

FIGURE 5: Subcellular localization of GFP-tagged CHP1: (A) subcellular localization of GFP-tagged wild-type CHP1 (a, b), EF3m (c), EF4m (d), and EF34m (e) expressed in PS120 cells (a) or in their stable transfectants of the wild-type NHE1 (b–e); (B) effect of various agents on the subcellular localization of the wild-type GFP-tagged CHP1 in NHE1 transfectants. Cells were placed in serum-free Dulbecco's modified Eagle's medium without phenol red for 5 h, and then 1 μ M PMA, 10% serum, or 2 units/ml thrombin were added. GFP fluorescence was observed under a fluorescent microscope equipped with a CoolSNAP imaging system (RS Photometrics) before (control) and 20 min after addition of the various agents.

acid, or PDGF-BB, which are all known to activate the exchange activity (Figure 5B, data not shown for some experiments). We also found that the plasma membrane localization of GFP fluorescence did not change upon addition of metabolic inhibitors (2-deoxyglucose plus oligomycin) that cause cell ATP depletion, thus inhibiting exchange activity (data not shown). Furthermore, we found no changes in the plasma membrane localization of GFP-tagged CHP1 mutants EF3m and EF4m after these various treatments (data not shown). These observations suggest that CHP1 is tightly associated with NHE1 in the plasma membrane and that this interaction is not affected by various stimuli.

All the cells expressing CHP1–GFP or its mutant derivatives exhibited high Na^+/H^+ exchange activity. The $^{22}\text{Na}^+$ uptake activity in cells clamped at acidic pH_i (5.6) by the $\text{K}^+/\text{nigericin}$ technique was in the range of 20–50 nmol/

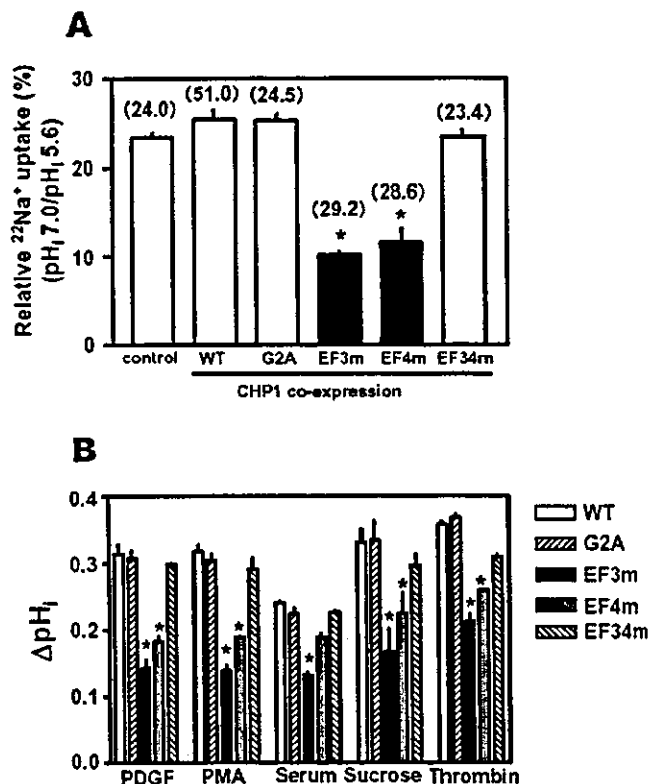


FIGURE 6: Exchange activity and regulation of NHE1 transfectants expressing various CHP1 mutants. Panel A shows ratios of EIPA-sensitive $^{22}\text{Na}^+$ uptake activities of cells coexpressing wild-type NHE1 and various CHP1 mutants at pH_i 7.0 and 5.6. Numbers in parentheses represent $^{22}\text{Na}^+$ uptake activity at pH_i = 5.6. Control cells were not transfected with CHP1 but stably expressing NHE1. Data are means \pm SD (n = 3; *, P < 0.05 versus cells not expressing exogenous CHP1). Panel B shows changes in pH_i measured using the ^{14}C benzoic acid equilibration method. The cells coexpressing NHE1 and various CHP1 variants were stimulated for 15 min at 37 $^\circ\text{C}$ with 10 ng/mL PDGF-BB, 1 μ M PMA, 10 $\mu\text{g}/\text{mL}$ lysophosphatidic acid, or 200 mM sucrose (hyperosmotic stress). Data are means \pm SD (n = 6; *, P < 0.05 versus cells expressing wild-type CHP1).

mg/min (data not shown). We compared the $^{22}\text{Na}^+$ uptake activities in cells expressing various CHP1 variants in the physiological pH_i range. As shown in Figure 6A, the ratio of $^{22}\text{Na}^+$ uptake at pH_i 7.2–5.6 was not significantly altered by expression of wild-type CHP1. Although a previous study (11) indicated that overexpression of CHP1 inhibits the NHE1 activity in the presence of serum, we observed no such CHP1-induced inhibition of the exchange activity. The reason for this discrepancy is unknown. Unlike the wild-type CHP1, the $^{22}\text{Na}^+$ uptake ratio was significantly reduced by EF3 or EF4 mutants. Consistent with this finding, we observed that mutations of EF3 or EF4 significantly reduced the cytoplasmic alkalization in response to PDGF-BB, thrombin, phorbol ester, serum, or hyperosmotic stress (sucrose) (Figure 6B). These observations suggest that mutation of EF3 or EF4 partly impairs the regulation of NHE1 by reducing pH_i sensitivity. In contrast, double mutation (EF34m) of CHP1 at EF3 and EF4 did not reduce the $^{22}\text{Na}^+$ uptake ratio or cytoplasmic alkalization (Figure 6A,B), consistent with the finding that this mutant CHP1 is not able to replace the endogenous CHP1 because of its weak interaction with NHE1. Finally, mutation of the myristoylation site (G2A) or EF1 did not affect pH_i -dependent regulation of NHE1.

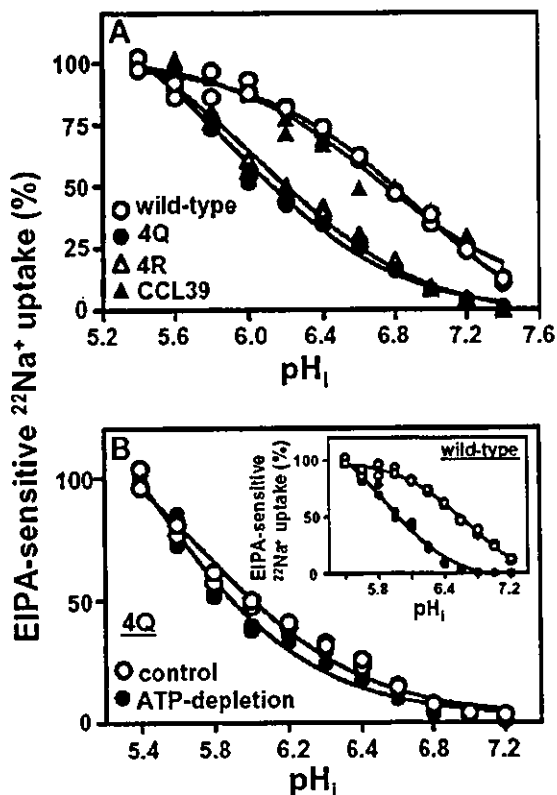


FIGURE 7: pH_i dependence of exchange activity in cells expressing some NHE variants. Panel A shows the pH_i dependence of ²²Na⁺ uptake in PS120 cells expressing wild-type NHE1 or CHP1 binding-defective mutants 4Q and 4R and CCL39 fibroblastic cells (the parental cell line of PS120). pH_i was clamped at various values with K⁺/nigericin. The maximal EIPA-sensitive ²²Na⁺ uptake activity measured at pH_i = 5.4 was high in cells expressing wild-type NHE1 (~50 nmol/mg/min), while it was lower but similar in cells expressing 4Q or 4R or in CCL39 cells (4.2, 4.2, or 4.1 nmol/mg/min, respectively). Data were normalized according to the maximal activity at pH_i = 5.4. Panel B shows the effects of ATP depletion on pH_i dependence of exchange activity in cells expressing 4Q or wild-type NHE1 (inset). Cells were depleted of ATP by treatment with the metabolic inhibitors 2-deoxyglucose (5 mM) and oligomycin (2 μg/mL). Data were normalized according to the maximal activity at pH_i = 5.4.

Properties of NHE1 Mutants Lacking CHP1 Binding. As described above, mutations of CHP1 partly impair pH_i-dependent regulation of NHE1. Therefore, it is of interest to determine how CHP1 binding affects the pH_i sensitivity of NHE1. Previously, we described two CHP1 binding-defective mutant exchangers, 4Q or 4R, in which Phe⁵²⁶, Leu⁵²⁷, Leu⁵³⁰, and Leu⁵³¹ of NHE1 were replaced by four glutamine or arginine residues, respectively (12) (see Figure 4B). In this study, by using extensive H⁺-killing selection, we obtained cells overexpressing 4Q or 4R and exhibiting relatively high activity (~4 nmol/mg/min at pH_i 5.4), thus allowing reliable measurement of the pH_i dependence of ²²Na⁺ uptake. As shown in Figure 7A, these mutations caused a marked acidic shift in the pH_i dependence (Figure 7A). As a control, we confirmed that CCL39 cells (the parental cell line of PS120) that exhibit exchange activity (V_{max}) comparable to 4Q or 4R show a pH_i dependence of exchange similar to that of PS120 cells overexpressing NHE1. In cells expressing these mutant exchangers, ATP depletion did not change the pH_i sensitivity of ²²Na⁺ uptake (Figure 7B). In addition, cytoplasmic alkalization in response to extracel-

lular stimuli, such as thrombin, PDGF-BB, hyperosmolarity, LPA, and PMA, was not observed in cells expressing 4Q or 4R (data not shown), consistent with the finding that these mutants exhibit an acidic shift of pH_i dependence.

DISCUSSION

In this study, we examined the role of CHP1, particularly its EF-hand Ca²⁺ binding motifs, in the pH_i-dependent regulation of NHE1. Our results indicated that a Ca²⁺ ion binds to each of EF3 and EF4 in CHP1 with an overall apparent K_d of ~90 nM and a Hill coefficient of ~1.0 (Table 1). This Ca²⁺ binding affinity was close to that of another family member, CaN-B (apparent K_d ≈ 70 nM) (36), although the apparent K_d values for Ca²⁺ in other EF-hand Ca²⁺ binding proteins vary widely (0.01–10 μM) (36–39). Although CHP1 potentially has four Ca²⁺ binding motifs, the two ancestral sites EF1 and EF2 do not bind Ca²⁺. This is in sharp contrast to CaN-B in which all four EF-hand motifs are able to bind Ca²⁺, although the two N-terminal sites, EF1 and EF2, have lower affinity for Ca²⁺ than the C-terminal sites, EF3 and EF4 (36). Intriguingly, the Ca²⁺ affinity of CHP1 increased markedly upon complex formation with the NHE1 fragment (aa 503–545). Consistent with this finding, ⁴⁵Ca²⁺ release from the complex was much slower than that from CHP1 alone. The extraordinarily high affinity of CHP1 for Ca²⁺ (~2 nM) suggests that the CHP1/NHE1 complex always contains two Ca²⁺ ions under physiological conditions.

