife was used to cut a circle into the slides about 0.5 cm in diameter, and encircling the sections. A drop of hydrofluoric acid (5%) was placed on the edge of this area, and this helped to separate the circle of parlodion from the slide. The slide was slowly immersed in a dish of distilled water and the circular section was floated off onto the surface. Using a forceps, copper specimen grids were placed over the sections, and the grids were picked up by taking a microscope slide covered with masking tape (rough side out), and pressing down on the grids with it then quickly flipping it over and bringing it out of the water. Grids were

allowed to dry, then sections were stained for 5 min with lead citrate. Sections were examined using a Hitachi HUE11 Transmission Electron Microscope, operated at 75 Kv.

RESULTS

Muscle Bath Experiments

Cisplatin-treated and control stomach smooth muscle strips were studied to determine if cisplatin treatment affects muscle spontaneous contractility, and its contractile responses to acetylcholine (ACh) and serotonin (5-HT). In physiological Tyrodes cisplatin-treated and control smooth muscle strips show a similar spontaneous contractility (Table 1), and contractile response to ACh (Figure 1) and 5-HT (Figure 2).

Muscle strips were studied in calcium-free Tyrodes to determine the effects of extracellular calcium depletion on spontaneous contractility and contractile response of the muscle strips to ACh and 5-HT. When muscle strips were incubated in calcium-free Tyrodes, both cisplatin-treated and control muscles show a gradual decrease in the frequency of spontaneous contractions as incubation time progressed (Table 1). A comparison of the response of cisplatin-treated and control muscle to ACh (Figure 1) reveals that in a calcium-free medium cisplatin-treated muscle is significantly more contractile than control muscle to ACh at 10^{-7} M. When the response of the muscle strips to 5-HT is measured in a calcium-free medium

(Figure 2), cisplatin-treated muscle is significantly more contractile than control muscle to 5-Ht at $3.6x10^{-6}$ M. Maximal contraction is achieved by cisplatin-treated muscle strips to 5-HT at $3.6x10^{-6}$ M, but with control muscle a maximal response is not obtained even at the highest 5-HT concentration used, $3.6x10^{-3}$ M.

When the response of control muscle strips in a physiological Tyrodes is compared to its response in a calcium-free solution, it was found that the strips contract significantly less in the calcium-free solution, to both ACh at 10^{-7} M ($P \leq .05$)(Figure 1), and 5-HT at $3.6x10^{-8}$ M ($P \leq .05$), $3.6x10^{-7}$ M ($P \leq .01$), and $3.6x10^{-6}$ M ($P \leq .01$)(Figure 2). The response of cisplatin-treated muscle in physiological Tyrodes compared to its response in calcium-free Tyrodes indicates that in a calcium-free medium the muscle contracts significantly more to ACh at 10^{-7} M ($P \leq .02$)(Figure 1), but there is no difference in its response to 5-HT (Figure 2).

In summary, the above data indicates that cisplatin-treated muscle displays normal spontaneous contractility both in physiological and calcium-free Tyrodes. Furthermore, cisplatin-treated muscle contracts like control muscle to ACh and 5-HT in a physiological solution, but in a calcium-free solution cisplatin-treated muscle is more contractile than the control muscle to both ACh and 5-HT. It was also found that in a calcium-free medium the response of normal muscle to both ACh and 5-HT is less than its

Table I. Spontaneous contractility of fundal strips[a].

		Average[b] number of spontaneous contractions ≥ 3 mg contractile force per 10 minute incubation time.						
	Time (min)	0-10	11-20	21-30	31-40	41-50	51-60	61-70
$Ca^{++}(+)$*	CONTROL	15	15					
	CDDP	14	12					
$Ca^{++}(-)$	CONTROL	21	15	6	4	1	1	0
	CDDP	25	18	17	12	11	5	1

[a]There are no significant differences in spontaneous contractility between cisplatin-treated and control muscle in physiological or calcium-free Tyrodes. Note the decrease in the number of contractions as a function of time in calcium-free Tyrodes.

[b]All data is based on values obtained from an average of six animals.

*Spontaneous contractility was not measured beyond 20 min.

Ca^{++} (+) = Physiological Tyrodes, Ca^{++}(-) = calcium-free Tyrodes, CDDP = Cisplatin-treated.

Figure 1. Dose response curves obtained from fundal Strips to Acetylcholine (ACh). The curves obtained from control and cisplatin-treated muscle when tested in physiological Tyrodes are not significantly different. When the muscles are tested in calcium-free Tyrodes, cisplatin-treated muscle shows a significantly greater response than control muscle at 10^{-7} M. ($P \leq .02$). $Ca^{++}(+)$ = Physiological Tyrodes, $Ca^{++}(-)$ = Calcium-free Tyrodes, CDDP = Cisplatin- treated. Each data point is based on values obtained from an average of six animals.

