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Inhibitory effect of organotin compounds on rat neuronal nitric oxide synthase through interaction with calmodulin

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Abstract

Organotin compounds, triphenyltin (TPT), tributyltin, dibutyltin, and monobutyltin (MBT), showed potent inhibitory effects on both L-arginine oxidation to nitric oxide and L-citrulline, and cytochrome *c* reduction catalyzed by recombinant rat neuronal nitric oxide synthase (nNOS). The two inhibitory effects were almost parallel. MBT and TPT showed the highest inhibitory effects, followed by tributyltin and dibutyltin; TPT and MBT showed inhibition constant (IC_{50}) values of around 10 μM. Cytochrome *c* reduction activity was markedly decreased by removal of calmodulin (CaM) from the complete mixture, and the decrease was similar to the extent of inhibition by TPT and MBT. The inhibitory effect of MBT on the cytochrome *c* reducing activity was rapidly attenuated upon dilution of the inhibitor, and addition of a high concentration of CaM reactivated the cytochrome *c* reduction activity inhibited by MBT. However, other cofactors such as FAD, FMN or tetrahydrobiopterin had no such ability. The inhibitory effect of organotin compounds (100 μM) on L-arginine oxidation of nNOS almost vanished when the amount of CaM was sufficiently increased (150–300 μM). It was confirmed by CaM–agarose column chromatography that the dissociation of nNOS–CaM complex was induced by organotin compounds. These results indicate that organotin compounds disturb the interaction between CaM and nNOS, thereby inhibiting electron transfer from the reductase domain to cytochrome *c* and the oxygenase domain.

Keywords: Neuronal nitric oxide synthase; Organotin compound; Triphenyltin; Tributyltin; Dibutyltin; Monobutyltin; Inhibitory effect; Calmodulin; Cytochrome *c* reduction

Organotin compounds have been used as heat stabilizers in vinyl chloride polymers, industrial catalysts for polyurethane foam and silicones, and biocides for bacteria and fungi. In particularly, tributyltin (TBT) and triphenyltin (TPT) have been widely used in antifouling paint for ships and aquaculture nets. Organotin compounds are toxic to marine species at low concentrations [1]. Molluscs are the most sensitive species, and shell malformations have been observed in oysters, as well as reduced growth and population decline [2,3]. Imposex, imposition of male characters in female organisms of some gastropod species, has been widely observed, and leads to reproductive failure and consequent population decline [4,5]. Recently, it was demonstrated that organotin compounds are neurotoxic and immunotoxic in mammals [6]. Contamination with organotin compounds has been reported in wild animals, especially in marine organisms [7–9]. TBT at levels up to 233 ng/g has been detected in mollusc products across Canada [10]. Organotin compounds also contaminate human food, especially shellfish, and water, mainly through contributions from industrial effluents and from leaching of polyvinyl chloride

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water pipes [7]. Humans exposed accidentally or occupationally to organotin compounds develop seizures, episodes of severe pain, and psychic disturbances [11,12]. A tolerable daily intake level for humans of 0.25 μg/kg body weight has been proposed [13]. Indeed, Kannan et al. [14] reported that butyltin compounds were detected in the order of monobutyltin (MBT) > dibutyltin (DBT) > TBT in human blood, and the average level of total butyltin compounds in blood collected from subjects in Michigan was 21 ng/ml, with a maximum of 101 ng/ml, which corresponds to the lower levels in sea mammalians. Industrial applications of organotin compounds and the consequent environmental release are of growing concern from the viewpoint of human health.

Nitric oxide synthase (NOS) catalyzes the synthesis of nitric oxide (NO) and L-citrulline from L-arginine with consumption of molecular oxygen and NADPH. NO contributes to a wide range of physiological functions including neurotransmission, blood pressure regulation, and immune responses. There are three isoforms of NOS, neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS), which share approximately 50% sequence homogeneity and have almost the same molecular architecture [15]. These NOSs are heme-thiolate proteins that catalyze the five electron-oxidation of L-arginine to NO and L-citrulline. These NOS molecules are composed of a C-terminal reductase domain that contains binding sites for NADPH, FAD, FMN, and CaM, and exhibits a high sequence identity to cytochrome P450 reductase, and a N-terminal oxygenase domain that contains binding sites for heme, tetrahydrobiopterin (H_4B), and L-arginine. CaM binding activates NO synthesis by enabling the reductase domain to transfer electrons to the oxygenase domain. The oxygenase domain provides the catalytic site for conversion of the substrate to the product and the reductase domain mediates transfer of electrons from NADPH to the oxygenase domain for the catalytic reactions. NOS functions in dimeric form and CaM promotes the dimerization [16–18]. An electron acceptor, such as cytochrome *c*, is reduced with NADPH in the reductase domain, and Ca–CaM is required for maximal activity to support electron transfer [19].