The high affinity for Ca²⁺ was also observed in mutant CHP1 proteins EF3m and EF4m, which have a single Ca²⁺ binding site, complexed with the NHE1 fragment. Increases in the affinity for Ca²⁺ by interaction with target proteins have also been reported for other Ca²⁺ binding proteins. For example, the Ca²⁺ binding affinity for calmodulin was increased 16- to 38-fold upon interaction with myosin light chain kinase (40), 2.6-fold with myristoylated alanine-rich protein kinase C substrate peptide (41), and 75-fold with the calmodulin binding peptide in CaN-A (36). CHP1 was reported to interact with other proteins, such as microtubules (25), CaN-A (24), DRAK2 (26), and KIF1Bβ2 (27), as well as members of the NHE1 family. Therefore, interaction with these proteins may also modify the Ca²⁺ binding affinity of CHP1.

Although mutation of CHP1 at either EF3 or EF4 impaired binding of 1 mol of Ca²⁺, it did not appear to affect the interaction of CHP1 with NHE1 as shown by *in vitro* binding of these mutant proteins, coimmunoprecipitation, and the plasma membrane localization of GFP-tagged CHP1. Therefore, these mutations do not appear to induce marked structural distortions. However, double mutation (EF34m) at both EF3 and EF4 impaired the interaction of CHP1 with NHE1. Consistent with this finding, Ca²⁺ removal by EGTA from the wild-type CHP1 reduced the interaction with NHE1 in a pull-down assay through the amyrose resin column (data not shown). Thus, the tight association of NHE1 with CHP1 requires binding of at least one Ca²⁺ on either EF3 or EF4. Furthermore, it should be noted that the effect of double mutation (EF34m) on regulation of NHE1 cannot be properly analyzed in cells that express endogenous CHP1.

We found that expression of NHE1, but not the CHP1 binding-deficient mutant derivatives 4Q and 4R, significantly

increased the amount of endogenous CHP1. However, coexpression of GFP-tagged CHP1 proteins (wild-type, EF3m, and EF4m) preserving the strong interaction with NHE1 greatly reduced the amount of endogenous CHP1. Thus, the amount of endogenous CHP1 in cells is strongly dependent on the number of available CHP1 binding sites provided from NHE1. Although the precise reason for this is unknown, it is likely that interaction with target proteins is required for stable expression of CHP1. That is, dissociation from the target proteins may promote CHP1 degradation.

In this study, we found that CHP1 binding-defective mutants of NHE1 (4Q and 4R) caused a marked acidic shift in the pH_i dependence of Na^+/H^+ exchange activity and completely impaired ATP depletion-induced inhibition and cytoplasmic alkalization in response to various stimuli. As we reported previously (32), not only mutation of the CHP1 binding region, but also deletions of different regions in the amino-terminus (subdomain I, amino acids 515–595) of the NHE1 cytoplasmic domain also markedly reduced pH_i sensitivity. Thus, subdomain I with bound CHP1 appears to be a key structure that permits the putative “ pH -sensor” to maintain a physiologically relevant conformation.

We found that mutation of EF3 or EF4 in CHP1 significantly reduced the Na^+/H^+ exchange activity in the physiological neutral pH_i range and reduced the cytoplasmic alkalization in response to various extracellular signals by decreasing the pH_i sensitivity of NHE1. Thus, mutation of each EF-hand in CHP1 somehow affects the pH_i -sensing of NHE1, although we could not evaluate the function of the double mutant CHP1 (EF34m) because of its weak interaction with NHE1. We found that EF3m, EF4m, and EF34m proteins migrated slowly on SDS–PAGE, suggesting that significant conformational changes of CHP1 occur upon mutation of each EF-hand. Such conformational changes appear to be due to removal of Ca^{2+} rather than the amino acid substitution itself, because incubation with EGTA resulted in similar slow migration of the wild-type CHP1 on SDS–PAGE (data not shown). Thus, the bound Ca^{2+} may play an important role in maintaining the CHP1 structure, thereby preserving the physiological pH_i sensitivity of NHE1.

According to the structural model of CHP1 deduced from the three-dimensional structure of CaN-B (43), EF-hand Ca^{2+} binding motifs would be located on the surface opposite the side where CHP1 binds to NHE1. It is likely that the surface of CHP1 with tightly bound Ca^{2+} controls the pH_i -sensing by interacting with other region(s) of NHE1. We observed that the pH_i sensitivity of NHE1 was markedly reduced by insertion of one amino acid residue (alanine) just to the N-terminal side (position aa 504 or 508) of the CHP1 binding site of NHE1, while the CHP1 binding ability was preserved (our unpublished observations). Therefore, the correct spatial orientation of CHP1 would be important for regulation of NHE. Recently, we reported that mutation of Arg⁴⁴⁰ in intracellular loop 5 (IL5), which connects transmembrane helices 10 and 11, markedly reduces the pH_i sensitivity of NHE1 (44). Thus, IL5 may interact with the CHP1 surface with tightly bound Ca^{2+} .

Many EF-hand Ca^{2+} binding proteins are known to regulate the functions of their target proteins in response to cytosolic Ca^{2+} mobilization. However, it is unlikely that CHP1 functions as such a Ca^{2+} sensor in the regulation of NHE1 because the affinity for Ca^{2+} ($K_d = \sim 2$ nM) for the

CHP1/NHE1 complex differs substantially from the range of intracellular Ca^{2+} concentrations (0.1–10 μ M). Instead, two EF-hand motifs of CHP1 together with tightly bound Ca^{2+} would serve as structurally important elements for preserving the normal function of NHE1, as discussed above. Such a structural role has also been suggested in the C-terminal EF-hand motifs in CaN-B (42) and in CaM (41). On the other hand, we reported previously that CaM interacts in a Ca^{2+} -dependent manner with the middle of the cytoplasmic domain of NHE1, which in the unstipulated state serves as an auto inhibitory domain decreasing the pH_i sensitivity of NHE1 (14, 15). The interaction of NHE1 with CaM is strictly Ca^{2+} -dependent, although it is much weaker than that with CHP1 (14). Previously, we proposed that NHE1 may be activated by Ca^{2+} -dependent interaction of CaM in response to Ca^{2+} -mobilizing signals (15). Our previous (14, 15) and several recent reports (45–48) reinforced the idea that CaM serves as an important regulatory protein in activation of NHE1 in response to hyperosmotic stress or Ca^{2+} -mobilizing agonists. NHE1 thus appears to be dually regulated by two Ca^{2+} binding proteins, CHP1 and CaM, similar to CaN-A. The former would preserve the physiological pH_i sensitivity of NHE1, whereas the latter would play a role in sensing cytosolic Ca^{2+} .

In summary, our current results suggest that the interaction of CHP1 with NHE1 is crucial for preserving the physiological pH_i sensitivity of NHE1 and that tightly bound Ca^{2+} serves as an important structural element that is required for this role. The significant effects of mutations in EF-hands on NHE1 regulation prompted us to generate a more efficient dominant negative mutant CHP1. In addition, the functional difference between CHP1 and CHP2, which we reported recently (13), provides important information for identification of the critical residues of CHP1. Further studies including analyses of the functions of mutated or chimerical CHP1 and determination of the crystal structure of CHP1/NHE1 complex are required to elucidate the molecular mechanism of CHP regulation of NHE1 and other NHE family members.

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Short communication

In vivo assessment of catechol *O*-methyltransferase activity in rabbit skeletal muscle

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Abstract

With the use of microdialysis technique in the anesthetized rabbit, we examined the catechol *O*-methyltransferase (COMT) activity at the skeletal muscle interstitium. We implanted a dialysis probe into the adductor muscle, and monitored dialysate catecholamines and their metabolites with chromatogram-electrochemical detection. Administration of COMT inhibitor (entacapone) decreased dialysate 3-methoxy 4-hydroxyphenylglycol (MHPG) levels. Local administration of dihydroxyphenylglycol induced increases in dialysate MHPG levels. These increases in dialysate MHPG levels were suppressed by the addition of entacapone. The concentration of MHPG in the skeletal muscle dialysate corresponded to the COMT activity in the skeletal muscle. Furthermore, local administration of norepinephrine or epinephrine increased nonmetanephrine or metanephrine levels in dialysate but not MHPG levels. Skeletal muscle microdialysis with local administration of catecholamine offers a new method for in vivo assessment of regional COMT activity.

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Keywords: Catecholamine; Catechol *O*-methyltransferase; Entacapone; Microdialysis; Skeletal muscle

Catechol *O*-methyltransferase (COMT) exerts a critical action on the inactivation of catecholamines and catechol estrogens (Boulton and Eisenhofer, 1998). COMT enzyme exists in almost all mammalian tissues and organs (Karhunen et al., 1994; Männistö and Kaakkola, 1999). The wide distribution of COMT in different tissues suggests an important physiological role for COMT activity. In vitro COMT activity has been widely assessed in various tissues (Männistö and Kaakkola, 1999; Tsunoda et al., 2002), while in vivo COMT activity has been assessed only in erythrocyte (Toumainen et al., 1996). To determine whether COMT activity is involved in cardiovascular regulation, we need information about in vivo COMT activity in organs and tissues.

A sophisticated technique using radiotracers has been employed for spillover of organ specific metabolite formed by COMT activity (3-methoxy 4-hydroxyphenylglycol, MHPG) (Lambert et al., 1995). This study suggested that majority of MHPG in plasma was derived from skeletal

muscle, with the exception of central nervous system. Dispersed organs, such as skeletal muscle, have a thin and diffuse sympathetic innervation, but skeletal muscle is one candidate suitable for investigating regional MHPG production (Tokunaga et al., 2003a,b). This organ is suited to microdialysis probe implantation. Recently we have developed the skeletal muscle microdialysis for the monitoring of catecholamines and their metabolites. At the skeletal muscle, the small amounts of dialysate norepinephrine and its metabolites could be determined by microdialysis with electrochemical detection.

In the present study, we examined whether COMT blocker affected regional norepinephrine kinetics at the skeletal muscle interstitial spaces. With the use of dialysis technique, the dialysate was sampled from the skeletal muscle, and dialysate catecholamines and their metabolites levels were measured with liquid chromatography. Further, the study was designed to examine regional *O*-methylation products evoked by local administration of catecholamine and determine whether these data provide information about in vivo regional COMT activity.

