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Cisplatin-induced inhibition of the calcium-calmodulin complex, neuronal nitric oxide synthase activation and their role in stomach distention

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Abstract Cisplatin (8 mg/kg; i.p.) treatment of Wistar rats produced no change in nitric oxide synthase (NOS) localization or its intensity for up to 5 days. However, immunohistochemicaly the levels of L-citrulline and the Ca^{2+}-calmodulin complex were decreased after only 3 days. An in vitro experiment using an analog of calmodulin, Mero-Calmodulin-1, showed that *cis*-diammine-diaquacisplatinum(II), a hydrolyzed form of cisplatin, inhibited the calmodulin conformational shift from occurring through a direct interaction with the calmodulin molecule. The results indicate that distention of the stomach was due to inhibition of neuronal NOS activation by a direct interaction between cisplatin and the calcium binding sites of the calmodulin molecule.

Key words Neuronal nitric oxide synthase · Calcium-calmodulin complex · Cisplatin · Stomach distention · Emesis

Introduction

Cisplatin (*cis*-diamminedichloroplatinum(II)), a broad-spectrum anticancer drug, has proven effective in the treatment of bladder, lung, ovarian [11, 31], head and neck [9], testicular [13], and breast [14, 20] cancers, and of certain types of leukemias [27].

Drawbacks of this chemotherapeutic drug are its severe toxic side effects, which include the production of free radicals, oxidative stress [12], lipid peroxidation [7], hypocalcemia, hypomagnesemia [26], tetany, nausea, vomiting, myelosuppression, nephrotoxicity [15], ototoxicity [38], peripheral neuropathy [30], embryotoxicity [6, 21], stomach distention and peptic ulcers [1, 4]. Nausea and vomiting is a dose-limiting effect in some patients receiving the drug with 60–80% of them having delayed emesis 48–72 h posttreatment [24, 33]. Distention of the stomach has been shown to parallel the nausea and vomiting [34]. Cisplatin induces inhibition of acetylcholine release from stomach nerve endings, which in turn results in a prolonged relaxation of stomach smooth muscle [4].

Studies on neuronal nitric oxide synthase (n-NOS) have shown that genetically engineered mice that have had the gene responsible for the production of n-NOS removed are normal except for bloating of the stomach due to prolonged constriction of the pyloric sphincter [17]. Stomach smooth muscle contraction is acetylcholine dependent [35], while pyloric sphincter smooth muscle relaxation is nitric oxide (NO) dependent [37], and both acetylcholine release and NO synthesis are calmodulin dependent [22, 36].

Calmodulin's ability to bind to calcium has been shown to be nonspecific [8]. Additionally, calmodulin has been shown to react with heavy metals like Cd^{2+}, Pb^{2+}, and Zn^{2+}, and these metal ions show biphasic activation curves [23]. At low concentrations they can induce activation of calmodulin, but at high concentrations there is no activation [23]. Monovalent ions have also been shown to have an inhibitory effect on calmodulin's ability to bind to calcium [23]. Once within the cell under low chloride ion concentrations, cisplatin has been shown to hydrolyze into monoaqua and diaqua species that have a single or double positive charge, respectively [1, 19, 28].

Since normal stomach motility is influenced in part by the NO neurotransmitter and this in turn is regulated by the calcium-calmodulin complex [36], the present study was undertaken to evaluate whether cisplatin prevents n-NOS activation through an inhibitory interaction with calmodulin similar to that seen with other heavy metal ions [23].

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Materials and methods

Animals

Male and female Wistar rats (Charles Rivers Laboratories, Wilmington, Mass.) weighing 175–200 g were kept on a 12-h light/12-h dark cycle with free access to laboratory animal feed and water. Rats were injected intraperitoneally (i.p.) with a bolus dose of cisplatin (8 mg/kg) in 0.85% NaCl, while the controls received the vehicle alone. Animals were either perfused with the fixatives or sacrificed by decapitation on the 4th or 5th day of treatment.

Tissue collection

Control and cisplatin-treated rats were given a lethal dose of sodium pentobarbital and perfused with a mixture of 0.5% glutaraldehyde and 4% formaldehyde in 0.1 *M* phosphate buffer (pH 7.4) using a Masterflex perfusion pump (Cole Parmer, Chicago, Ill.). Tissues were then excised and postfixed at 4 °C for 8–12 h in the same fixative [40]. Tissues from decapitated animals were quickly excised and placed in a mixture of 0.5% glutaraldehyde and 4% paraformaldehyde in 0.1 *M* phosphate buffer (pH 7.4) at 4 °C for 8–12 h.