The inhibition of NOS enzymatic activity has severe consequences in animals. Thus, it is important to investigate the effects of chemical compounds, which might be taken up by animals, on the NOS activity. In this study, the effect of organotin compounds on nNOS activity was examined using TBT and TPT, as well as DBT and MBT, which are metabolites of TBT in animals [20,21], as substrates (Fig. 1). We found that organotin compounds inhibited nNOS activity in a reconstituted system, and the inhibitory effect was attributed to interference with the interaction between Ca–CaM and nNOS.

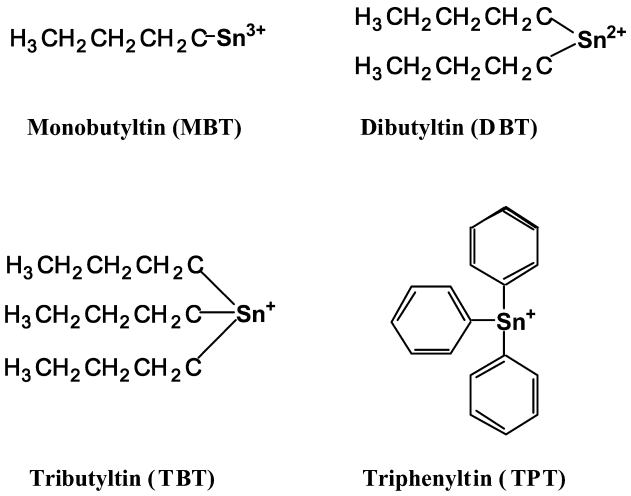

Fig. 1. Structures of organotin compounds.

Materials and methods

Materials. L-Arginine and L-citrulline were obtained from Nacalai Tesque (Kyoto, Japan). [2,3,4,5-^{3}H]L-Arginine monohydrochloride and L-[ureido-^{14}C]citrulline were from Amersham–Pharmacia Biotech (Uppsala, Sweden). (6R)-5,6,7,8-Tetrahydro-L-biopterin dihydrochloride was from Dr. B. Schircks Laboratories (Jona, Switzerland). [2,3,4,5-^{3}H]L-Arginine was purified with an HPLC cation-exchange column using a 0–0.2 M ammonium hydroxide gradient, according to the method of Iwanaga et al. [22]. Monobutyltin (MBT) was obtained from Acros Organics (Geel, Belgium) and tributyltin (TBT), dibutyltin (DBT), and triphenyltin (TPT) were from Tokyo Chemical Industry (Tokyo, Japan), respectively.

Preparation of nNOS. Plasmid containing rat nNOS cDNA in Bluescript SK(−) was kindly donated by Dr. Synder of Johns Hopkins Medical School, Baltimore, and pCWori was a gift from Dr. Dahlquist of Oregon University, Oregon [23,24]. The construction of pCWnNOS and protein expression were carried out according to the method of Roman et al. with some modification [25,26]. Briefly, *Escherichia coli* BL21 transfected with pCWnNOS was cultured in Terrific broth containing 50 μg/mL ampicillin. After lysozyme treatment and subsequent pulse sonication of the *E. coli*, the soluble fraction was loaded on a DEAE–Sephacel column and nNOS was eluted with an NaCl gradient from 0 to 200 mM. The nNOS fraction was subjected to FPLC (Amersham-Pharmacia Biotech, Uppsala, Sweden) with sequential chromatographies using 2′,5′-ADP–Sepharose 4B (Amersham Bioscience) and CaM–agarose columns (Sigma Chemical, St. Louis, MO). Purified nNOS was concentrated to about 60 μM in a MACROSEP (Pall Filtron, Northborough). The purity of nNOS was confirmed by SDS–PAGE and the preparation was stored in −80 °C. The concentration of nNOS was determined from the dithionite-reduced CO-differential spectrum, using $\Delta\varepsilon(444\text{–}490\ \text{nm}) = 91\ \text{mM}^{-1}\text{cm}^{-1}$[27].