Male Japanese white rabbits weighing 2.6–3.1 kg each were anesthetized with pentobarbital sodium (30–35 mg/kg,

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i.v.). The level of anesthesia was maintained with a continuous intravenous infusion of pentobarbital sodium (1–2 mg/kg/h). After tracheotomy, the animals were ventilated with room air mixed with oxygen. Body temperature was maintained with a heated pad and lamp. All protocols were performed in accordance with the American Physiological Society guidelines for the use of animals. After a longitudinal skin incision was made in the left groin, the dialysis probes were implanted in the left adductor muscle with a fine guiding needle.

For skeletal muscle dialysis, we designed a transverse dialysis probe. The dialysis fiber (13 mm length, 0.31 mm O.D. and 0.2 mm I.D.; PAN-1200, 50,000 molecular mass cutoff, Asahi Chemical, Tokyo, Japan) was glued at both ends into a polyethylene tube (25 cm length, 0.5 mm O.D. and 0.2 mm I.D.) (Akiyama et al., 1991; Tokunaga et al., 2003a,b). The dialysis probe was perfused with Ringer solution at a speed of 10 μ l/min using a microinjection pump (CMA 102, Carnegie Medicin, Stockholm, Sweden). Dialysate catecholamines and their metabolite concentrations were measured by high-performance liquid chromatography with electrochemical detection (Takauchi et al., 1997; Tokunaga et al., 2003a,b; Yamazaki et al., 1995).

Basal dialysate norepinephrine, dihydroxyphenylglycol (DHPG) and MHPG levels were presented in Table 1. Entacapone (COMT blocker) was intraperitoneally administered (10 mg/kg) (Illi et al., 1995; Scheinin et al., 1998). Administration of entacapone decreased the MHPG level of dialysate but increased the DHPG levels of dialysate. The dialysate norepinephrine levels were not affected by entacapone. These changes were preserved 2 h after administration of entacapone.

To examine regional COMT activity, we measured the formation of MHPG evoked by local administration of exogenous DHPG via dialysis probe. We determined doses of DHPG based on the dialysate DHPG concentration in the previous experiments (Akiyama and Yamazaki, 2001). Local administration of DHPG (25, 250 ng/ml) dose-dependently increased the MHPG levels of dialysate (Fig. 1). These increases in the MHPG levels were prevented by pretreatment with entacapone.

In this study, exogenous DHPG dose-dependently increased the MHPG levels of dialysate. Exogenous DHPG via the dialysis probe easily traversed the cell membrane and reached skeletal muscle (Goldstein et al., 1998). In contrast, entacapone significantly decreased the MHPG levels of

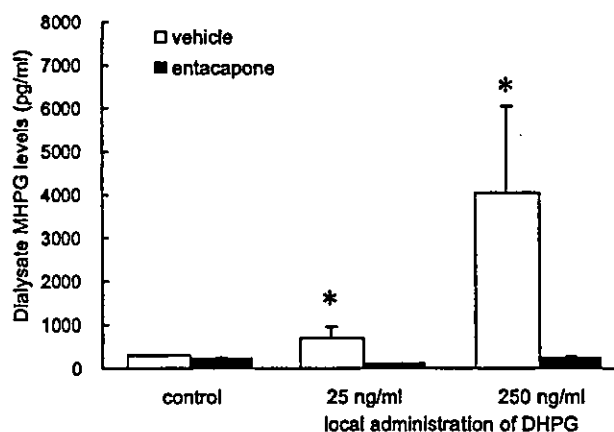


Fig. 1. Effects of exogenous dihydroxyphenylglycol (DHPG) infusion on the 3-methoxy 4-hydroxyphenylglycol (MHPG) production. Local administration of DHPG dose-dependently increased the MHPG levels of dialysate. These increases in the MHPG levels were prevented by pretreatment with entacapone. Values are means \pm SE ($n=5$). * $P<0.05$ vs. control.

dialysate. These data suggest that regional COMT activity corresponds to the production of dialysate MHPG levels. Furthermore, entacapone induced a decrease in the dialysate MHPG level accompanied by an increase in the dialysate DHPG but not norepinephrine level. Therefore we consider that regional DHPG is one possible substrate for MHPG production, and that the concentration of MHPG or MHPG/DHPG ratio in the skeletal muscle dialysate might correspond to the COMT activity in the skeletal muscle.

Earlier studies suggested species and organ differences in extraneuronal uptake and COMT activity (Scheinin et al., 1998; Tsunoda et al., 2002). Extraneuronal norepinephrine uptake and COMT activity were well examined in rabbit heart with the findings suggesting that rabbit heart hardly metabolizes isoprenaline to methoxyphenaline (Lindmar and Löffelholz, 1974). Thus rabbit heart seems to have a very poorly developed extraneuronal system, including weak COMT activity, for the uptake and metabolism of catecholamines (Trendelenburg, 1978). On the other hand, rabbit aortic strips have a high capacity for COMT activity (Levin, 1974). From these and previous data (Tokunaga et al., 2003a,b), the ratio of MHPG/DHPG in myocardium and skeletal muscle were 1.0 ± 0.2 and 7.9 ± 1.3 , respectively. Rabbit skeletal muscle seems to have a well-developed COMT activity. In the skeletal muscle sympathetic innervation was not dense, and the DHPG levels were less than that of heart (Tokunaga et al., 2003a,b). Therefore, other compounds or plasma DHPG might be involved in the regional formation of MHPG in the skeletal muscle.

MHPG is produced by extraneuronal *O*-methylation of DHPG formed intraneuronally from norepinephrine or by the extraneuronal combination of COMT and monoamine oxidase (MAO) on norepinephrine and epinephrine (Akiyama and Yamazaki, 2001; Eisenhofer et al., 1988). Therefore, MHPG is mainly yielded from DHPG, norepinephrine or epinephrine at the skeletal muscle. Furthermore,

Table 1
Basal dialysate NE, DHPG, and MHPG levels in rabbit skeletal muscle

	Before entacapone	After entacapone
NE (pg/ml)	8 \pm 1	10 \pm 1
DHPG (pg/ml)	27 \pm 4	53 \pm 11*
MHPG (pg/ml)	198 \pm 12	147 \pm 18*

NE, norepinephrine; DHPG, dihydroxyphenylglycol; MHPG, 3-methoxy 4-hydroxyphenylglycol. Values are means \pm SE. $n=5$.

* $P<0.05$ vs. values before entacapone.

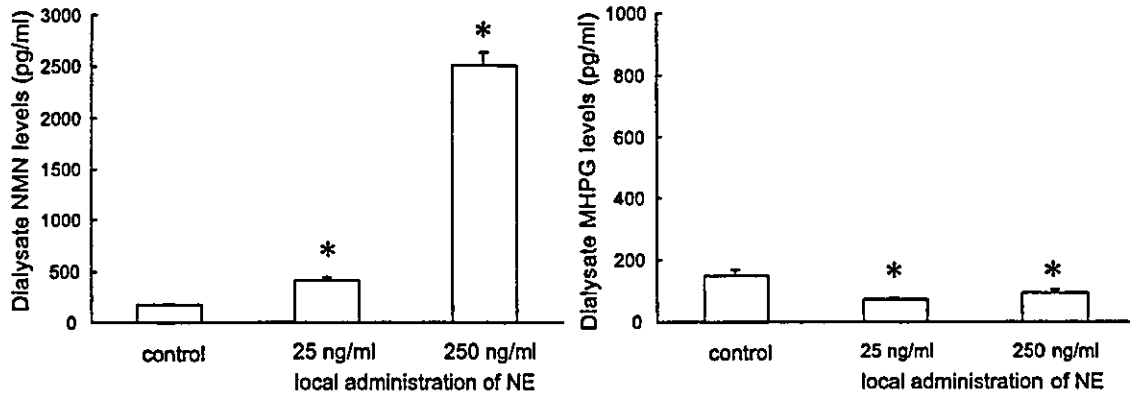


Fig. 2. Effects of exogenous norepinephrine (NE) infusion on the 3-methoxy 4-hydroxyphenylglycol (MHPG) and normetanephrine (NMN) production. Local administration of NE dose-dependently increased the NMN levels of dialysate but not MHPG levels. Values are means \pm SE ($n=5$). * $P<0.05$ vs. control.

O-methylation of catechol compounds includes MHPG, normetanephrine and metanephrine. We examined the relation between catecholamines and their metabolites. To compare norepinephrine and epinephrine with DHPG infusion, norepinephrine or epinephrine infusion with similar doses of DHPG was administered. Local administration of norepinephrine increased the normetanephrine levels of dialysate but not the MHPG levels (Fig. 2). Local administration of epinephrine increased the metanephrine levels of dialysate but not the MHPG levels (Fig. 3). Our data suggest that only DHPG is a possible substrate for MHPG production. Local administration of norepinephrine or epinephrine produced normetanephrine or metanephrine but not MHPG. Or rather, norepinephrine or epinephrine caused a decrease in the dialysate MHPG level. These data are consistent with data on the origins of plasma MHPG in rats, which indicated that most MHPG arises from *O*-methylation of the DHPG by intraneuronal deamination of norepinephrine (Eisenhofer et al., 1994).

Our data indicate that COMT exerts an important role on the degradation of catecholamines in the skeletal muscular interstitium. Muscular catecholamines derive from circulating blood and surrounding sympathetic nerve systems (Tokunaga et al., 2003a,b). Therefore, COMT activity in

the skeletal muscle may be related to regional or systemic sympathetic nerve activity. The relationship between regional COMT activity and sympathetic nerve activity remains to be further examined. Muscle sympathetic nerve activity is involved in the regulation of vascular tone and glucose metabolism in the skeletal muscle (Lundvall and Edfeldt, 1994; Spraul et al., 1994). Further studies concerning the physiological role of regional COMT activity on vascular or metabolic control are warranted.

To our knowledge, this is the first report on the *in vivo* assessment of COMT activity by direct measurement of dialysate MHPG, normetanephrine, and metanephrine obtained from skeletal muscle. Local administration of DHPG increased the MHPG levels of dialysate. These increases in MHPG were prevented by pretreatment with a COMT inhibitor. Therefore we consider that the concentration of MHPG in the skeletal muscle dialysate might correspond to the COMT activity in the skeletal muscle. Measurement of MHPG/DHPG ratio or MHPG formation evoked by DHPG infusion in skeletal muscle may be particularly appropriate for providing information about regional COMT activity. Thus skeletal muscle microdialysis with local administration of catecholamine offers a new method for *in vivo* assessment of regional COMT activity.