After fixation, all tissues were placed overnight in 0.1 *M* phosphate buffer (pH 7.4) containing 15% sucrose, and cryosectioned (7–10 μm). Cryosections were picked up on gelatin-coated coverslips.

Nitric oxide synthase localization

Cryosections were washed in 50 m*M* Tris (pH 7.4) for 10 min at room temperature, and incubated in 50 m*M* Tris (pH 7.4) containing 1 mg/ml NADPH (Sigma, St Louis, Mo.), 0.3 m*M* Nitro Blue tetrazolium (Sigma), and 0.2% Triton X-100 (Sigma) at 37 °C for 3 h. Sections were dehydrated through a graded series of alcohol, cleared in Hemode (Fisher Scientific, Chicago, Ill.), and mounted on glass slides with Permount (Fisher Scientific, Chicago, Ill.) [10, 40].

L-Citrulline localization

Immunocytochemical localization of citrulline was done according to previously established protocols [32, 40]. Cryosections from perfused fixed animals were rinsed for 10 min in 0.01 *M* phosphate-buffered saline (PBS; pH 7.4) and treated with 5% normal goat serum (NGS) (Jackson Immunoresearch, West Grove, Pa.) in 0.01 *M* PBS (pH 7.4) for 10 min. These sections were then incubated overnight at room temperature with an antibody to L-citrulline (1:20 000 dilution; Arnel Products, New York, N.Y.) in 0.01 *M* PBS (pH 7.4), containing 1% NGS, and 0.1% sodium azide. The sections were then rinsed in 0.01 *M* PBS for 10 min and 5% NGS for 10 min, and incubated for 30–60 min in a solution of biotinylated goat antirabbit IgG (1:15 dilution; Sigma) in 0.01 *M* PBS (pH 7.4), containing 1% NGS and 0.1% sodium azide. Sections were washed for 10 min in 0.01 *M* PBS (pH 7.4) followed by a 10-min wash in 1% hydrogen peroxide in 0.1 *M* PBS (pH 7.4) to block endogenous peroxidases. After washing, the sections were incubated for 30–60 min in avidin peroxidase solution (1:15 dilution) with 0.01 *M* PBS containing 0.1% sodium azide, and exposed to freshly prepared 0.075% diaminobenzidine (DAB) with 0.0002% hydrogen peroxide in 50 m*M* Tris (pH 7.6). These sections were thoroughly rinsed in 50 m*M* Tris followed by 0.01 *M* PBS and mounted on glass slides with glycerin jelly [32, 40]. Control sections involved incubation in media without the primary or secondary antibody.

Calcium-calmodulin complex localization

Care was taken to prepare all solutions for the following experiments with double-distilled deionized water and calcium-free reagents. Immunocytochemical localization of the Ca^{2+}-calmodulin complex was done using an established protocol [29] with the minor modification that tissues were incubated in normal donkey serum (NDS) to minimize crossreactivity with rat tissue by the secondary antibody. Sections were washed with 0.01 *M* PBS (pH 7.0) and incubated with a 1:50 dilution of mouse monoclonal anticalmodulin antibody (Chemicon International, Temecula Calif.) in 0.01 *M* PBS (pH 7.0) containing 1% NDS, and 0.1% sodium azide for 60 min at 37 °C. After primary incubation sections were washed with 0.01 *M* PBS (pH 7.0) and incubated for 30 min in 5% NDS containing 0.01 *M* PBS (pH 7.0). Tissues were then incubated for 60 min at 37 °C with FITC-conjugated donkey antimouse IgG (1:200 dilution; Jackson Immunoresearch, West Grove, Pa.) in 0.01 *M* PBS (pH 7.0), containing 1% NDS, and 0.1% sodium azide. Sections were then washed with 0.01 *M* PBS, and mounted on glass slides with slow fade (Molecular Probes, Eugene, Oreg.). Sections incubated in 0.01 *M* PBS (pH 7.0) containing 5% NDS instead of the primary antibody were used as controls [29].

Photomicroscopy

Transmission images were taken using a Zeiss Photomicroscope II with neutral density filters. Epifluorescent images were taken using a Zeiss 10 laser scanning confocal microscope (LSM) and a Matrix Multicolor computerized camera unit. All fluorescent images were taken in confocal mode, using a blue argon laser (488 nm) for excitation and a long-pass barrier filter of 520 nm.