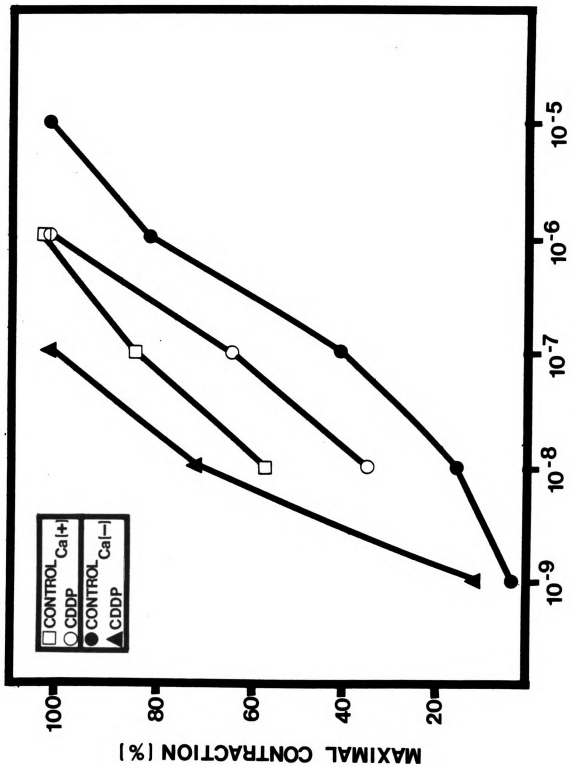
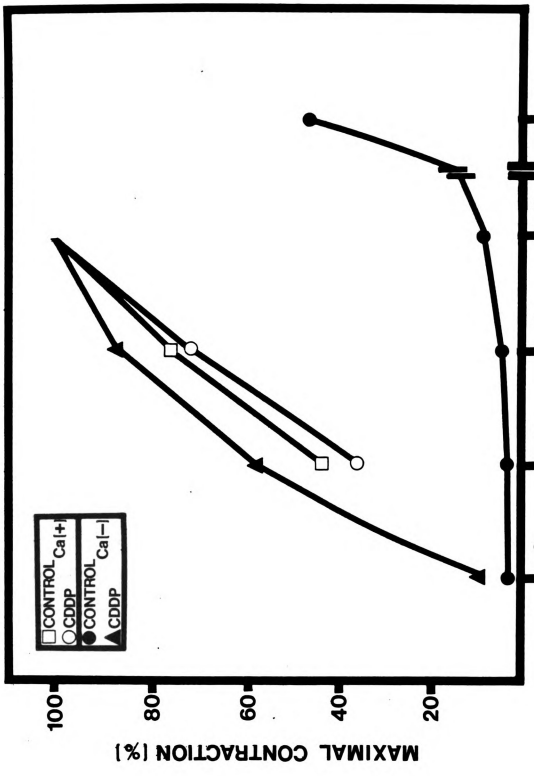

MAXIMAL CONTRACTION [%]
100
80
60
40
20
ACh [M]
10-9
10-8
10-7
10-6
10-5
□CONTROL Ca[+]
○CDDP
●CONTROL Ca[−]
▲CDDP

Figure 2. Dose response curves obtained from fundal strips to serotonin (5-HT). The curves obtained from control and cisplatin-treated muscle when tested in physiological Tyrodes are not significantly different. When the muscles are tested in calcium-free Tyrodes, cisplatin-treated muscle shows a significantly greater response than control muscle at 3.6×10^{-6} M. ($P \leq .01$). $Ca^{++}(+)$ = Physiological Tyrodes, $Ca^{++}(-)$ = Calcium-free Tyrodes, CDDP = Cisplatin-treated. Each data point is based on values obtained from an average of six animals.


MAXIMAL CONTRACTION [%]
100
80
60
40
20
□CONTROL Ca[+]
○CDDP
●CONTROL Ca[−]
▲CDDP
3.6x10-9
3.6x10-8
3.6x10-7
3.6x10-6
3.6x10-3
5-HT [M]

response in physiological Tyrodes.

Acetylcholinesterase Localization

To determine if acetylcholinesterase (AChE) inhibition could be the cause of cisplatin induced smooth muscle hypercontractility, AChE was histochemically localized in gastric tissues from cisplatin-treated and control rats, and staining intensity and distribution was compared between experimental groups. Results from these experiments indicate that AChE levels and distribution are identical between tissues from cisplatin-treated and control animals. In all tissue samples, AChE staining was localized in the muscular layers of the stomach wall.

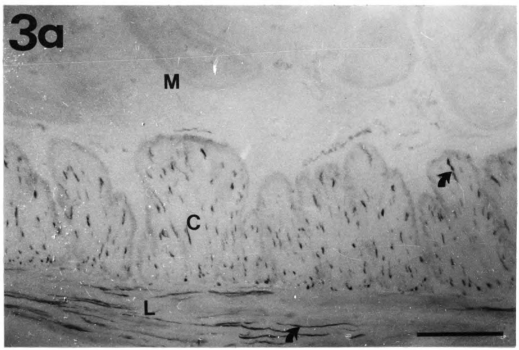
In the cardiac region of the stomach of drug-treated (Figure 3a.) and control animals (Figure 3b.), the muscularis mucosa was weakly AChE positive, the reaction product being homogenous throughout this layer and not localized specifically to neural structures resolvable with the light microscope. The longitudinal and circular muscle layers of the muscularis were weakly AChE positive throughout, however, certain localized areas were intensely AChE positive. Such areas included sections of nerve fibers, nerve fascicles, and ganglia.