Preparation of CaM. Rat calmodulin cDNA was kindly donated by Drs. Hayashi and Taniguchi of the Institute for Comprehensive Medical Science, Fujita Health University, Aichi, Japan. CaM was extracted from *E. coli* transfected with a plasmid containing the cDNA and purified using phenyl–Sepharose CL-4B column chromatography [28]. The purified CaM was lyophilized and stored at −80 °C.

Assay of cytochrome c and ferricyanide reduction activities. The standard reaction mixture was composed of 50 mM Hepes (pH 7.5), 2 mM $CaCl_2$, 200 mM NaCl, 1 μM FAD, 1 μM FMN, 100 nM CaM, and 50 μM cytochrome *c* (from horse heart, Sigma) or 100 μM potassium ferricyanide. Ten microliters of inhibitor solution in ethanol

was added to the reaction mixture (1 mL). nNOS (5 pmol) was mixed with 1 mL of the reaction mixture, and 10 μL of 5 mM NADPH was added to initiate the reaction. Rates of reduction of cytochrome *c* and ferricyanide were measured at 25 °C by monitoring the absorbance changes at 550 nm ($\Delta\varepsilon = 21\ mM^{-1}cm^{-1}$) for cytochrome *c*, and at 420 nm ($\Delta\varepsilon = 1.02\ mM^{-1}cm^{-1}$) for ferricyanide [29,30].

Assay of L-citrulline formation activity. The assay for L-citrulline production activity was performed as described [22]. The reaction mixture contained 50 mM Hepes (pH 7.5), 2 mM $CaCl_2$, 20 μM H_4B, 1 μM FAD, 1 μM FMN, 150 or 300 nM CaM, 0.1 mM DTT, 40 μM L-arginine, and 0.5 μCi [2,3,4,5-^{3}H]L-arginine in 0.2 mL. Ten microliters of ethanol solution of inhibitor was added to the mixture. nNOS (5 pmol) was added to 200 μL of the reaction mixture, and the reaction solution was pre-incubated at 25 °C for 30 s. The reaction was started by adding 10 μL of 5 mM NADPH and continued for 2 min. It was stopped by vigorous mixing of the solution with 100 μL of a stop solution composed of a mixture (2:3, v/v) of 2-propanol and an aqueous solution which contained 50 mM phosphoric acid, 50 mM sodium dihydrogenphosphate, and 37.5 mM SDS. After centrifugation at 400*g* for 10 min, the supernatant was loaded on an ODS-column (4 × 250 mm, LiChrospher 100 RP-18, Cica-Merck, Tokyo, Japan) in an HPLC system consisting of a PU980 HPLC pump (JASCO, Tokyo, Japan), a column oven (CO-965 JASCO), and a Gilson 204 fraction collector. Amino acid metabolites were eluted with a mixture (26:74, v/v) of 2-propanol and an aqueous solution containing 20 mM phosphoric acid, 20 mM sodium dihydrogenphosphate, and 15 mM SDS at a flow rate of 0.4 mL/min at the column temperature of 55 °C. The radioactivity of the separated metabolites was measured with a liquid scintillation counter (Aloka, LSC-700).

Detection of the dissociation of NOS–CaM by organotin compounds using CaM–agarose. Columns (0.3 × 0.3 cm) of CaM–agarose were preequilibrated at room temperature with 50 mM Hepes (pH 7.5), 2 mM $CaCl_2$, 200 mM NaCl, 1 μM FAD/FMN, 40 μM L-arginine, 10 μM H_4B, and 0.1 mM DTT. One hundred microliters of 2 μM NOS was loaded on each column and washed with more than 10 volumes of the above buffer. Elution of nNOS bound to CaM–agarose was examined with 100 μL of the above buffer containing 100 μM organotin compounds. Ten microliters of the input sample and 10 μL of the eluted sample were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) (6%) and bands were visualized by silver-staining (Wako Pure Chemical Industries, Osaka, Japan).