Preparation of hydrolyzed cisplatin

Cisplatin (3 mg) was added to 10 ml of double-distilled deionized water and incubated at 37 °C for 2 weeks [25]. The product of this hydrolysis was a mixture of monoaqua-cisplatin (*cis*-diammine-monoaqua-monochlorplatinum(II)) and diaqua-cisplatin (*cis*-diammine-diaquaplatinum(II)). Diaqua-cisplatin [3] was prepared by adding 2.5 mg of a synthesized form of the molecule, to 10 ml of double-distilled deionized water.

Calcium-calmodulin binding: in vitro studies

MeroCalmodulin-1 (MeroCam) is a covalent adduct of calmodulin, isolated from bovine brain, and dye Mc4.19 that fluoresces when it undergoes a conformational shift associated with Ca^{2+} activation [16].

Lyophilized MeroCam was dissolved in double-distilled deionized water to a concentration of 123 n*M*. Fluorescence was measured using a Perkin Elmer 650–40 fluorescence spectrophotometer with a 150 Xenon lamp. Each cuvette was filled with 1.0 ml of the solution. Excitation and emission slits were kept at 15 nm and 5 nm, respectively. Excitation was at 532 nm and 608 nm, emission was monitored at 623 nm. Data were plotted using the ratio of 608 nm to 532 nm. The measurements were made at three different times and the results were the same on each occasion.

Determination of calcium contamination of the MeroCam sample was done by taking 1 ml of a 123 n*M* solution of MeroCam and adding alternating concentrations 50 μl 1 m*M* EGTA, 50 μl 1 m*M* $CaCl_2$, 50 μl 1 m*M* EGTA, and 100 μl 1 m*M* $CaCl_2$. Binding studies were conducted by adding increasing concentrations of 10 μ*M* diaqua-cisplatin, $CaCl_2$, double-distilled deionized water, or the hydrolyzed product of cisplatin containing a mixture of monoaqua- and diaqua-cisplatin [8, 16] at regular intervals in a 123 n*M* MeroCam solution.

Results

Nitric oxide synthase localization

Using histochemical methods the presence of n-NOS was demonstrated in the muscular plexus of the stomach smooth

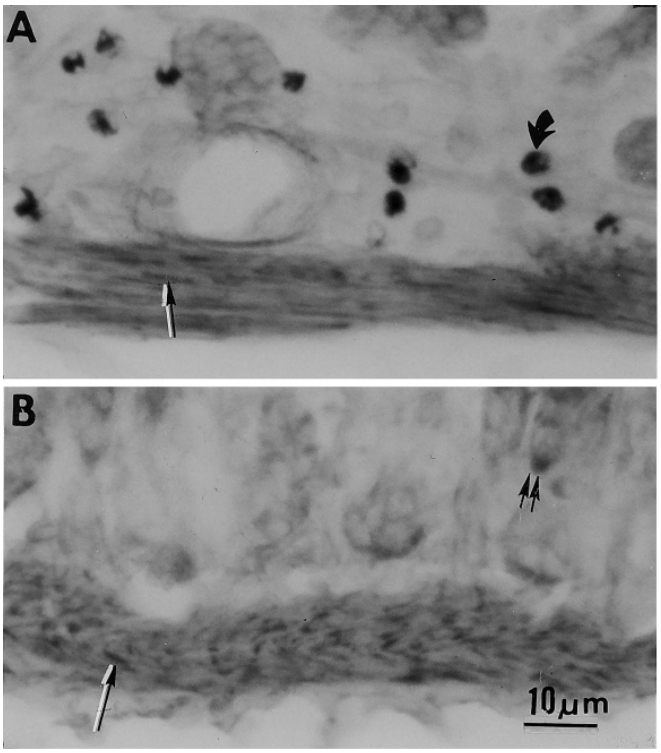

Fig. 1A, B Light micrographs of NOS localization in the pyloric region of the stomach. In control tissues (**A**) there is intense localization of macrophage NOS (m-NOS) within macrophages (*curved arrow*). Cisplatin-treated tissues (**B**), however, show no localization for macrophage NOS. Note that both control tissues and cisplatin-treated tissues show localization of n-NOS within the nerve fibers (*single arrows*) of the stomach smooth muscle, and within the cytoplasm of stomach villi (*double arrows*)