In the pyloric region of the stomach of drug-treated (Figure 4a.) and control rats, the muscularis mucosa was AChE negative, and the muscularis and nerve cells stained similarly to that of the cardiac stomach region.

The muscularis of the pyloric sphincter in the drug-treated and control animals (Figure 4b.) stained similarly to that of the cardiac and pyloric regions of the stomach.

From the above experiments it can be concluded that cisplatin treatment causes no change in AChE levels or distribution in the cardiac, pyloric, and pyloric sphincter tissues of the rat stomach.

Figure 3. Light micrographs showing acetylcholinesterase staining in the cardiac regions of the stomach of (a) a cisplatin-treated and (b) a control rat. Structures most heavily stained are nerve fibers (arrows) in the muscularis. M, muscularis mucosa; C, circular muscle layer; L, longitudinal muscle layer. Bars = 100 um.


3a
M
C
L

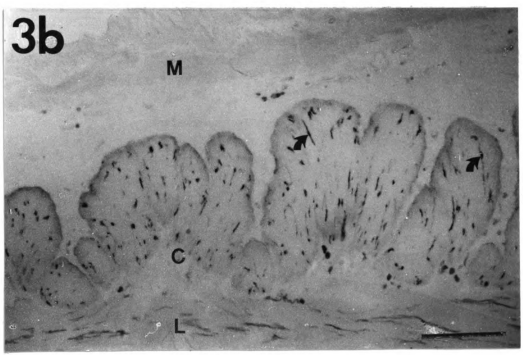
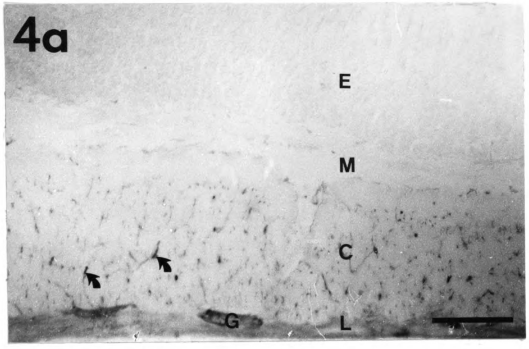

3b
M
C
L

Figure 4a. Light micrograph showing acetylcholinesterase staining in the pyloric regions of the stomach of a cisplatin- treated rat. Structures most heavily stained are nerve fibers (arrows) and ganglia in the muscularis. E, epithelium; M, muscularis mucosa; C, circular muscle layer; L, longitudinal muscle layer. Bar = 100 um.

Figure 4b. Light micrograph showing acetylcholinesterase staining in the pyloric sphincter muscularis of a control rat. Structures mot heavily stained are nerve fibers (arrows). Bar = 100 um.


4a
E
M
C
G
L


4b

^{3}H-Choline Uptake Experiments

^{3}H-Choline (^{3}H-Ch) uptake into the stomachs of cisplatin-treated rats was compared to that of normal rats, to determine if acetylcholine metabolism in the enteric nervous system is altered by cisplatin treatment. Radioactivity was measured in the cardiac, pyloric, and pyloric sphincter regions of the stomach at 5 minutes, 3 and 12 hours after ^{3}H-Ch injection.

In the cardiac region of the stomach (Figure 5) radioactivity counts are significantly greater in cisplatin-treated animals than in controls at 5 min, 3 and 12 hours after ^{3}H-Ch injection. The radioactivity is significantly increased with time in both the drug treated and experimental groups.

In the pyloric region of the stomach (Figure 6) there are no significant differences in the radioactivity between cisplatin-treated and control tissues, and for both groups there is no statistically significant change in tissue radioactivity with time.

In the pyloric sphincter (Figure 7) radioactivity counts in cisplatin-treated rats are significantly higher than those in control rats at 5 min and 12 hours after ^{3}H-Ch injection, and in both the drug-treated and control animals radioactivity decreases with time.

In summary, the data from the above experiments indicates that ^{3}H-Ch uptake is significantly higher in the cardiac and pyloric sphincter tissues of cisplatin-treated rats as compared to controls. When tissue radioactivity was

Figure 5. Radioactivity counts (mean + S.D.) in the cardiac region of the stomach of rats at 5 min, 3 and 12 hour intervals after ^{3}H-Choline injection. Note that tissues from cisplatin-treated (CDDP) rats show greater radioactivity counts than the controls at all time periods. (* ≤ P, .02, ** ≤ P, .01). Each data point is based on counts obtained from an average of 12 animals.