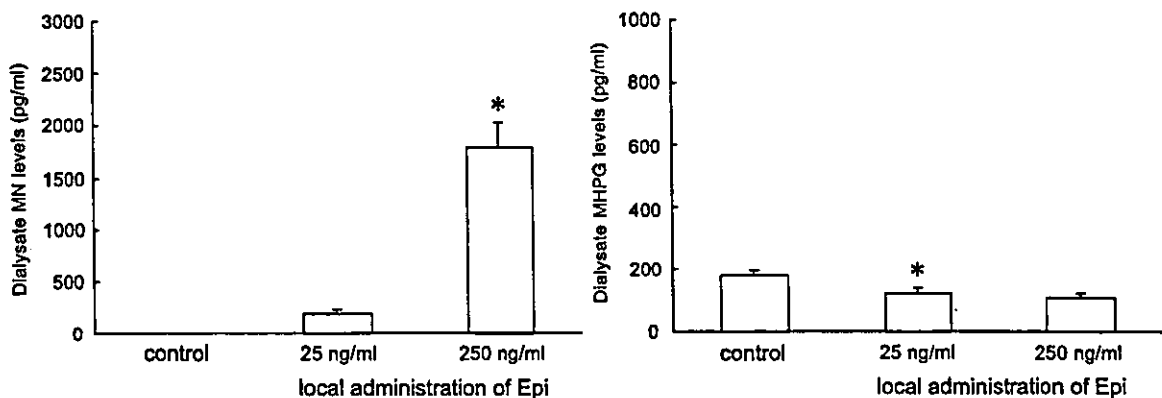


Fig. 3. Effects of exogenous epinephrine (Epi) infusion on the metanephrine (MN) and 3-methoxy 4-hydroxyphenylglycol (MHPG) production. Local administration of Epi increased the MN levels of dialysate but not MHPG levels. Values are means \pm SE ($n=5$). * $P<0.05$ vs. control.

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Effects of Ca²⁺ channel antagonists on acetylcholine and catecholamine releases in the in vivo rat adrenal medulla

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Akiyama, Tsuyoshi, Toji Yamazaki, Hidezo Mori, and Kenji Sunagawa. Effects of Ca²⁺ channel antagonists on acetylcholine and catecholamine releases in the in vivo rat adrenal medulla. *Am J Physiol Regul Integr Comp Physiol* 287: R161–R166, 2004. First published March 18, 2004; 10.1152/ajpregu.00609.2003.—To elucidate the types of voltage-dependent Ca²⁺ channels controlling ACh and catecholamine releases in the in vivo adrenal medulla, we implanted microdialysis probes in the left adrenal medulla of anesthetized rats and investigated the effects of Ca²⁺ channel antagonists on ACh, norepinephrine, and epinephrine releases induced by nerve stimulation. The dialysis probes were perfused with Ringer solution containing a cholinesterase inhibitor, neostigmine. The left splanchnic nerves were electrically stimulated at 2 and 4 Hz before and after intravenous administration of Ca²⁺ channel antagonists. ω -Conotoxin GVIA (an N-type Ca²⁺ channel antagonist, 10 μ g/kg) inhibited ACh release at 2 and 4 Hz by ~40%, norepinephrine release at 4 Hz by ~50%, and epinephrine release at 2 and 4 Hz by ~45%. A fivefold higher dose of ω -conotoxin GVIA (50 μ g/kg) did not further inhibit these releases. ω -Conotoxin MVIIC (a P/Q-type Ca²⁺ channel antagonist, 50 μ g/kg) inhibited ACh and epinephrine releases at 4 Hz by ~30%. Combined ω -conotoxin GVIA (50 μ g/kg) and MVIIC (250 μ g/kg) inhibited ACh release at 2 and 4 Hz by ~70% and norepinephrine and epinephrine releases at 2 and 4 Hz by ~80%. Nifedipine (an L-type Ca²⁺ channel antagonist, 300 and 900 μ g/kg) did not change ACh release at 2 and 4 Hz; however, nifedipine (300 μ g/kg) inhibited epinephrine release at 4 Hz by 20%, and nifedipine (900 μ g/kg) inhibited norepinephrine and epinephrine releases at 4 Hz by 30%. In conclusion, both N- and P/Q-type Ca²⁺ channels control ACh release on preganglionic splanchnic nerve endings while L-type Ca²⁺ channels do not. L-type Ca²⁺ channels are involved in norepinephrine and epinephrine releases on chromaffin cells.

anesthetized rats; microdialysis; norepinephrine; epinephrine; preganglionic autonomic nerve endings

Ca²⁺ INFLUX through the voltage-dependent Ca²⁺ channels induces the release of transmitters from neuronal or secretory cells by initiating exocytosis from vesicles. Voltage-dependent Ca²⁺ channels have been classified into L-, N-, P-, Q-, R-, and T-types (12, 25, 30). To better understand the mechanism controlling the release of transmitters, it is important to determine the type of Ca²⁺ channels involved in the release of the transmitters on neuronal or secretory cells.

In the in vivo adrenal medulla, catecholamine release is controlled by central sympathetic neurons through preganglionic splanchnic nerves. Splanchnic nerve endings make synaptic-like contacts with chromaffin cells (9). ACh released from splanchnic nerve endings consequently evokes catecholamine release from chromaffin cells by activation of cholin-

ergic receptors. Thus, in vivo catecholamine release requires Ca²⁺ influx through the voltage-dependent Ca²⁺ channels at two different sites in the adrenal medulla: splanchnic nerve endings and chromaffin cells. Numerous studies have investigated the nature of Ca²⁺ channels controlling transmitter release from postganglionic autonomic nerve endings (8, 11, 32, 33, 36, 37). Little information is, however, available on the type of Ca²⁺ channels controlling the ACh release from preganglionic autonomic nerve endings including splanchnic nerve endings. Moreover, although the types of Ca²⁺ channels controlling catecholamine release have been investigated using isolated chromaffin cells in various species (5, 6, 13, 16, 21, 23, 24), it remains unknown whether endogenous ACh induces Ca²⁺ influx through the same types of Ca²⁺ channels on chromaffin cells.

We have recently developed a dialysis technique to simultaneously monitor ACh and catecholamine releases in the in vivo adrenal medulla (2). This method makes it possible to characterize Ca²⁺ channels controlling ACh release from splanchnic nerve endings and catecholamine release from adrenal medulla in the in vivo state. In the present study, we applied the microdialysis technique to the adrenal medulla of anesthetized rats and investigated the effects of Ca²⁺ channel antagonists on dialysate ACh and catecholamine responses induced by the electrical stimulation of splanchnic nerves.

MATERIALS AND METHODS

Animal preparation. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85–23, revised 1996). Adult male Wistar rats weighing 380–450 g were anesthetized with pentobarbital sodium (50–55 mg/kg ip). A cervical midline incision was made to expose the trachea, which was then cannulated. The rats were ventilated with a constant-volume respirator using room air mixed with oxygen. The left femoral artery and vein were cannulated for monitoring arterial blood pressure and administration of anesthetic, respectively. The level of anesthesia was maintained with a continuous intravenous infusion of pentobarbital sodium (15–25 mg·kg⁻¹·h⁻¹ iv). Electrocardiogram was monitored for recording heart rate. A thermostatic heating pad was used to keep the esophageal temperature within a range of 37–38°C. With the animal in the lateral position, the left adrenal gland and left splanchnic nerve were exposed by a subcostal flank incision, and the left splanchnic nerve was transected. Shielded bipolar stainless steel electrodes were applied to the distal end of the nerve, which was then stimulated with a digital stimulator (SEN-7203, Nihon Kohden) with a rectangular pulse (10 V and 1 ms in duration).

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Dialysis technique. The materials of the dialysis probe were the same as those used in our previous dialysis experiments (1, 2). Briefly, each end of the dialysis fiber (0.31 mm OD, and 0.20 mm ID; PAN-1200 50,000 mol wt cutoff, Asahi Chemical) was inserted into the polyethylene tube (25-cm length, 0.5 mm OD, and 0.2 mm ID; SP-8) and glued. The length of the dialysis fiber exposed was 3 mm.

The left adrenal gland was gently lifted, and the dialysis probe was implanted in the medulla of the left adrenal gland along the long axis by using a fine guiding needle. The dialysis probe was perfused with Ringer solution containing a cholinesterase inhibitor, neostigmine (10 μ M), at a speed of 10 μ l/min using a microinjection pump (CMA/100, Carnegie Medicin). Ringer solution with no buffer consisted of (in mM) 147.0 NaCl, 4.0 KCl, and 2.25 $CaCl_2$. One sampling period was 2 min (1 sample volume = 20 μ l). We started the protocols followed by a stabilization period of 3–4 h and sampled dialysate taking the dead space volume into account.

Dialysate ACh, norepinephrine (NE), and epinephrine (Epi) concentrations were measured as indexes of ACh and catecholamine releases in the adrenal medulla. Half of the dialysate sample was used for the measurement of ACh, and the remaining half for the measurement of NE and Epi. ACh and catecholamine assays were separately conducted using each high-performance liquid chromatography with electrochemical detection as previously described (3, 4).

Experimental design. The experiment was performed based on the previous experiment showing that dialysate ACh and catecholamine responses were reproducible on repetition of stimulation (2). The left splanchnic nerves were electrically stimulated for 2 min at 30-min intervals. Three dialysate samples were continuously collected per electrical stimulation: one before, one during, and one after stimulation. Stimulations at two different frequencies (2 and 4 Hz) were performed before and after intravenous administration of Ca^{2+} channel antagonists.

We tested three types of Ca^{2+} channel antagonists (25): the N-type Ca^{2+} channel antagonist ω -conotoxin GVIA, the P/Q-type Ca^{2+} channel antagonist ω -conotoxin MVIIC, and the L-type Ca^{2+} channel antagonist nifedipine. We determined the first doses of Ca^{2+} channel antagonists based on the dose used in the earlier experiments (7, 14, 26, 29, 37) and tested ω -conotoxin GVIA (10 μ g/kg) in six rats, ω -conotoxin MVIIC (50 μ g/kg) in six rats, and nifedipine (300 μ g/kg) in six rats. Second, we tested a fivefold higher dose of ω -conotoxin GVIA (50 μ g/kg) in six rats, a combination of fivefold higher doses of ω -conotoxin GVIA (50 μ g/kg) and MVIIC (250 μ g/kg) in six rats, and a threefold higher dose of nifedipine (900 μ g/kg) in six rats. We did not test a higher dose of ω -conotoxin MVIIC singly because a high dose of ω -conotoxin MVIIC loses its selectivity for P/Q-type and inhibits N-type Ca^{2+} channels (18).

Nifedipine was administered twice before 2- and 4-Hz stimulation, but ω -conotoxin GVIA and MVIIC were administered once before 2-Hz stimulation because the ω -conotoxin family has long-lasting blocking actions (8, 18, 36). We assessed the responses to nerve stimulation 30, 20, and 10 min after administration of ω -conotoxin GVIA, ω -conotoxin MVIIC, and nifedipine, respectively, when heart rate and arterial pressure had already been stabilized.

At the end of the experiment the rats were killed with pentobarbital sodium, and the implant sites were examined. The dialysis probes were confirmed to have been implanted in the adrenal medulla, and no bleeding or necrosis was found macroscopically.