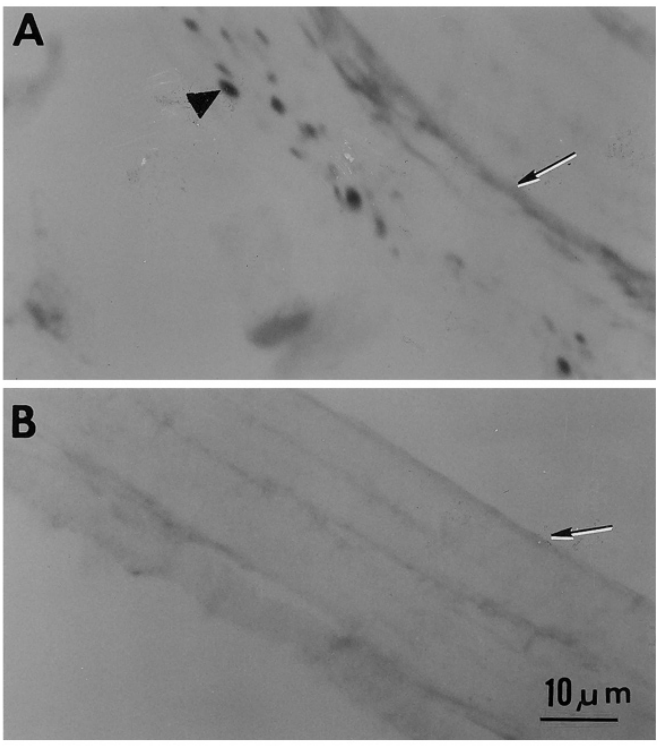

Fig. 2A, B Light micrographs of immunohistochemical localization of L-citrulline, observed as intense brown staining only in the stomach smooth muscle of the control rats (**A**). No such staining was observed after cisplatin treatment (**B**). *Arrowhead* neural ganglia, *arrows* nerve fibers

muscle in both the cisplatin-treated and control animals (Fig. 1). Nerve fibers of the muscular plexus were localized as dark blue striations within the smooth muscle of the stomach (Fig. 1). In tissues from control animals macrophages were intensely positive for NOS within the stomach (Fig. 1), but tissues from cisplatin-treated animals did not show any NOS within their macrophages (Fig. 1). Cross-sections of the villi from the stomachs of both the control and cisplatin-treated animals showed an intense reaction (Fig. 1).

L-Citrulline localization

Immunohistochemical studies demonstrated an intense reaction for L-citrulline in the neural ganglia, muscular plexus, and nerve fibers within the smooth muscle of the stomach and pyloric sphincter, and the stomach villi of control animals (Fig. 2).

Pyloric sphincter and stomach tissues from cisplatin-treated animals showed minimal to no localization of L-citrulline within the neural plexus and the neural ganglia of the smooth muscle (Fig. 2). Localization of L-citrulline was also depressed in cross-sections of the stomach villi of cisplatin-treated animals.

Calcium-calmodulin complex localization

Control animal tissues showed intense immunohistochemical localization of the Ca^{2+}-calmodulin complex in the smooth muscle of the pyloric sphincter as well as in Brunner's glands and both the longitudinal and circular muscle layers of the gastroduodenal junction (Fig. 3). The intensity of fluorescence of the nerve fibers within the deep muscular plexus was so great that nerve endings could not be easily distinguished from nerve fibers (Fig. 3).

When compared with tissues from control animals, tissues from cisplatin-treated animals showed a marked decrease in the localization of the Ca^{2+}-calmodulin complex within the smooth muscle of the pyloric sphincter and Brunner's glands and longitudinal and circular muscle layers at the gastroduodenal junction (Fig. 3). While nerve endings of the deep muscular plexus were labeled by the antibody, the nerve fibers themselves showed little to no labeling (Fig. 3).

Calcium-calmodulin binding: in vitro studies

After the addition of 50 μ*M* of EGTA, the excitation ratio of the MeroCam solution decreased by a factor of one (Fig. 4). This decrease was reversed by addition of 50 μ*M* $CaCl_2$.