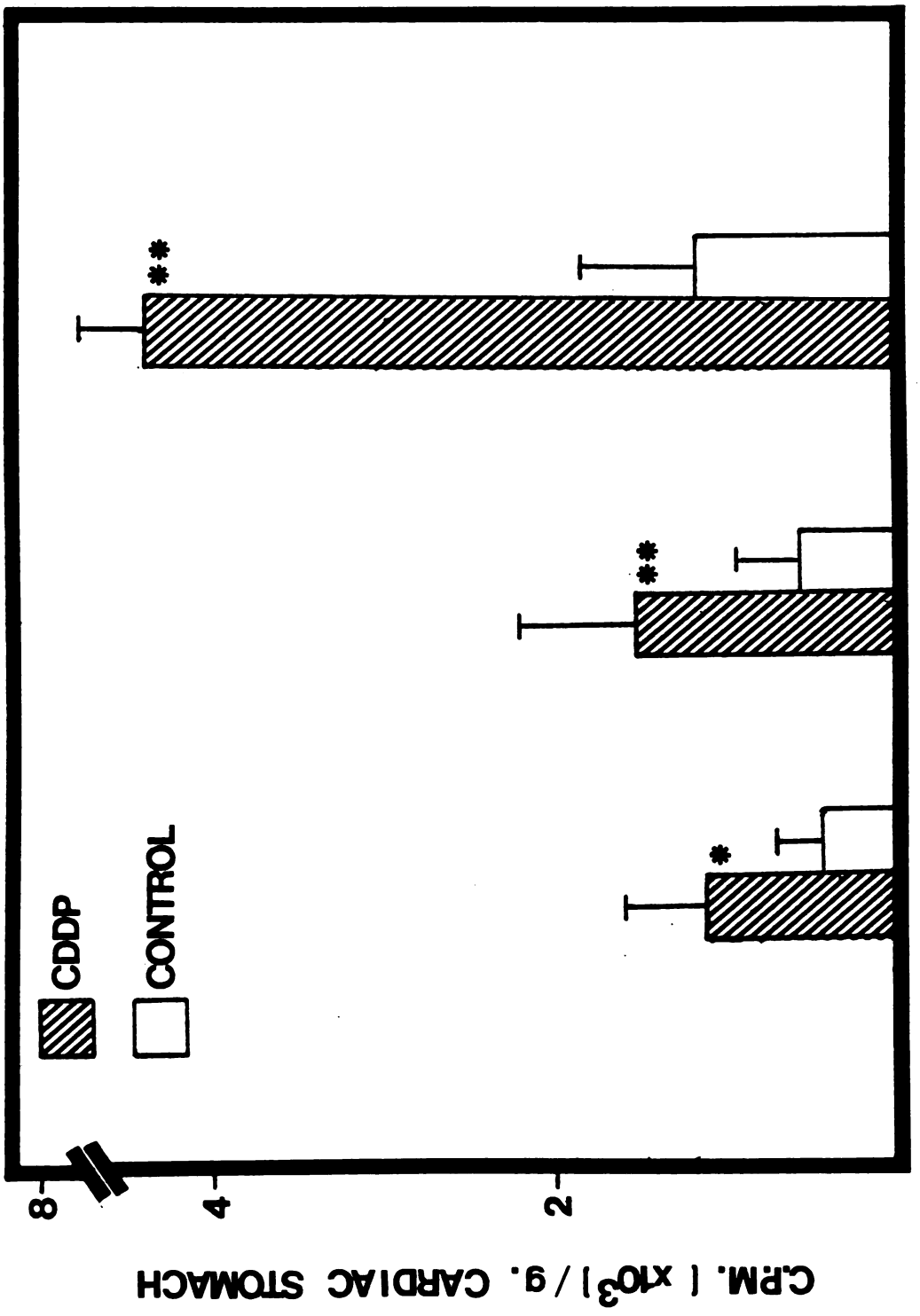

C.P.M. [x10³] / g. CARDIAC STOMACH
8
4
2
CDDP
CONTROL
*
**
**
5 MIN.
3 HRS.
12 HRS.
TIME AFTER ³H-CHOLINE INJECTION

Figure 6. Radioactivity counts (mean + S.D.) in the pyloric region of the stomach of rats at 5 min, 3 and 12 hour intervals after ^{3}H-Choline injection. Note that there are no significant differences between values obtained from cisplatin-treated (CDDP) rats and control rats. Each data point is based on counts obtained from an average of 12 animals.