Results

Inhibitory effect of organotin compounds on L-arginine oxidase and cytochrome c reductase activities of nNOS

The inhibitory effects of organotin compounds, MBT, DBT, TBT, and TPT, on L-arginine oxidation to NO and L-citrulline (NOS activity) and cytochrome *c* reduction by nNOS were examined. When 100 μM MBT, DBT, TBT, and TPT was added in the presence of 150 nM CaM, NOS activity in terms of L-citrulline formation decreased to 8%, 25%, 33% and 24% of the control activity, respectively. Cytochrome *c* can accept an electron from the FMN moiety of nNOS. The inhibitory effect of organotin compounds on the cytochrome *c* reducing activity of nNOS was also measured at the inhibitor concentration of 100 μM. The organotin compounds MBT, DBT, TBT, and TPT inhibited the cytochrome *c* reducing activity of nNOS to 10–40% of the original value. The inhibition of cytochrome *c* reductase activity can be considered as arising from the disturbance of electron transfer from NADPH to FMN in the reductase domain of nNOS, since the inhibitory actions of organotin compounds on NOS activity and cytochrome *c* reducing activity of nNOS were similar to each other (Fig. 2). MBT and TPT exhibited the highest inhibitory effect in each case, followed by tributyltin and dibutyltin. TPT and MBT both have inhibition constant (IC_{50}) values of around 10 μM.

The dose dependency of the inhibitory effect of organotin compounds on cytochrome *c* reducing activity was examined. The activity was steeply decreased by MBT and TPT to 20 μM, but only gradually decreased by DBT and TBT (Fig. 3). Plots of inhibitory activity versus concentration gave K_i values for TPT, MBT,

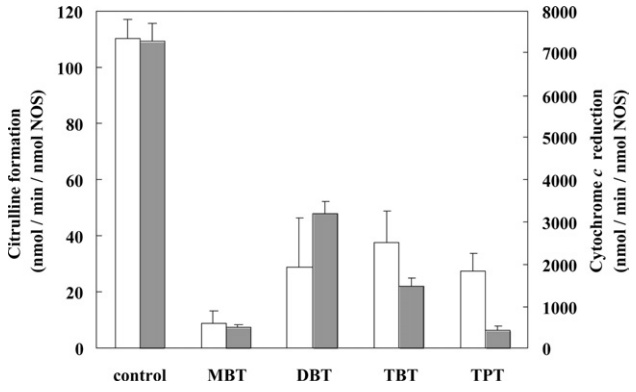

Fig. 2. Inhibitory effect of organotin compounds on L-citrulline forming activity and cytochrome *c* reducing activity of nNOS. Inhibition of L-citrulline forming activity (white bars) and cytochrome *c* reducing activity (gray bars) by monobutyltin (MBT), dibutyltin (DBT), tributyltin (TBT), and triphenyltin (TPT) is shown. Organotin compounds were added at 100 μM to the reaction solution. Details of the reaction conditions are given in Materials and methods. The values are averages of three measurements and the lines show standard errors.

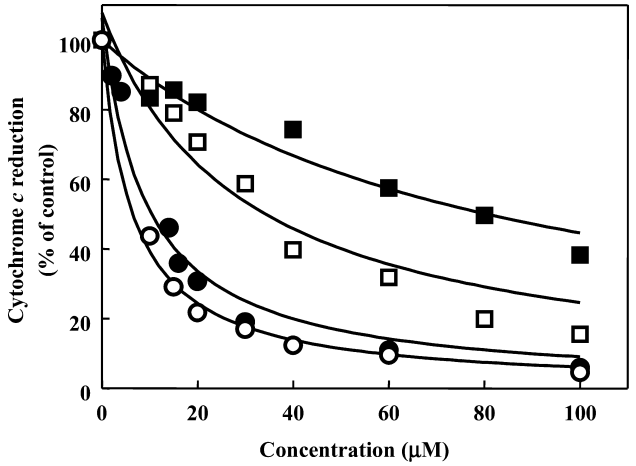

Fig. 3. Effects of concentration of DBT (closed squares), TBT (open squares), MBT (closed circles), and TPT (open circles) on cytochrome *c* reducing activity of nNOS. The reaction solution contained 100 nM calmodulin and 5 nM nNOS. Details of the reaction conditions are given in Materials and methods.

TBT, and DBT of 6 ± 1, 11 ± 2, 34 ± 4, and 80 ± 10 μM (average ± SE of three experiments), respectively, on the assumption that equimolar complexes were formed between nNOS and organotin compounds.