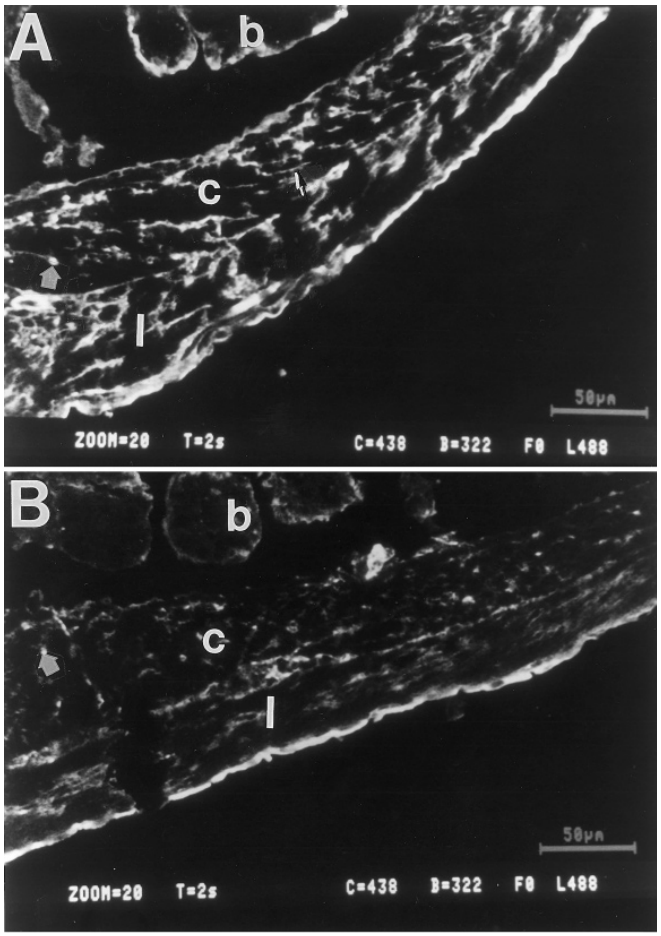

Fig. 3A, B Light micrographs indicating the presence of the Ca^{2+}-calmodulin-complex taken using a Zeiss 10 laser scanning confocal microscope in confocal mode with a 488-nm argon laser as the light source. Normal control tissue (**A**) demonstrates intense fluorescence within the gastroduodenal junction. Cisplatin-treated tissues (**B**) show a marked decrease in fluorescence (*c* circular muscle layer, *l* longitudinal muscle layer, *thin arrows* nerve fibers, *thick arrows* nerve endings, *b* (Brunner's glands)

Fluorescence was alternately decreased and increased by subsequent additions of 50 µ*M* EGTA and 50 µ*M* $CaCl_2$ respectively (Fig. 4).

MeroCam when subjected to increasing concentrations of calcium showed a 2.5-point increase in its excitation ratio, when compared with the baseline solution which received only double-distilled deionized water, with a peak in fluorescence at 80 µ*M* $CaCl_2$ (Fig. 5).

MeroCam that received increasing concentrations of the mixture of mono- and diaqua-cisplatin showed an initial two-point excitation ratio peak at 40 µ*M* but the excitation ratio declined afterwards ending with only a half-point increase in the excitation ratio compared with the baseline solution (Fig. 5).

With varying concentrations of diaqua-cisplatin fluorescence decreased dramatically with a 2.5-point decrease in its excitation ratio compared with the baseline solution (Fig. 5).

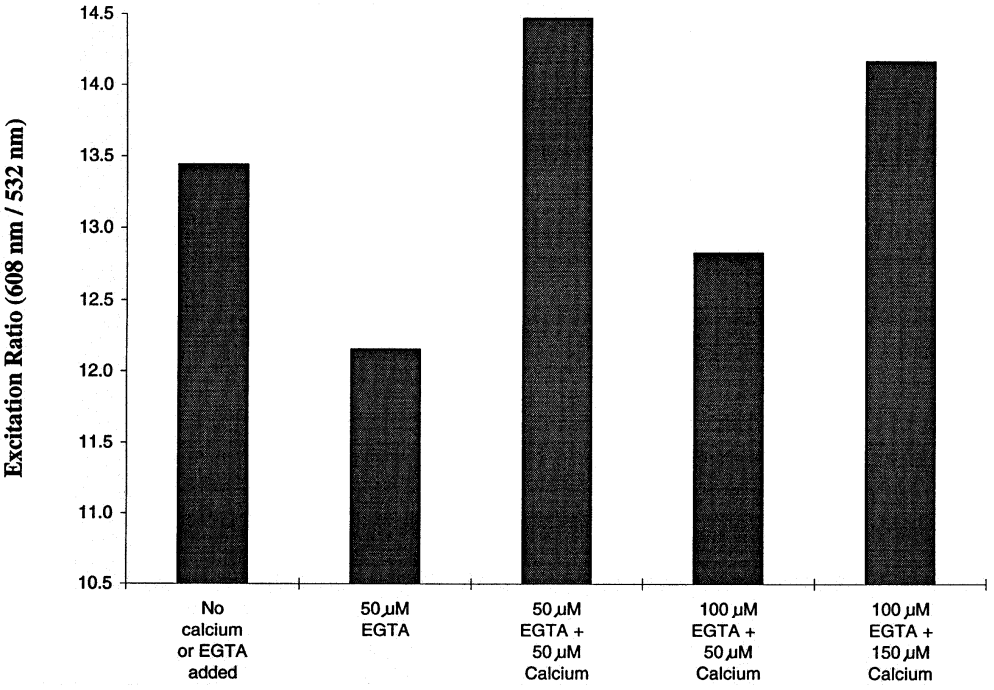

Fig. 4 Graph showing the fluorescence of 123 n*M* MeroCam when treated with alternating doses of 1 m*M* EGTA and 1 m*M* $CaCl_2$. Fluorescence of MeroCam decreased after the addition of EGTA. This decrease was reversed by subsequent addition of $CaCl_2$. This effect of EGTA was repeated through two more alternating additions of EGTA and $CaCl_2$