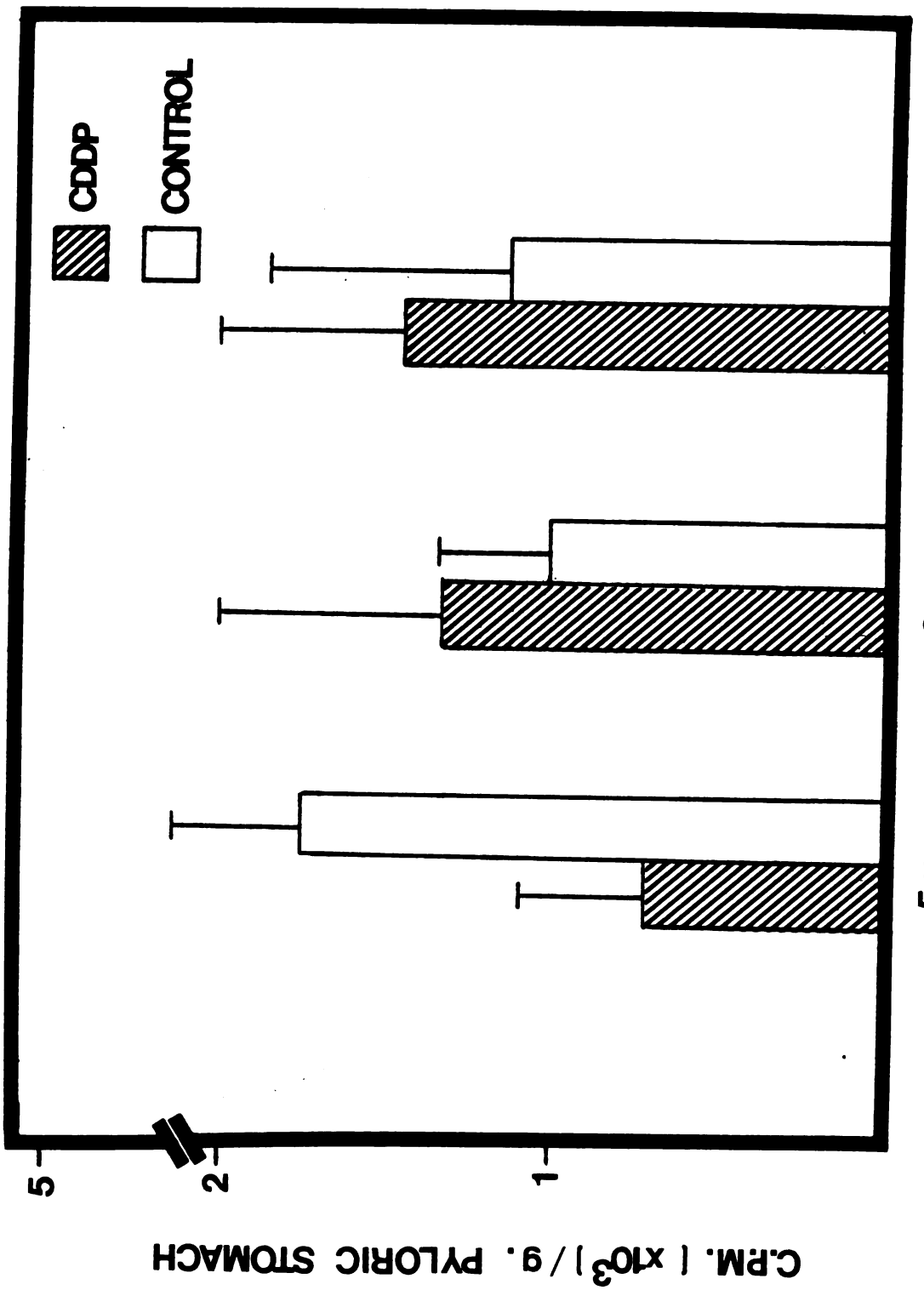
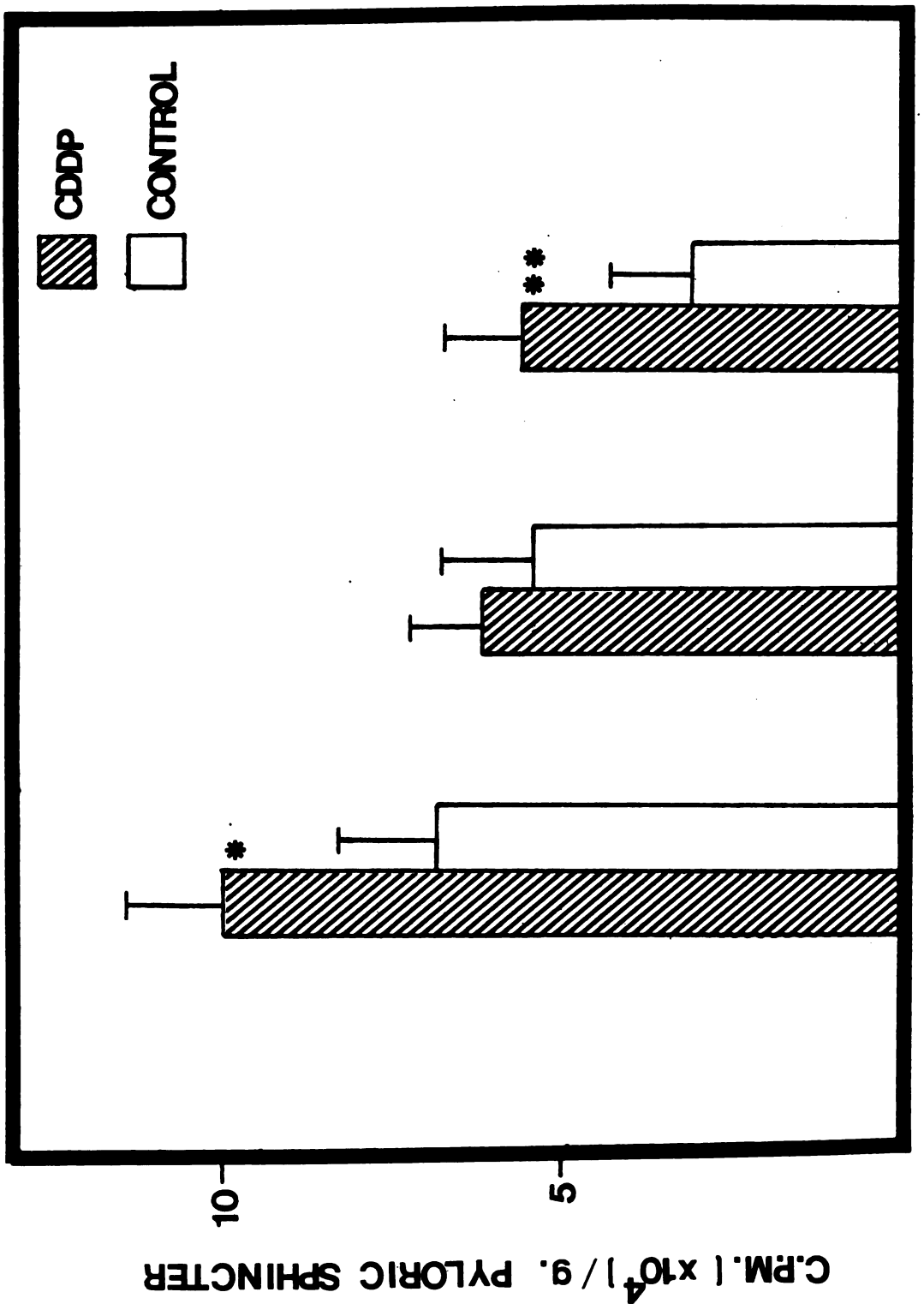