Effect of CaM on the inhibitory effect of organotins on cytochrome c reductase activity of nNOS

nNOS requires cofactors, FMN, FAD, H_4B, and Ca–CaM, for full activity. There is a possibility that organotin compounds might disturb the interaction between a cofactor(s) and nNOS. In order to examine the effects of MBT on the interaction, we removed one cofactor at a time from the complete reaction system composed of 0.1 μM FAD/FMN, 0.1 mM DTT, 10 μM H_4B, and 100 μM CaM, and 5 nM nNOS for cytochrome *c* reduction reaction. The removal of FAD/FMN, H_4B or DTT from the incubation mixture did not greatly affect the cytochrome *c* reducing activity of nNOS, but the removal of CaM decreased the activity to 10% of the original (Table 1). It should be noted that the removal of the cofactors, in this experiment, does not mean the complete removal of those cofactors from the NOS, but rather the absence of externally added cofactors from the reaction mixture. The addition of 25 μM MBT to the complete reaction system showed a similar effect on the activity to the removal of CaM. This result suggests that MBT may interfere with the interaction between CaM and nNOS.

It cannot be ignored that MBT might irreversibly damage the nNOS molecule and such damage might cause an inhibitory effect similar to the removal of CaM. The reversibility of the inhibitory effect was therefore examined by rapid dilution of MBT. The time course of cytochrome *c* reduction before and after the dilution of MBT is shown in Fig. 4A. The broken line shows the time course of cytochrome *c* reduction in the presence of 10 μM MBT, 100 nM CaM, and 10 nM nNOS (10 μM MBT solution), and the full line from 0 to 20 s shows that in the presence of 20 μM MBT, 100 nM CaM, and 20 nM nNOS, where the concentration of nNOS is twice that in the former case (20 μM MBT solution). The slope with 20 μM TBT solution (full line) is about half that with 10 μM solution (broken line), meaning that the presence of 20 μM MBT in 20 nM nNOS solution inhibits the nNOS activity to almost 1/4 of that in the presence of 10 μM MBT. At the reaction time of 20 s, a solution containing 100 nM CaM was added to the reaction solution containing 20 μM MBT, so that the composition of the reaction solution became the same as that of the 10 μM solution. The slope of 20 μM MBT solution after dilution was almost the same as that of 10 μM MBT solution. However, dilution containing other cofactors such as FAD, FMN or H_4B did not show such effect. These experimental results clearly show that the effect of MBT could be reversed by rapid dilution with the solution containing CaM, and MBT does not cause irreversible damage to nNOS.

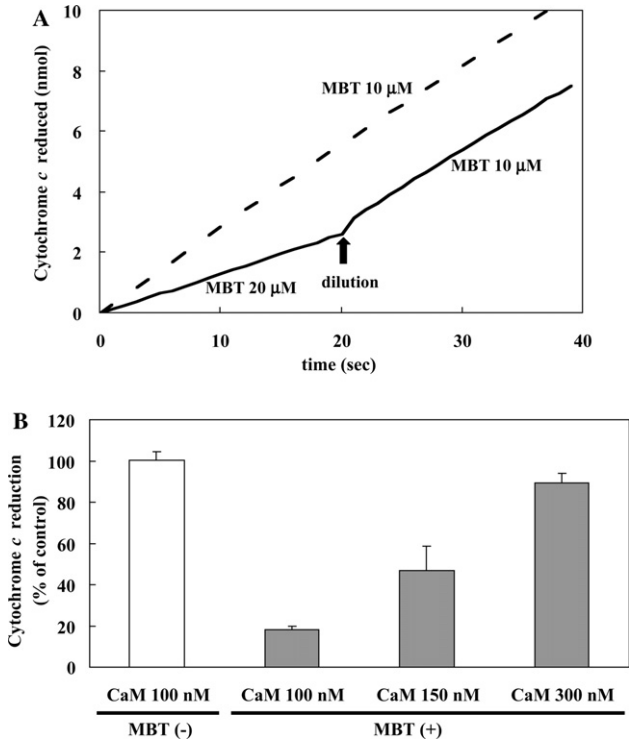

Fig. 4. The recovery of cytochrome *c* reducing activity of nNOS upon dilution (A) or addition of calmodulin (CaM) (B). In (A), the broken line shows the time course of cytochrome *c* reduction in the presence of 10 μM MBT, 100 nM CaM, and 10 nM nNOS and the full line from 0 to 20 s shows that in the presence of 20 μM MBT, 100 nM CaM, and 20 nM nNOS. At the reaction time of 20 s, a solution containing 100 nM CaM was added to the reaction solution corresponding to the full line and the resulting composition of the reaction solution became the same as that of the broken line reaction solution. In (B), cytochrome *c* reducing activity of nNOS (10 nM) was measured with CaM (100–300 nM) in the presence or absence of MBT (40 μM). The details of reaction conditions are given in Materials and methods.