Drugs. Drugs were mixed fresh for each experiment. Neostigmine methylsulfate (Shionogi), ω -conotoxin GVIA (Peptide Institute), and ω -conotoxin MVIIC (Peptide Institute) were dissolved and diluted in Ringer solution. Nifedipine (Sigma Chemical) was dissolved in ethanol and diluted in Ringer solution.

Statistical methods. To examine the effects of nerve stimulation and Ca^{2+} channel antagonists, we analyzed heart rate and mean arterial pressure and dialysate ACh, NE, and Epi responses by using one-way ANOVA with repeated measures. When statistical significance was detected, the Newman-Keuls test was applied (35). Statis-

tical significance was defined as $P < 0.05$. Values are presented as means \pm SE.

RESULTS

Effects of Ca^{2+} channel antagonists on heart rate and mean arterial pressure. ω -Conotoxin GVIA (10 μ g/kg) decreased heart rate from 418 ± 9 to 328 ± 13 beats/min ($P < 0.05$) and mean arterial pressure from 115 ± 2 to 74 ± 2 mmHg ($P < 0.05$). ω -Conotoxin GVIA (50 μ g/kg) did not further decrease heart rate and mean arterial pressure. ω -Conotoxin MVIIC decreased heart rate from 408 ± 3 to 390 ± 5 beats/min ($P < 0.05$) but did not change mean arterial pressure. Combined ω -conotoxin GVIA and MVIIC decreased heart rate from 415 ± 10 to 327 ± 4 beats/min ($P < 0.05$) and mean arterial pressure from 124 ± 2 to 57 ± 2 mmHg ($P < 0.05$). Nifedipine (300 μ g/kg) decreased mean arterial pressure from 113 ± 4 to 86 ± 4 mmHg ($P < 0.05$) but did not change heart rate. Nifedipine (900 μ g/kg) decreased mean arterial pressure from 124 ± 3 to 73 ± 2 mmHg ($P < 0.05$).

Effects of Ca^{2+} channel antagonists on ACh and catecholamine releases. ACh could not be detected in dialysate before or after stimulation. Thus we expressed dialysate ACh concentration during stimulation as an index of ACh release induced by stimulation. In contrast, substantial amounts of NE and Epi were observed in dialysate before stimulation. Intravenous administration of Ca^{2+} channel antagonists did not affect these basal NE and Epi releases (Table 1). Dialysate NE and Epi concentrations increased by nerve stimulation and rapidly declined after the stimulation. Thus we subtracted the dialysate NE and Epi contents before stimulation from those during stimulation and expressed these values as indexes of NE and Epi releases induced by stimulation.

Effects of ω -conotoxin GVIA. ω -Conotoxin GVIA (10 μ g/kg) significantly inhibited ACh release at 2 Hz from 6.2 ± 0.9 to 3.6 ± 0.5 nM, ACh release at 4 Hz from 12.2 ± 1.7 to 7.9 ± 1.2 nM, NE release at 4 Hz from 34 ± 6 to 17 ± 3 nM, Epi release at 2 Hz from 81 ± 13 to 42 ± 3 nM, and Epi release at 4 Hz from 180 ± 21 to 94 ± 7 nM. However, inhibition of NE release at 2 Hz was not statistically significant (Fig. 1A). A fivefold higher dose of ω -conotoxin GVIA (50 μ g/kg) did not

Table 1. Basal dialysate NE and Epi concentrations before and after administration of Ca^{2+} channel antagonists

	NE, nM	Epi, nM
<i>ω-Conotoxin GVIA (10 and 50 μg/kg) (n = 12)</i>		
Before administration	4.9 \pm 0.9	20.6 \pm 2.9
After administration	3.8 \pm 0.6	21.0 \pm 2.6
<i>ω-Conotoxin MVIIC (50 μg/kg) (n = 6)</i>		
Before administration	4.1 \pm 1.0	20.2 \pm 2.6
After administration	4.6 \pm 0.9	24.0 \pm 3.5
<i>ω-Conotoxin GVIA (50 μg/kg) + MVIIC (250 μg/kg) (n = 6)</i>		
Before administration	4.4 \pm 1.3	17.5 \pm 3.8
After administration	3.1 \pm 0.7	20.8 \pm 4.4
<i>Nifedipine (100 and 300 μg/kg) (n = 12)</i>		
Before administration	4.0 \pm 0.6	17.4 \pm 2.2
After administration	3.3 \pm 0.9	17.7 \pm 2.9

Values are means \pm SE; n, no. of rats. NE, norepinephrine; Epi, epinephrine.

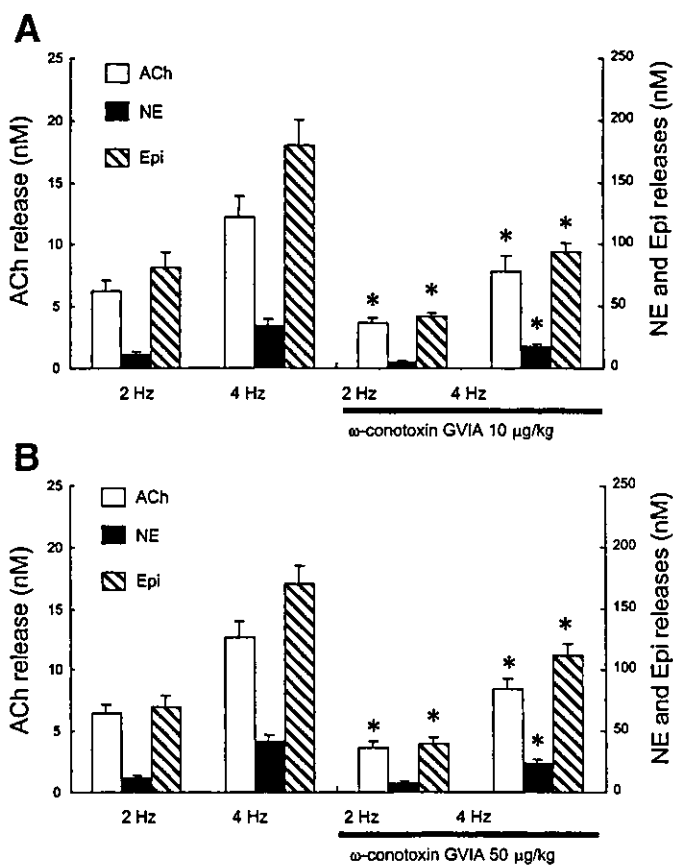


Fig. 1. Effects of ω -conotoxin GVIA on ACh, norepinephrine (NE), and epinephrine (Epi) releases. ω -Conotoxin GVIA (10 μ g/kg) inhibited ACh release at 2 and 4 Hz, NE release at 4 Hz, and Epi release at 2 and 4 Hz (A). A 5-fold higher dose of ω -conotoxin GVIA (50 μ g/kg) did not further inhibit these releases (B). Values are means \pm SE from 6 rats. * P < 0.05 vs. ACh, NE, or Epi release at same frequency before administration.

further inhibit release. ω -Conotoxin GVIA (50 μ g/kg) significantly inhibited ACh release at 2 Hz from 6.5 ± 0.7 to 3.7 ± 0.5 nM, ACh release at 4 Hz from 12.6 ± 1.4 to 8.5 ± 0.8 nM, NE release at 4 Hz from 41 ± 6 to 24 ± 4 nM, Epi release at 2 Hz from 70 ± 10 to 40 ± 6 nM, and Epi release at 4 Hz from 170 ± 15 to 112 ± 10 nM (Fig. 1B).

Effects of ω -conotoxin MVIIC. ω -Conotoxin MVIIC (50 μ g/kg) significantly inhibited ACh release at 4 Hz from 11.7 ± 2.5 to 8.5 ± 2.1 nM and Epi release at 4 Hz from 170 ± 38 to 129 ± 35 nM. Inhibitions of ACh and Epi releases at 2 Hz and NE release at either frequency were not statistically significant (Fig. 2).

Effects of combined ω -conotoxin GVIA and MVIIC. Combined ω -conotoxin GVIA (50 μ g/kg) and MVIIC (250 μ g/kg) significantly inhibited ACh release at 2 Hz from 6.7 ± 0.6 to 1.9 ± 0.3 nM, ACh release at 4 Hz from 12.1 ± 1.3 to 3.8 ± 0.6 nM, NE release at 2 Hz from 11.1 ± 1.1 to 1.2 ± 0.3 nM, NE release at 4 Hz from 36 ± 5 to 8 ± 2 nM, Epi release at 2 Hz from 88 ± 9 to 13 ± 3 nM, and Epi release at 4 Hz from 187 ± 20 to 49 ± 9 nM (Fig. 3).

Effects of nifedipine. Nifedipine (300 μ g/kg) did not change ACh release at either frequency but significantly inhibited Epi release at 4 Hz from 172 ± 31 to 135 ± 23 nM. Inhibitions of Epi release at 2 Hz and NE release at either frequency were not statistically significant (Fig. 4A). A threefold higher dose of

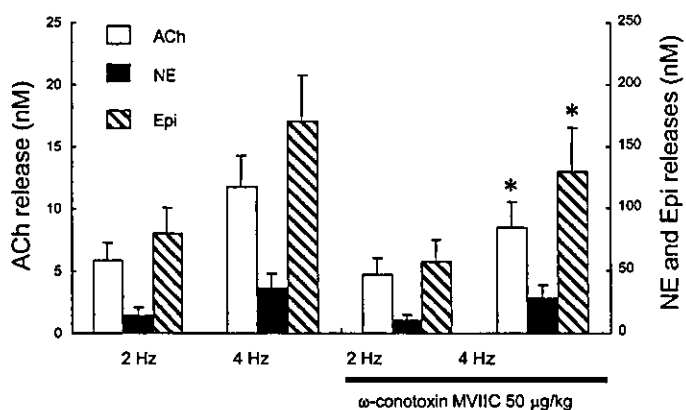


Fig. 2. Effects of ω -conotoxin MVIIC on ACh, NE, and Epi releases. ω -Conotoxin MVIIC (50 μ g/kg) inhibited ACh and Epi releases at 4 Hz. Values are means \pm SE from 6 rats. * P < 0.05 vs. ACh, NE, or Epi release at same frequency before administration.

nifedipine (900 μ g/kg) did not change ACh release but significantly inhibited Epi release at 4 Hz from 188 ± 24 to 128 ± 15 nM and NE release at 4 Hz from 33 ± 5 to 24 ± 4 nM. Inhibitions of NE and Epi releases at 2 Hz were not statistically significant (Fig. 4B).