CDDP
CONTROL
5
2
1
C.P.M. (x10³) / g. PYLORIC STOMACH
5 MIN.
3 HRS.
12 HRS.
TIME AFTER ³H-CHOLINE INJECTION

Figure 7. Radioactivity counts (mean + S.D.) in the pyloric sphincter of rats at 5 min, 3 and 12 hour intervals after ^{3}H-Choline injection. Note that tissues from cisplatin-treated rats show greater radioactivity counts than the controls at the 5 min and 12 hour time intervals (* $\leq$ P, 0.5, ** $\leq$ P, .01). Each data point is based on counts obtained from an average of 12 animals.


CDDP
CONTROL
10
5
C.P.M. (x10⁴) / g. PYLORIC SPHINCTER
5 MIN.
3 HRS.
12 HRS.
TIME AFTER ³H-CHOLINE INJECTION

measured as a function of time following ^{3}H-Choline injection, it was found that in the cardiac region of the stomach there was an increase in radioactivity with time whereas with the sphincter tissues there was a decrease.

Autoradiography

In ^{3}H-Ch uptake experiments only cardiac stomach tissue was processed for autoradiography, to determine the specificity of the ^{3}H-Ch uptake. Autoradiograms show similar results in tissues taken from both control and drug-treated animals at all time periods after ^{3}h-Ch injection.

Light and electron microscope autoradiograms show silver grains located over the nuclear and cytoplasmic membranes of the smooth muscle cells (Figure 8), and over the synaptic endings (Figure 9). Though grains over muscle cells seem specific to cell membranes, those found over synaptic endings cannot be attributed to specific structures within the endings because the size of the grains is much larger than the neurotransmitter vesicles and mitochondria. Since all sections have few synaptic endings, and low grain counts, no quantitative data can be obtained comparing cisplatin- treated and control tissue.

The above autoradiographic data indicates that ^{3}H-Ch uptake into the cardiac region of the stomach is associated with cellular membranes and the axonal endings.

Figure 8. electron microscope autoradiogram indicating ^{3}H-Choline incorporation into smooth muscle cell membranes. Note the silver grains over the cytoplasmic (arrows) and nuclear (arrowhead) membranes. This cell is from the gastric muscularis of a cisplatin-treated rat injected with ^{3}H-Choline and sacrificed 12 hours later. The electron dense granules within the cell and the intercellular spaces are the calcium-pyroantimonate precipitate. N, nucleus. Bar = .5 um.