Table 1
Effect of deletion of cofactors from the complete reaction mixture for cytochrome *c* reduction of nNOS

	Complete	−CaM	$-H_4B$	−FAD/FMN	−DTT
MBT (−)	8340 ± 320 (100)	618 ± 22.2 (7.4)	8458 ± 950 (101)	7457 ± 719 (90.5)	8185 ± 796 (98.1)
MBT (+)	1433 ± 508 (17.2)	400 ± 8.5 (4.8)	1256 ± 258 (15.1)	1315 ± 6.0 (15.8)	1136 ± 253 (13.6)

Each activity is presented as nmol/min/nmol NOS ± SE, and parentheses show the relative value with MBT (−) complete activity taken as 100. The composition of the reaction mixture and the reaction conditions were as described in Materials and methods.

Next, we examined the possibility that MBT might interfere with the binding of CaM to nNOS. In the presence of 100 nM CaM, the addition of 40 μM MBT decreased the activity to 20% of the original, but the addition of CaM to the concentration of 150 and 300 nM restored the activity to 50% and 90% of that in the absence of 40 μM MBT, respectively (Fig. 4B). These results suggest that MBT might compete with CaM for binding to nNOS.

CaM is an important factor in the sequential electron transfer process from NADPH to FAD, FMN, and heme in nNOS [31]. The effect of MBT on the electron transfer process from NADPH to FAD can be detected by monitoring the ferricyanide reduction activity of nNOS [30]. The experiments were performed under the same conditions as those in Fig. 2, where the CaM concentration and nNOS concentration were 100 and 5 μM, respectively. The inhibitory effect of MBT (100 μM) on ferricyanide reducing activity was not so marked (data not shown). The result is consistent with the reported effect of CaM on this step.

Effect of CaM on the inhibitory effect of organotins on NOS activity

Next, we examined the effect of CaM on inhibition of NOS activity (L-citrulline forming activity of nNOS) by organotin compounds. NOS activity in the presence of 150 nM CaM (Fig. 5, white bars) was decreased by the addition of MBT, DBT, TBT, and TPT (100 μM) to 8%, 25%, 33%, and 24 % of the control, respectively. However, the activity recovered markedly upon addition of a further 150 nM CaM to the reaction solution. In this case, NOS activity without any inhibitor increased by about 35%, showing that 150 nM CaM was not sufficient for full activity of 25 nM nNOS (Fig. 5). Further addition of CaM to 500 nM did not significantly increase the activity from that with 300 nM CaM (data not shown). It is remarkable that the increase of the CaM concentration from 150 to 300 nM removes the inhibitory effect of the organotin compounds on NOS activity. These results clearly show that the organotin compounds disturb the interaction between CaM and nNOS, but do not interact with the L-arginine binding site.

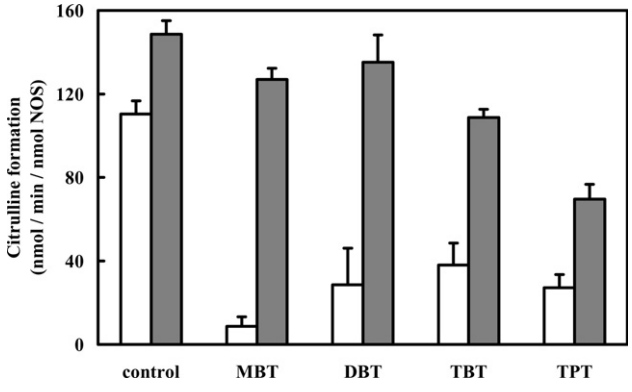

Fig. 5. The effect of addition of calmodulin (CaM) on the inhibition by organotin compounds of the L-citrulline forming activity of nNOS. Organotin compounds were added at 100 μM to the reaction solution containing 25 nM nNOS and 150 (white bars) or 300 nM calmodulin (gray bars). Details of the reaction conditions are given in Materials and methods. The values are averages of three measurements and the lines show standard errors. The activity of 150 nM CaM are the same data shown in Fig. 2.