DISCUSSION

Effects of Ca²⁺ channel antagonists on ACh release from splanchnic nerve endings. In the present study, ω -conotoxin GVIA (10 μ g/kg) inhibited ACh release at both 2 and 4 Hz by approximately 35–40%. A fivefold higher dose of ω -conotoxin GVIA (50 μ g/kg) did not further inhibit ACh release. ω -Conotoxin MVIIC (50 μ g/kg) inhibited ACh release at 4 Hz by ~30%. Combined ω -conotoxin GVIA (50 μ g/kg) and MVIIC (250 μ g/kg) inhibited ACh release at both 2 and 4 Hz by ~70%. N- and P/Q-type Ca²⁺ channels could be present on the splanchnic nerve endings and be involved in ACh release. P/Q-type Ca²⁺ channels may play a role in ACh release at a high frequency of stimulation. ACh release response was resistant to nifedipine (300 and 900 μ g/kg) at both 2 and 4 Hz. L-type Ca²⁺ channels could not be present on splanchnic nerve

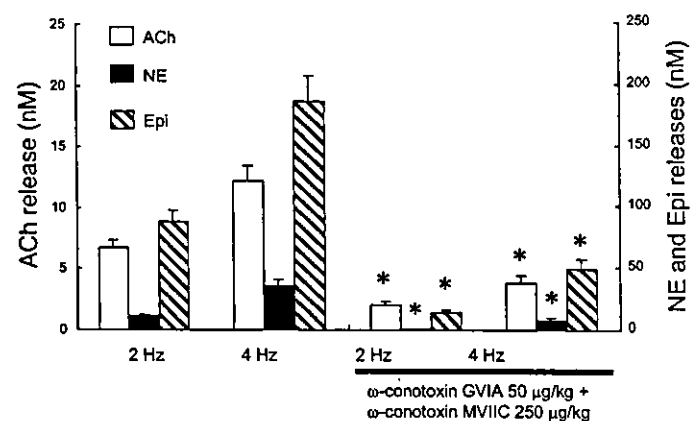


Fig. 3. Effects of combined ω -conotoxin GVIA and MVIIC on ACh, NE, and Epi releases. Combined ω -conotoxin GVIA (50 μ g/kg) and MVIIC (250 μ g/kg) inhibited ACh, NE, and Epi releases at 2 and 4 Hz. Values are means \pm SE from 6 rats. * P < 0.05 vs. ACh, NE, or Epi release at same frequency before administration.

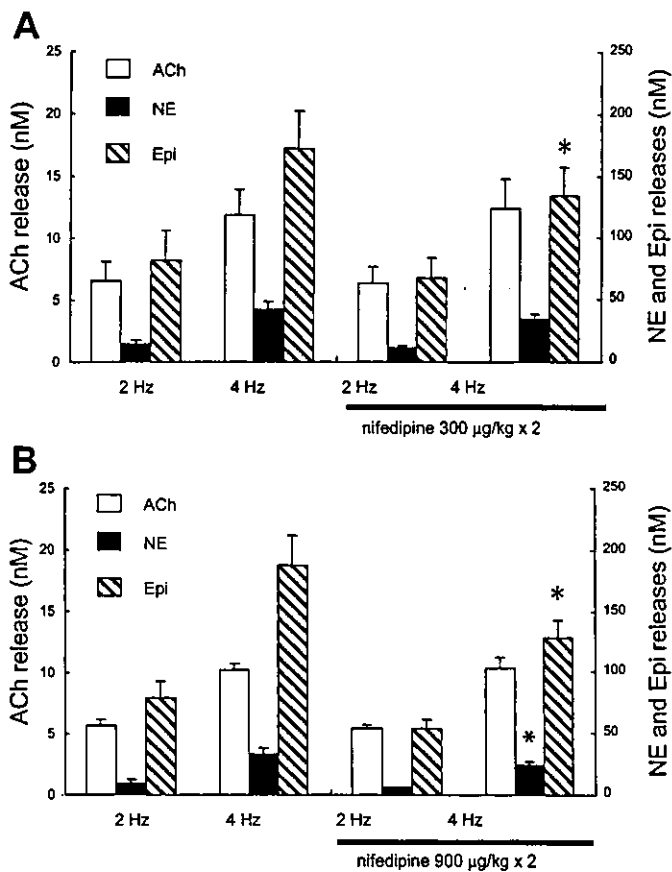


Fig. 4. Effects of nifedipine on ACh, NE, and Epi releases. Nifedipine (300 µg/kg) did not change ACh release at either frequency but inhibited Epi release at 4 Hz (A). Nifedipine (900 µg/kg) did not change ACh release at either frequency but inhibited NE and Epi releases at 4 Hz (B). Values are means \pm SE from 6 rats. * $P < 0.05$ vs. ACh, NE, or Epi release at same frequency before administration.

endings or not play a major role in ACh release. This is the first direct study to demonstrate the type of Ca^{2+} channels controlling ACh release from splanchnic nerve endings.

In isolated rat adrenal glands, catecholamine release induced by field stimulation is sensitive to P/Q-type Ca^{2+} channel antagonist, whereas that induced by exogenous ACh is insensitive (27). This indirect study suggested the involvement of P/Q-type Ca^{2+} channels in ACh release but failed to show the involvement of N-type Ca^{2+} channels. In isolated bovine adrenal glands, a direct measurement study showed that a reduction of the extracellular Ca^{2+} concentration inhibits 3H -labeled ACh release induced by field stimulation, but N- and L-type Ca^{2+} channel antagonists do not (28). Thus our findings are in part consistent with these direct and indirect studies but inconsistent as to the involvement of N-type Ca^{2+} channels.

This discrepancy might be ascribed to the experimental method. The contribution of Ca^{2+} channels may vary with the type of method used to evoke ACh release. In these studies, ACh release was evoked by electrical field stimulation of isolated adrenal glands, which is known to induce ACh release but not direct depolarization of chromaffin cells (34). In the present study, ACh release was evoked in the *in vivo* state by electrical stimulation of splanchnic nerves. The type of Ca^{2+} channels involved in ACh release may vary with the frequency,

amplitude, or time period of stimulation. Actually, in the present study, we observed the involvement of P/Q-type Ca^{2+} channels at only high-frequency stimulation, while it has been reported in perfused rat adrenal glands that N-type Ca^{2+} channels are involved in the maintenance of catecholamine release in response to long splanchnic nerve stimulation (31). The time period of 2 min in the present study seems to be longer than those in earlier studies but could be within the physiological range. Moreover, the blocking action of ω -conotoxin GVIA is time dependent as well as dose dependent and irreversible (8, 11, 32, 36). The maximum functional effect of ω -conotoxin GVIA has been observed to be at least 15 min after administration. We evaluated the effect of ω -conotoxin GVIA 30 min after intravenous administration, when heart rate and mean arterial pressure had already been stabilized. The evaluation early after administration might lead to underestimation of the inhibitory effects of ω -conotoxin GVIA.

There are many similarities between synaptic transmission from splanchnic nerves to chromaffin cells and sympathetic ganglionic transmission (17). In isolated guinea pig paravertebral ganglia, an electrophysiological study has shown that both N- and P-type Ca^{2+} channel antagonists reduce cholinergic synaptic conductance, whereas L-type Ca^{2+} channel antagonist does not (19). In isolated rat superior cervical ganglia, both N- and P-type Ca^{2+} channel antagonists inhibit the rise in Ca^{2+} concentration in the terminal boutons (22). Moreover, in isolated rat superior cervical ganglia, 3H -labeled ACh release induced by high K^+ is inhibited by both N- and P-type Ca^{2+} channel antagonists but unaffected by L-type Ca^{2+} channel antagonist (15). Our findings are similar to these findings obtained from isolated sympathetic preganglionic nerves.

The inhibition by ω -conotoxin GVIA (50 µg/kg) was almost the same as that by ω -conotoxin GVIA (10 µg/kg). Moreover, the inhibition by combined ω -conotoxin GVIA (50 µg/kg) and MVIIC (250 µg/kg) was almost algebraically the sum of the individual inhibition by ω -conotoxin GVIA (10 µg/kg) and MVIIC (50 µg/kg). These results suggest that fivefold higher doses of ω -conotoxin GVIA and MVIIC are sufficient to cause inhibition of Ca^{2+} channels. However, ~30% of ACh release was resistant to combined ω -conotoxin GVIA (50 µg/kg) and MVIIC (250 µg/kg). Other types of Ca^{2+} channels except for N- and P/Q-types may be involved in ACh release from splanchnic nerve endings. Further examination could be needed.

Effects of Ca^{2+} channel antagonists on catecholamine release from chromaffin cells. In the present study, nifedipine (300 µg/kg) did not change ACh release at 2 and 4 Hz but inhibited Epi release at 4 Hz by ~20%. A threefold higher dose of nifedipine (900 µg/kg) did not change ACh release at 2 and 4 Hz but inhibited NE and Epi releases at 4 Hz by ~30%. Adrenal chromaffin cells are divided into two populations: NE- and Epi-storing cells (10). L-type Ca^{2+} channels could be present on the surface of both NE- and Epi-storing cells and play a role in NE and Epi releases.

Approximately 70% of catecholamine release was resistant to nifedipine (900 µg/kg). This result suggests that other types of Ca^{2+} channels except for L-type are present on chromaffin cells and involved in NE and Epi releases, although we cannot exclude the possibility of incomplete inhibition of L-type Ca^{2+} channels. Species differences in the types of Ca^{2+} channels controlling Ca^{2+} influx and catecholamine release have been

shown with rat, cat, and bovine chromaffin cells (5, 6, 13, 24). In patch-clamp studies of isolated rat chromaffin cells, Ca²⁺ inward current elicited by depolarization is sensitive to both L- and N-type Ca²⁺ channel antagonists (16, 21). The study measuring Ba²⁺ current by patch-clamp technique has shown the existence of L-, N-, and P/Q-type Ca²⁺ channels on rat chromaffin cells and the following distribution of Ca²⁺ channels in decreasing order: L-type > N-type > P/Q-type (13). In the present study, ω -conotoxin GVIA (10 and 50 μ g/kg) inhibited NE release at 4 Hz and Epi release at 2 and 4 Hz by approximately 45–50%. ω -Conotoxin MVIIC (50 μ g/kg) inhibited Epi release at 4 Hz by ~30%. Combined ω -conotoxin GVIA (50 μ g/kg) and MVIIC (250 μ g/kg) inhibited NE and Epi releases at 2 and 4 Hz by approximately 75–85%. However, these Ca²⁺ channel antagonists simultaneously inhibited ACh release to almost the same extent. It is difficult to determine how much Ca²⁺ antagonists are acting on chromaffin cells when Ca²⁺ channel antagonists inhibit ACh release. Thus, although much of these inhibitions of catecholamine release may be considered to be consequences of the inhibition of ACh release, we cannot exclude the possibility that N- or P/Q-type Ca²⁺ channels may be involved in the *in vivo* catecholamine release on chromaffin cells.