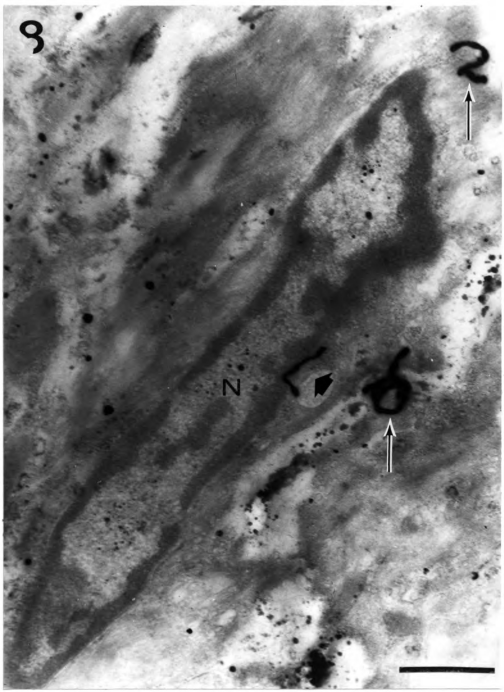
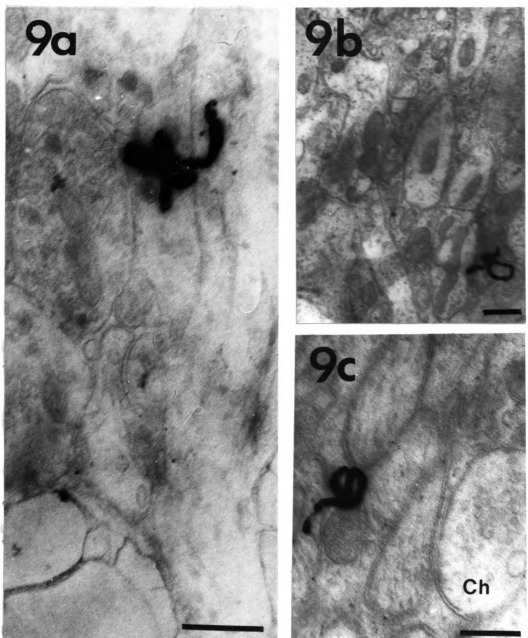

9
2
N
6

Figure 9 (a., b., and c.). Electron microscope autoradiograms indicating ^{3}H-Choline incorporation into axonal endings. These endings are from the gastric muscularis of cisplatin-treated rats injected with ^{3}H-Choline and sacrificed 5 min later. Ch, cholinergic terminal? Bars = .2 um.


9a
9b
9c
Ch

DISCUSSION

Smooth muscle strips from the fundus of cisplatin-treated rats were studied *in vitro* in physiological Tyrodes. It was found that the muscle is spontaneously contractile and contracts like untreated muscle to acetylcholine (ACh) and serotonin (5-HT). The finding that drug-treated muscle is spontaneously contractile indicates that the muscle probably can produce slow wave membrane potentials, giving rise to action potentials and muscle contraction, as has been demonstrated with normal smooth muscle (31).

To determine the effects of extracellular calcium depletion on cisplatin-treated muscle, muscle strips were studied *in vitro* in a calcium-free medium. Both cisplatin-treated and control muscle showed a gradual decrease in spontaneous contractility as incubation time increased in the calcium-free medium. This decrease occurred presumably through inhibition of both components of the slow wave membrane depolarizations, which initiate spontaneous contractions, and which are thought to be dependent on extracellular calcium (31). In the calcium-free medium cisplatin-treated muscle showed a greater response to both ACh and 5-HT than did control muscle. The leftward shift of the ACh and 5-HT dose response curves observed

with cisplatin-treated muscle, as compared to control curves, is a response characteristic of the supersensitivity phenomenon (13).

In cholinergic systems, deviation supersensitivity occurs when acetylcholinesterase (AChE) activity has been inhibited, which results in greater than normal quantities of ACh in contact with muscle receptors (36). Contrary to the deviation type supersensitivity, states of non-deviation supersensitivity are induced in smooth muscle by performing procedures which chronically interrupt neurotransmission to the muscle. Such procedures include denervation, or treatment with ganglionic blocking agents, reserpine, or colchicine (36). In non-deviation supersensitivity, the muscle would have a lower excitation threshold to all agents which initiate contraction through membrane depolarization (36). Non-deviation supersensitivity is termed non-specific because the drugs which induce the heightened response act by independent receptor systems on the effector organ (12). In the present experiment the supersensitivity is non-specific because a heightened contractile response was elicited by both ACh and 5-HT, which are drugs that act independently of each other to cause contraction in the rat fundus (16). Since cisplatin-treated gastric muscle has normal levels of AChE, and is supersensitive to both ACh and 5-HT, it is concluded that cisplatin-induced supersensitivity is of the non-deviation type.

The finding that cisplatin-induced supersensitivity occurred only in a calcium-free medium indicates that the extracellular calcium depletion was an important variable in eliciting the supersensitive response. In the calcium-free Tyrodes normal muscle contracted less to both ACh and 5-HT as compared to its response in physiological Tyrodes, and the muscle showed maximal contraction (38 mg force) to ACh, but not to 5-HT. This behavior occurred presumably because in the rat gastric fundus the contraction in response to ACh utilizes intracellular calcium stores, whereas a contraction to 5-HT is dependent on extracellular calcium (34). In the calcium-free Tyrodes cisplatin-treated muscle contracted more to ACh and the same to 5-HT as compared to its response in physiological Tyrodes, and the muscle showed a maximal contraction to both ACh and 5-HT. The behavior of cisplatin-treated muscle indicates that extracellular calcium depletion did not have an inhibitory effect on the contractility of the muscle to ACh and 5-HT, whereas with normal muscle the contractility was decreased. These data suggest that there may be a change in the calcium metabolism in cisplatin-treated muscle as compared to that of normal muscle. In smooth muscles displaying a non-deviation type supersensitivity, such changes in calcium homeostasis have been reported, and could be responsible for the altered contractile behavior (8).