Dissociation of nNOS from nNOS–CaM complex by organotin compounds

The dissociation of nNOS from nNOS–CaM complex in a solution of organotin compounds was detected at room temperature by using CaM–agarose. Two hundred picomoles of nNOS was loaded on the column, which was eluted with a buffer containing 100 μM organotin compound. Lane 1 in Fig. 6 shows the result of SDS–PAGE of the input sample alone, exhibiting a band of about 150 kDa due to nNOS. A faint band of nNOS was detected in lanes 3–6 which show the eluate from the CaM–agarose column with 100 μM organotin compounds, MBT (lane 3), DBT (lane 4), TBT (lane 5), and TPT (lane 6). The 150 kDa band intensities in lanes 3–6 were at most 10% of the input (lane 1). As

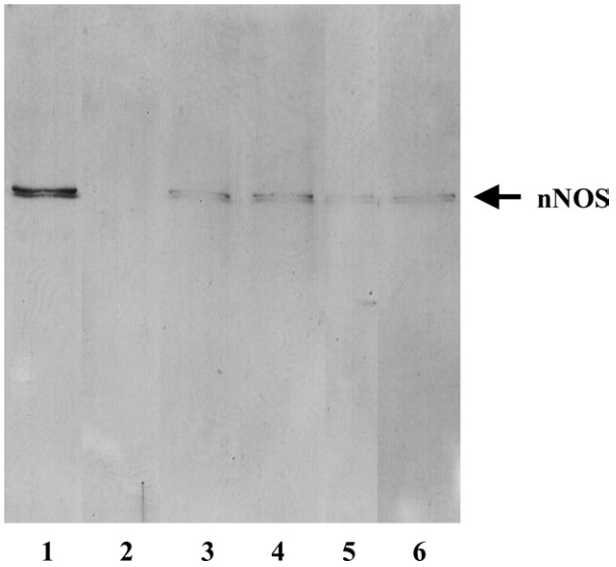

Fig. 6. Dissociation of nNOS–CaM complex induced by organotin compounds. One hundred microliters of 2 μM NOS was loaded on CaM–agarose columns and the columns were washed extensively. nNOS bound to CaM agarose was eluted with 100 μL buffer containing 100 μM organotin compound. Ten microliters of the input sample and 10 μL of the eluates were subjected to SDS–PAGE (6%) and bands were visualized by silver-staining. Lane 1 shows a band of the input sample to the column at the position corresponding to about 150 kDa. Lanes 3–6 show nNOS eluted from the CaM–agarose column with 100 mM organotin compounds, MBT (lane 3), DBT (lane 4), TBT (lane 5), and TPT (lane 6). Lane 2 shows the eluate in the absence of organotin compounds.

shown in lane 2, no observable nNOS was eluted in the absence of organotin compounds. These results indicate that organotin compounds induce dissociation of nNOS from nNOS–CaM agarose complex.

Discussion

In this study we examined the effect of organotin compounds on nNOS activity using recombinant rat nNOS. Here, we present the first evidence that organotin compounds inhibit nNOS activity by blocking the interaction of CaM and nNOS. Inhibitors of nNOS are used to study the functions of NO in living organisms. *N*-Methyl-L-arginine is a widely used inhibitor of NOS, and any physiological response that is inhibited by *N*-methyl-L-arginine might be mediated by NO [32]. Several other arginine derivatives are known to inhibit the activity by competition with arginine at the arginine binding site of NOS [33]. In contrast, the NOS reaction requires electrons from NADPH, and chemicals which interfere with the electron flow can inhibit NOS function. It is well known that NOS reaction is strictly regulated by Ca^{2+}, which binds to CaM, and Ca^{2+}–CaM activates the NOS reaction. It is also known that nNOS requires Ca–CaM for the reduction of cytochrome *c* by NADPH at the reductase domain [19]. MBT decreased the cytochrome *c* reducing activity to a level similar to that without CaM in the reaction system. This suggests that MBT might interfere with the interaction between CaM and nNOS. Most of the activity was recovered by the addition of a high concentration of CaM, as shown in Fig. 4. This is consistent with the reversible recovery of the cytochrome *c* reducing activity by the dilution of MBT. Ferricyanide reduction, which is conducted at the FAD binding site, was unaffected by all organotin compounds examined. These results clearly show that organotin compounds compromise the ability to donate electrons to cytochrome *c* and the oxygenase domain by interfering with the interaction between nNOS and CaM (Fig. 7). Guan and Iyanagi [30] reported that CaM binding to the reductase domain facilitates intramolecular one-electron transfer from FAD to FMN. This is consistent with our finding that organotin compounds inhibited cytochrome *c* reduction, but not ferricyanide reduction. In other words, organotin compounds inhibit electron transfer from FAD to FMN, which is facilitated by CaM. The inhibitory effect of individual organotin compounds on the cytochrome *c* reducing activity of nNOS corresponded well to that on the activity of L-citrulline and NO formation from L-arginine and molecular oxygen, which is reasonable, because both inhibitions originate from the disturbance of CaM binding to nNOS. Some peptides and proteins inhibit CaM binding to nNOS and prevent the formation of NO [34,35]. Several chemicals, including calmidazolium and melatonin, have also been reported to disturb CaM binding to NOS and to inhibit electron transfer between the two domains, resulting in inhibition of the NOS activity [36–38]. The anti-estrogenic drug tamoxifen has been reported to inhibit both the L-arginine oxidase and cytochrome *c* reductase activities of nNOS by interfering with CaM binding to nNOS [38].