The inhibition of NE release at 2 Hz by ω -conotoxin GVIA (10 and 50 μ g/kg) and the inhibition of NE release at 4 Hz by ω -conotoxin MVIIC (50 μ g/kg) were not statistically significant despite significant inhibitions of ACh and Epi releases. In the same preparation, we have shown that cholinergic antagonists almost inhibited NE and Epi releases induced by nerve stimulation (1, 2). However, the correlation between ACh and NE releases was poorer than that between ACh and Epi releases when stimulation frequency was raised stepwise (2). Insignificant inhibitions of NE release may be ascribed to this poor correlation.

In the present study, Ca²⁺ channel antagonists did not affect basal dialysate NE and Epi levels. In our previous study of the same preparation, these basal levels were not affected by neostigmine, hexamethonium, or atropine (1). We then concluded that these basal dialysate NE and Epi levels reflect noncholinergic catecholamine release. N-, P/Q-, and L-type Ca²⁺ channels may not play a major role in basal noncholinergic catecholamine release from adrenal medulla.

Methodological considerations. We administered neostigmine locally to adrenal medulla through a dialysis probe. Cholinesterase inhibitor was necessary to monitor endogenous ACh even during splanchnic nerve stimulation because released ACh is rapidly degraded by acetylcholinesterase before reaching the dialysis fiber. In the same preparation, local administration of neostigmine enhanced the dialysate catecholamine response to nerve stimulation by approximately threefold, but dialysate ACh and catecholamine responses are correlated with the stimulation frequency of splanchnic nerves in the presence of neostigmine (2). Thus dialysate ACh and catecholamine responses are likely to be correlated with the amount of Ca²⁺ influx from voltage-dependent Ca²⁺ channels even in the presence of neostigmine.

Intravenous administration of Ca²⁺ channel antagonists induced changes in heart rate or mean arterial pressure. These changes might affect ACh and catecholamine releases through a baroreflex mechanism. Moreover, these hemodynamic changes might decrease the spillover of ACh or catecholamine

from adrenal medulla and affect the dialysate ACh or catecholamine concentrations (20). In our preparation, however, splanchnic nerves had been transected before control sampling, and basal dialysate catecholamine concentrations did not change before or after administration. Thus effects of these hemodynamic changes could be negligible when we considered the effects of Ca²⁺ channel antagonists on nerve stimulation-induced dialysate responses.

In conclusion, we applied dialysis technique to the adrenal medulla of anesthetized rats and investigated the effects of Ca²⁺ channel antagonists on ACh and catecholamine releases induced by electrical stimulation of splanchnic nerves. Both N- and P/Q-type Ca²⁺ channels control ACh release on preganglionic splanchnic nerve endings while L-type Ca²⁺ channels do not. L-type Ca²⁺ channels are involved in norepinephrine and epinephrine releases on chromaffin cells.

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Portable X-ray generator utilizing a cerium-target radiation tube for angiography

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Abstract

The development of a portable X-ray generator with a cerium-target tube and its application to angiography are described. The portable X-ray generator consists of a main controller, a unit with a Cock–Croft circuit and an X-ray tube, and a personal computer. Negative high voltages are applied to the cathode electrode in the X-ray tube, and the tube voltage and current are regulated by the controller or the computer. The X-ray tube is a glass-enclosed double-focus diode with a cerium target and a 0.5 mm-thick beryllium window. The maximum tube voltage and current were 60 kV and 0.8 mA, respectively. The focal-spot sizes were 4 mm × 4 mm (large) and 1 mm × 1 mm (small), respectively. Angiography was performed with a computed radiography system using iodine-based microspheres. The tube voltage, the current, the distance between the imaging plate and the X-ray source, and the spot size were 60 kV, 0.4 mA, 1.5 m, and small, respectively. In this angiography, we observed coronary arteries and fine blood vessels of about 50 μm or less with high contrasts.

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Keywords: Cerium-target X-ray tube; Cerium characteristic X-rays; K-absorption edge; High contrast angiography; Microangiography

1. Introduction

In conjunction with single crystals, synchrotrons generate monochromatic X-rays. These rays play an important role in parallel radiography and have been employed to perform high-contrast micro-angiography [1] and phase imaging [2–4]. However, it is difficult to obtain sufficient machine times for various research projects including medical applications.

So far, several different flash X-ray generators have been developed [5,6], and soft generators [7–12] with photon energies of lower than 150 keV can be employed to perform biomedical radiography. In order to produce monochro-

matic X-rays, plasma flash X-ray generators [13–16] are useful, since quite intense and sharp characteristic X-rays such as lasers have been produced from weakly ionized linear plasmas of nickel, copper and molybdenum, while bremsstrahlung rays are hardly detected at all. Using these generators, the characteristic X-ray intensity substantially increased with corresponding increases in the charging voltage.

Since K-series characteristic X-rays from cerium target are absorbed effectively by iodine-based contrast mediums, a cerium-target X-ray tube is very useful in order to perform high-contrast angiography. On the other hand, cerium is a rare earth element and has a high reactivity, and it is difficult to design the target. However, the development of a cerium-target tube for high-contrast angiography has long been wished for.

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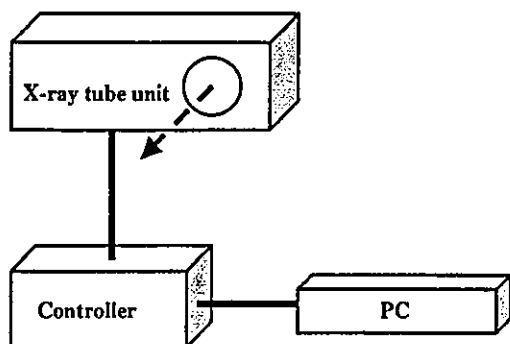


Fig. 1. Block diagram of the portable X-ray generator with a cerium-target radiation tube, which is specially used for angiography using iodine-based contrast mediums. The negative high voltage is applied to the cathode electrode, and the tube current is regulated by the filament temperature. Although the X-ray tube is a double-focus type, we usually employ a small focus in order to measure the radiographic characteristics and to perform angiography.

In the present research, we developed a portable X-ray generator with a cerium-target tube, used to perform preliminary study on angiography achieved with cerium K-series characteristic X-rays.

2. Generator

Fig. 1 shows the block diagram of the X-ray generator, which consists of a main controller, an X-ray tube unit with a Cockcroft circuit and a cerium-target tube, and a personal computer. The negative high-voltage is applied to the cathode electrode, and the anode (target) is connected to the ground potential. In this experiment, the tube voltage was regulated from 40 to 65 kV, and the tube current was regulated within 0.8 mA by the filament temperature. The exposure time is controlled in order to obtain optimum X-ray intensity, and the X-ray tube is a double-focus type with focal-spot dimensions of approximately 4 mm × 4 mm (large spot) and 1 mm × 1 mm (small spot), respectively. The maximum

tube current is determined by the spot dimensions, and the currents of small and large spots are 0.4 and 0.8 mA, respectively.

3. Characteristics

3.1. X-ray intensity

X-ray intensity was measured by a Victoreen 660 ionization chamber at 1.0 m from the X-ray source using a small spot with an exposure time of 1.0 s (Fig. 2). At a constant tube current of 40 μA , the X-ray intensity increased when the tube voltage was increased. The intensity was roughly in proportion to the tube current at a constant tube voltage of 60 kV. In this measurement, the intensity with a tube voltage of 60 kV and a current of 90 μA was 2.14 $\mu\text{C}/\text{kg}$ at 1.0 m from the source with errors of less than 0.2%.

3.2. X-ray source

In order to measure images of the X-ray source, we employed a pinhole camera with a hole diameter of 50 μm in conjunction with a computed radiography (CR) system (Fig. 3) [17]. When the tube voltage was increased, the spot intensity increased slightly, and spot dimensions seldom varied and had values of approximately 1 mm × 1 mm.

3.3. X-ray spectra

In order to measure X-ray spectra, we employed a cadmium tellurium detector (CDTE2020X, Hamamatsu Photonics Inc.) (Fig. 4). Compared with a germanium detector, this detector has lower energy resolutions. When the tube voltage was increased, both the characteristic X-ray intensity and the maximum photon energy of bremsstrahlung X-rays increased. According to insertion of a monochromatic cerium oxide filter, quasi-monochromatic X-rays were obtained.

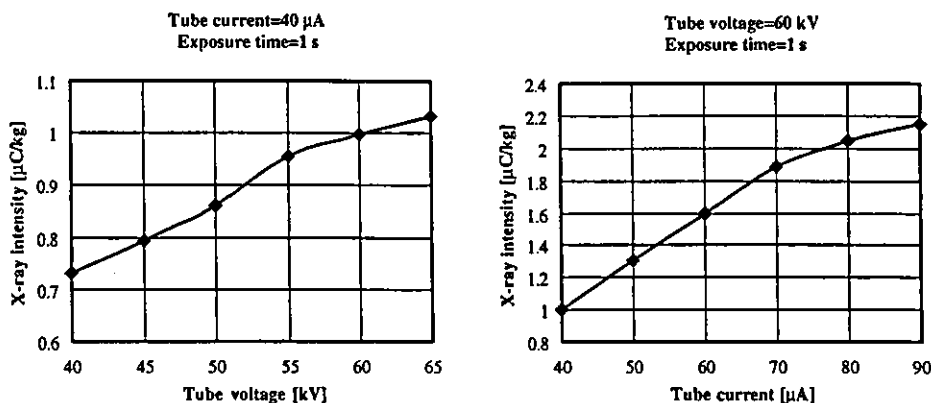


Fig. 2. X-ray intensity measured at 1.0 m from the X-ray source according to changes in the tube voltage and current. In the measurement, we employed an ionization chamber without using a monochromatic filter.

V_t : Tube voltage

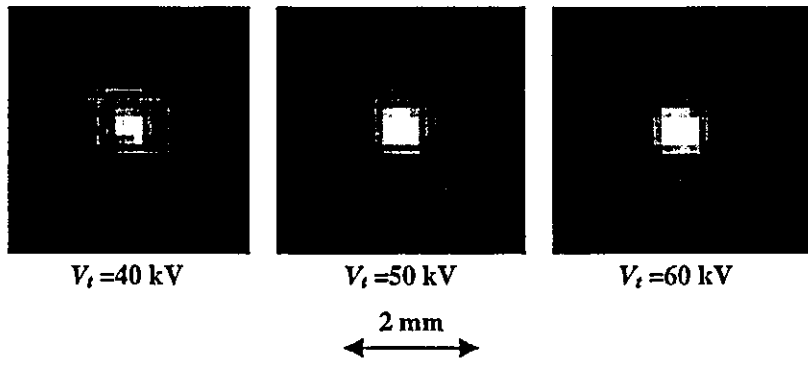


Fig. 3. Images of the X-ray source measured by a 100 mm diameter pinhole with changes in the tube voltage.