Supersensitized aortic strips from reserpine-treated animals show a reduction in the affinity (22) or number (11)

of membrane-calcium binding sites, though the total tissue content of calcium is not significantly altered (15,22). In a calcium-free solution reserpine-treated muscle retains more calcium and shows greater response to norepinephrine and epinephrine than controls (15,22). It is suggested that in supersensitized muscle there is an increase in the lability or size of the intracellular calcium pool (22). In supersensitized vas deferens muscle an increase in the membrane threshold potential occurs (17), and such an increase could be due to an alteration in membrane-calcium binding in the muscle (19). In supersensitized muscle the occurrence of an increased membrane threshold potential would explain the increased sensitivity to drugs which initiate contraction by depolarizing the cell membrane (17). Changes in membrane-calcium binding are also thought to affect the gating of ionic channels (6), which in turn would affect muscle excitability.

In the present experiment since extracellular calcium depletion did not cause an inhibitory effect on the response of cisplatin-treated muscle to ACh and 5-HT, it is possible that cisplatin-treatment, like reserpinization, may cause an increase in the lability or size of the intracellular calcium pool in gastric muscle.

Non-specific supersensitivity in muscle has only been induced by blocking nerve transmission to the muscle (12,37). With the supersensitized muscle, it is not clear if alterations in calcium homeostasis are a direct

effect of the interruption in nerve function. This uncertainity arises because in most studies concerning supersensitivity and calcium homeostasis, and interruption of nerve transmission is inflicted by reserpinization. Reserpine is a drug which is used to block nerve function, but which could also exert direct effects on calcium metabolism in the smooth muscle (37). Unlike reserpinization, cisplatin-treatment has not been demonstrated to cause an interruption of nerve transmission, however cisplatin may exert considerable effects on calcium homeostasis.

Cisplatin causes an efflux of calcium from isolated mitochondria (2), and possible mitochondrial damage as seen in ultrastructural studies (2). Mitochondria could play a role as reserve calcium sinks in some smooth muscles (23), and if cisplatin causes efflux of calcium from mitochondria in smooth muscle cells, then cellular calcium stores and muscle contractility could be affected. Cisplatin-treatment has also been shown to cause a decrease in the amount of calcium bound to the plasma membrane of kidney cells (3). If this drug-effect is occurring in gastric smooth muscle, cell membranes could become more excitable, and therefore non-specifically supersensitive. Cisplatin could be depleting the cellular calcium stores indirectly by causing serum hypocalcemia. a drug effect reported in laboratory animals (31) and man (29).

The evidence cited above suggests that cisplatin

induced muscle supersensitivity could be due solely to effects of the drug on calcium homeostasis in the gastric muscle. Irrespective of this possiblity, is was important to establish if cisplatin causes an inhibition of nerve function to gastric muscle, since such neurotoxicity is normally associated with agents that cause non-deviation type supersensitivity in smooth muscle (12,37).

To determine if cisplatin is toxic to the gastric innervation, ^{3}H-Choline (^{3}H-Ch) uptake and metabolism was studied in enteric nerves of cisplatin-treated rats. Autoradiograms of the cardiac stomach from ^{3}H-Ch injected rats indicated that at the light and electron microscope level the isotope was localized over smooth muscle cell nuclear and cytoplasmic membranes, and axonal endings. The presence of the label over synaptic endings was expected since cholinergic nerve terminals have a high affinity uptake system for choline (27). This high affinity system is sodium and energy dependent (25,26), but there is also uptake based on passive diffusion which only accounts for a few percent of synaptic choline (18). It is therefore not obvious which uptake system was operating in the drug-treated animals. Because acetylcholine synthesis is not directly coupled to choline uptake (29), it is not possible to determine if acetylcholine synthesis from ^{3}H-Ch had occurred in these neurons. ^{3}H-Ch has been demonstrated to be incorporated into nuclear and plasma membranes through metabolism of ^{3}H-Ch into membrane

phospholipids (14), which explains the isotope localization within the cell membranes, as observed in the present study.

The autoradiographic data indicates that an undermined proportion of gastric ^{3}H-Ch uptake may be due to incorporation of the isotope into cellular membranes. Due to the uncertainty about the specificity of the ^{3}H-Ch uptake, no conclusions can be made concerning the significance of the gastric tissue radioactivity counts observed in cisplatin-treated and control rats.

In conclusion, the results of the present study indicate that cisplatin-treatment may alter clacium homeostasis in gastric smooth muscle. If such an effect is general to all smooth muscles and nerves, this could have a profound effect on neuro-muscular functions of the cisplatin-treated animal.

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