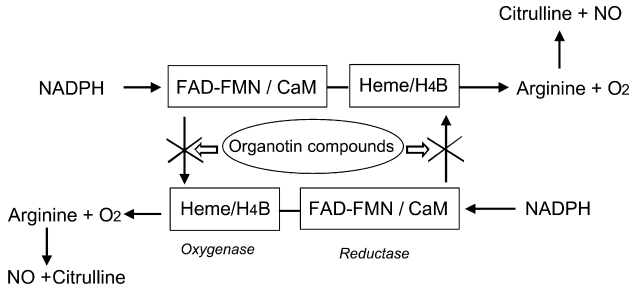

Fig. 7. Postulated mechanism for the inhibitory effect of organotin compounds on nNOS.

In order to confirm the interference of organotin compounds with the interaction between nNOS and CaM molecules, we loaded purified nNOS on a CaM–agarose column. After extensive washing, some of the nNOS could be eluted from the columns with a solution containing 100 μM organotin compounds, as shown in Fig. 6. This result clearly demonstrates the dissociation of nNOS from CaM–NOS complex by organotin compounds. However, the eluted nNOS was less than 10% of the input amount. It was expected that almost all the bound nNOS might be eluted by 100 μM MBT, based on the dependence of nNOS activity on MBT concentration. The data in Fig. 3 were obtained in the presence of 100 nM CaM, but the effective CaM concentration in the agarose column is expected to be much higher, though its value cannot be easily calculated. At higher concentrations of CaM, the effects of inhibitors became much weaker, as shown in Fig. 4 and Fig. 5. Another possible reason for the weak effect of organotin compounds might be absorbance of the compounds on the agarose resin, which would reduce the effective inhibitor concentration.

Ca–CaM regulates not only nNOS but also a number of other enzyme systems. Stimulation of Ca-ATPase of rat brain synaptic membranes by CaM is inhibited by organotin compounds in the order TBT > triethyltin > TMT. The inhibition was removed by increasing of the CaM concentration, indicating that the inhibitory mechanism may be similar to that for nNOS shown in the present study [39,40]. CaM plays many important roles in the regulation of synaptic functions, synthesis of neurotransmitters, activation of kinase, and modulation of membrane receptors. The disturbance of CaM binding to regulated proteins, including NOS, by organotin compounds is likely to have a serious effect on signaling systems. Further examination of the effect of organotin compounds on CaM binding to other regulatory proteins than nNOS is necessary. We found that

double reciprocal plots of the activity in the presence of MBT against CaM concentration did not fit well to a simple competitive inhibition mechanism (data not shown). It is well known that NOS functions in the dimeric form, and nNOS molecules in the dimeric form may bind to two CaM molecules; organotin compounds might interfere with this process. The amount of nNOS in the dimeric form can be measured by using low-temperature SDS–PAGE, and it has been shown that L-arginine and H_4B synergistically support homodimer formation, while CaM has no influence on it [41]. Our preliminary experiments on the effects of organotin compounds on the dimerization of nNOS using low-temperature SDS–PAGE showed that the compounds had no significant effect on the monomer–dimer equilibrium of nNOS (data not shown). Allosteric-type inhibition of nNOS by organotin compounds could be involved instead. Further studies are in progress.

Organotin compounds are serious environmental pollutants. An endocrine-disruptive effect of these compounds has been reported in wild animals, especially in marine organisms [42]. Contamination with organotin compounds is roughly equivalent to 0.1–10 μM in the whole animal body [8]. This is almost the same as the concentration used in this study. In humans, organotin compounds are ingested through food, marine animals, and cooked food that has been in contact with plastic packaging or with plastic gloves worn by workers in the food industry [43]. Organotin compounds are reported to pass through the blood–brain barrier, but their concentrations in brain are yet not known. Organotin compounds might well influence signaling systems, including the nNOS system, in the brain, and they might be neurotoxic.

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