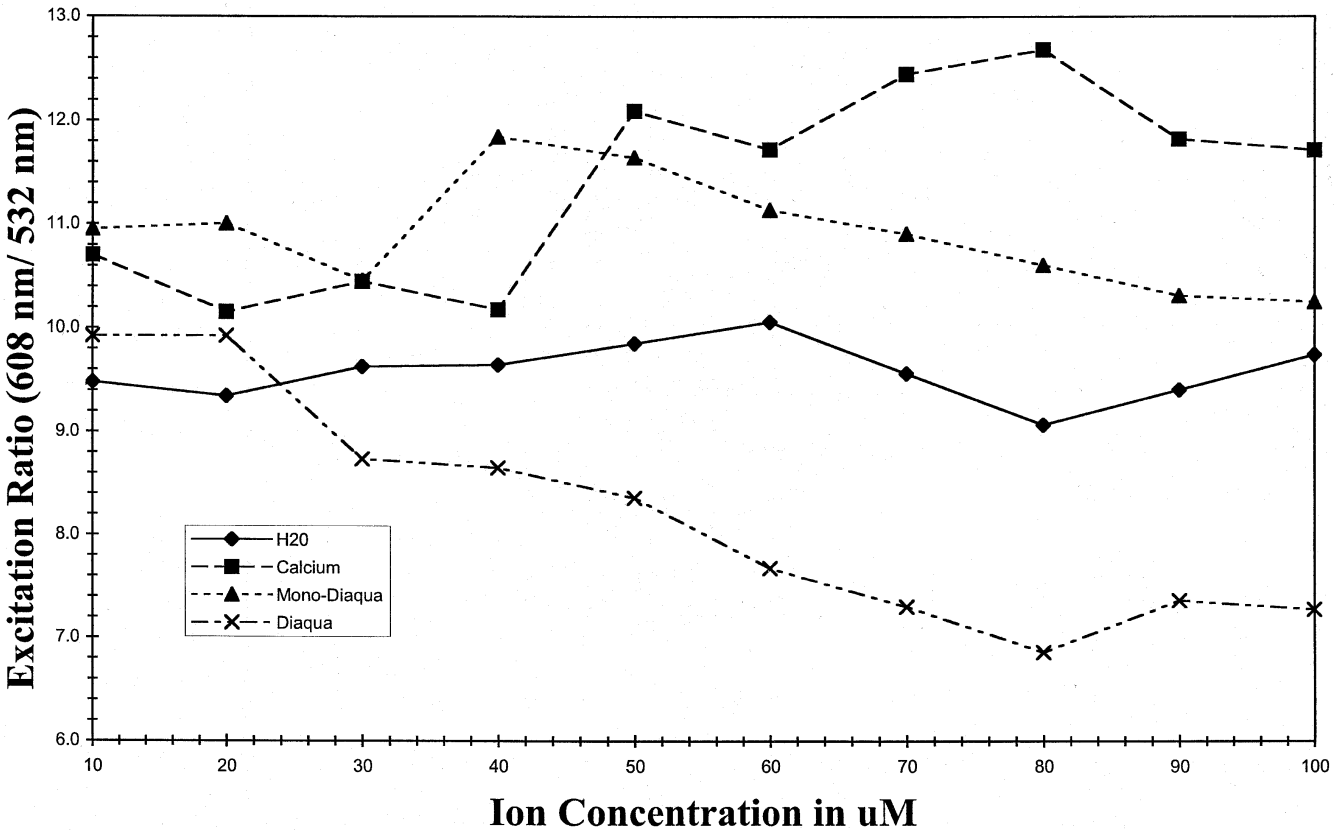

Fig. 5 Graph showing the fluorescence of 123 n*M* MeroCam when treated with varying concentrations of calcium, diaquacisplatin, double-distilled deionized water, and a mixture containing monoaqua- and diaqua-cisplatin. Note that the fluorescence of MeroCam decreased after diaqua-cisplatin treatment with the greatest decrease obtained at a concentration of 80 μ*M*

Discussion

The mechanisms of action of cisplatin are far from clear. It is generally accepted that cisplatin binds to DNA to prevent its replication or transcription, yet repair mechanisms have been shown to reverse this process [5]. Microtubular and microfilamentous depolymerization has also been suggested as a means of inhibiting cytokinesis [2]. However, these mechanisms do not explain the toxicities associated with this drug. Of these, nausea and vomiting is the dose-limiting factor [4]. We have learned to control these symptoms in part through diuresis and the use of antiemetic drugs but the cause of these toxicities still eludes us. Cisplatin treatment causes distention of the stomach in rodents as they do not show antiemetic responses [34]. Distention of the stomach has been shown to parallel the nausea and vomiting that is associated with the clinical use of cisplatin [34]. Thus, alleviation of stomach distention may hold the key to preventing the symptoms of nausea and vomiting associated with it, but the mechanism by which cisplatin induces distention of the stomach needs to be worked out before this can be achieved.

Normal stomach motility is controlled in part by two different neurotransmitter systems, one involving acetylcholine and the other NO. Both systems are initiated by an action potential-induced depolarization of the nerve plasma membrane that triggers the opening of calcium-gated channels [22]. The subsequent increase in cytosolic calcium causes an increase in the formation of the calcium-calmodulin complex. In the case of stomach smooth muscle contraction, this complex phosphorylates synapsin I, which in turn causes the uncoupling of acetylcholine vesicles from the cytoskeletal structure of stomach nerve terminals [22]. Once free from the cytoskeletal structure the vesicles proceed to the synaptic cleft where they are released, opening the sodium channels of the surrounding smooth muscle. This in turn triggers contraction of myofibrils causing muscle contraction (Fig. 6A) [22].

The calcium-calmodulin complex causes pyloric sphincter relaxation, however, by activating n-NOS [36]. Once activated, n-NOS converts L-arginine into L-citrulline and NO [32]. NO is then immediately released into the synaptic cleft where it easily diffuses into the surrounding smooth muscle and activates guanylate cyclase [22, 35]. This in turn causes an increase in the levels of cGMP which leads to smooth muscle relaxation.

Inhibition of acetycholine activity and accumulation of acetylcholine vesicles in the nerve terminals of stomach smooth muscle has been demonstrated after cisplatin treatment [4]. Histochemical localization of NOS showed that levels of the enzyme in the smooth muscle of both the pyloric sphincter and stomach were unaffected by cisplatin treatment. However, the level of L-citrulline

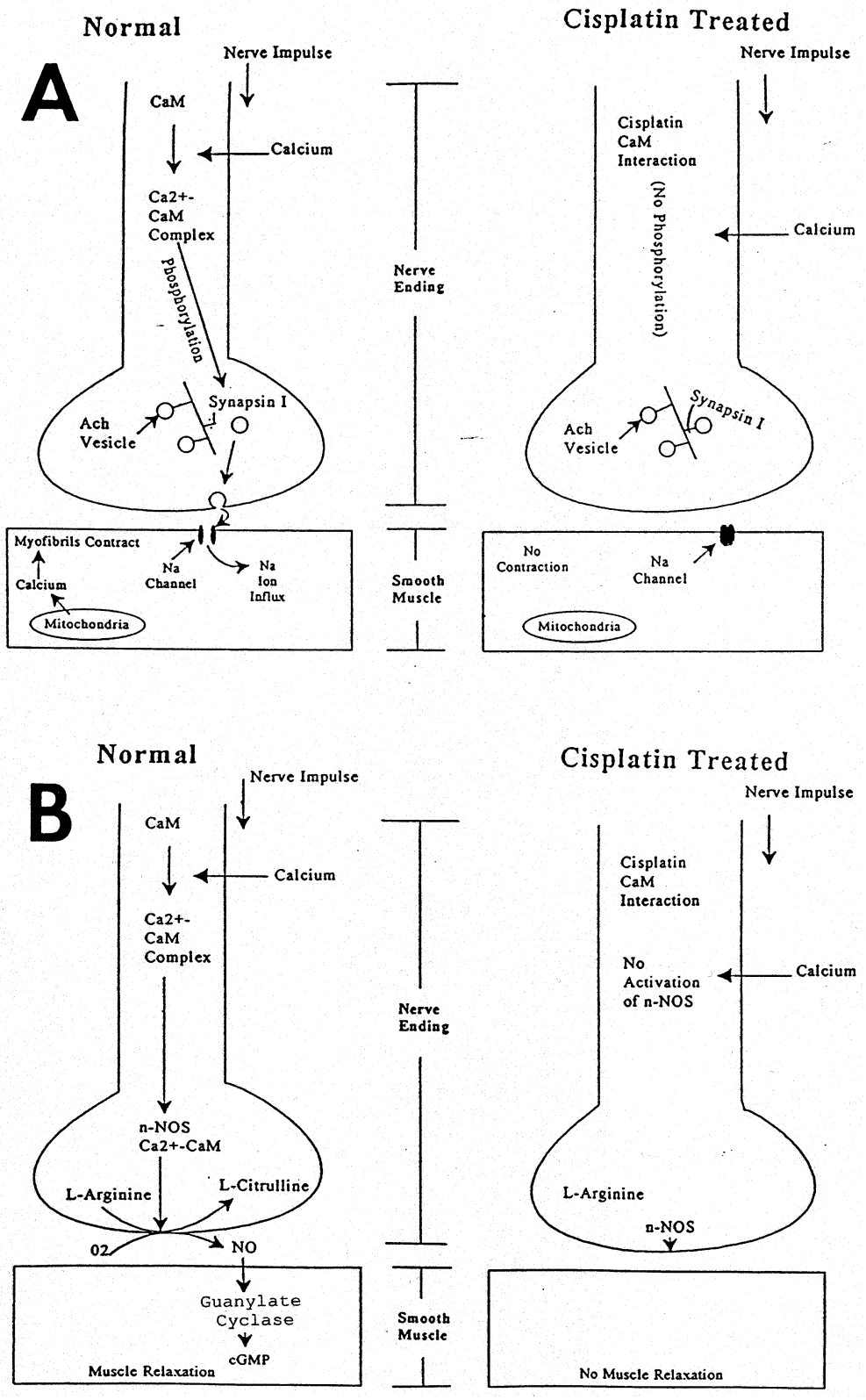

Fig. 6A, B Schematic representation of the interaction of acetylcholine (**A**) and NO (**B**) with the gastrointestinal smooth muscle and a possible mechanism by which cisplatin also interacts (*Ach* acetylcholine *CaM* calmodulin)

present in the neural ganglia and muscular plexus of the smooth muscle from cisplatin-treated animals was depressed compared with control animals. Thus, cisplatin treatment appears to inhibit the activation of n-NOS in the pyloric sphincter.

Acetylcholine release and n-NOS activation are regulated by the calcium-calmodulin complex [22, 36], and our studies show that cisplatin depresses the level of this complex. It is possible that this disruption of the calcium-calmodulin complex is responsible for the inhibition of

acetylcholine release and NO production that are seen with cisplatin treatment.

Cisplatin hydrolyzes into monoaqua (monovalent) and diaqua (divalent) species rapidly within the cell. Cations have been shown to interfere with the ability of calmodulin to bind to calcium, and the monovalent and divalent species of cisplatin have been shown to be the most toxic forms of the drug [1, 18, 19, 28].

In vitro tests on the effects of cisplatin's monoaqua and diaqua species have shown that addition of diaqua cisplatin causes a distinct and immediate decrease in the amount of calmodulin undergoing a conformational shift, whereas increased calcium levels cause an increase in the level of calmodulin undergoing a conformational shift associated with calcium binding [8, 16, 23]. These results suggest that diaqua cisplatin with its divalent charge inhibits the ability of calmodulin to undergo its conformational shift by competitively binding to the sites where calcium would normally bind. Thus, it is possible that n-NOS activation and acetylcholine vesicle release are prevented by a competitive inhibition of the calmodulin molecule.

Without normal n-NOS activation and acetylcholine vesicle release, stomach motility would be compromised. Specifically, stomach smooth muscle would undergo prolonged relaxation [4], and pyloric sphincter smooth muscle would become hypercontractile [17] with the end result being stomach distention which may induce nausea and vomiting [4, 17, 39]. Thus, a treatment that could reverse the effect of cisplatin on calmodulin might be extremely beneficial in the therapeutic application of the drug.

A possible method for the alleviation of the nausea and vomiting associated with the drug's toxic effects on stomach motility is the artificial elevation of calcium levels prior to and during cisplatin treatment. In rodents, such treatments have been shown to reduce stomach distention and ulceration, and return normal contractility to stomach smooth muscle [2]. This ability of artificially high calcium levels to reduce cisplatin-induced distention of the stomach is most likely due to a competition between the increased calcium and diaqua cisplatin for calmodulin binding sites.

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