V_t : Tube voltage

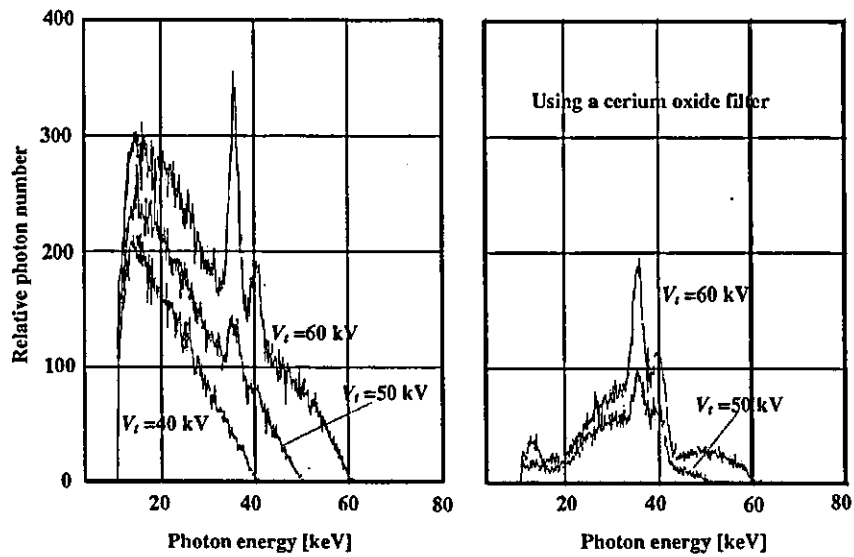


Fig. 4. X-ray spectra measured by a cadmium tellurium detector with changes in the tube voltage.

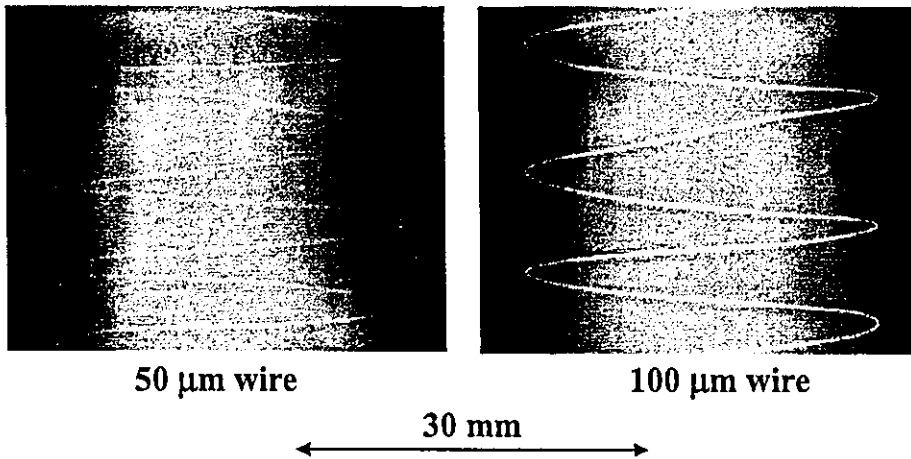


Fig. 5. Radiograms of tungsten wires around a rod made of PMMA used for estimating the image resolution.

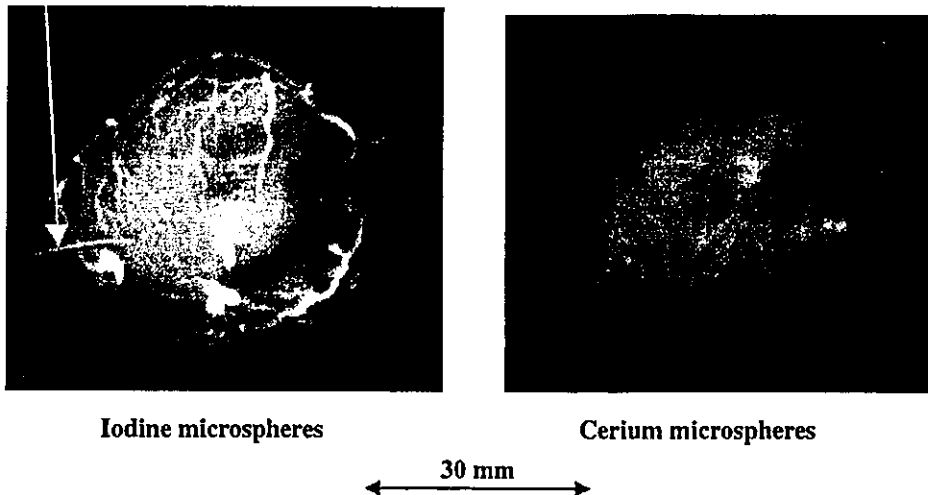
50 μm tungsten wire

Fig. 6. Angiograms of rabbit hearts using (a) iodine and (b) cerium microspheres.

4. Angiography

The angiography was performed by a CR system (Konica Regius 150) using the monochromatic filter, and the distance (between the X-ray source and the imaging plate) and the tube voltage were 1.5 m and 60 kV, respectively.

Firstly, rough measurements of image resolution were made using wires. Fig. 5 shows radiograms of tungsten wires coiled around rods made of polymethyl methacrylate (PMMA). Although the image contrast increased with increases in the wire diameter, a 50 μm diameter wire could be observed.

50 μm tungsten wire

Fig. 7. Angiograms of the external ear of a rabbit using iodine-based microspheres. In this angiography, we employed a 50 μm tungsten wire to roughly determine the diameters of blood vessels.

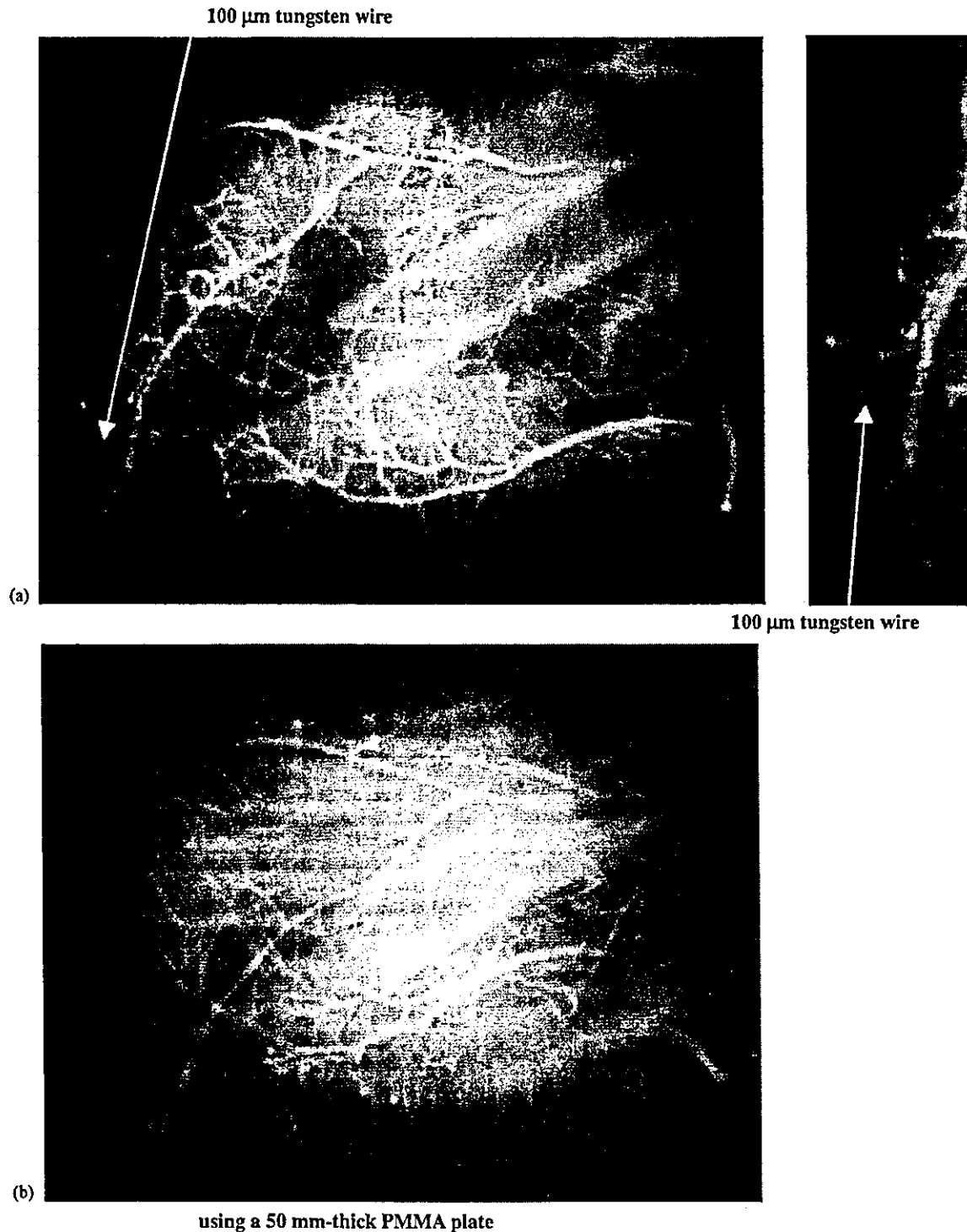


Fig. 8. Angiograms of an extracted heart of a dog. (a) Normal image and (b) image using a 50 mm PMMA plate set in front of the heart, facing the X-ray source.

Angiograms of rabbit hearts are shown in Fig. 6. These two images were obtained using iodine and cerium microspheres of 15 μm in diameter. In case where the cerium spheres were employed, the coronary arteries were barely visible. Fig. 7 shows an angiogram of the external ear of a rabbit using iodine spheres, and fine blood vessels

of about 50 μm are clearly visible. In angiography of a larger heart extracted from a dog, using iodine spheres, a 50 mm thick PMMA plate was set in front of the heart facing X-ray source, and image contrast of coronary arteries decreased slightly with increases in the plate thickness (Fig. 8).

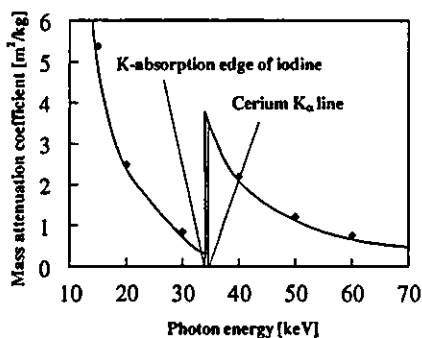


Fig. 9. Relation between the mass absorption coefficient of iodine and the photon energy of cerium K_{α} line.

5. Discussion

Cerium is a rare earth element and has a high reactivity. However, the average photon energy of K_{α} lines is 34.566 keV, and iodine spheres with a K-absorption edge of 33.155 keV absorb the lines easily (Fig. 9). Next, since the spheres easily transmit bremsstrahlung X-rays with energies of lower than the edge, it is important that the rays be absorbed as much as possible before angiography in order to increase the image contrast.

In rough measurement of image resolution, we obtained resolutions of 50 μm or less, and high-contrast blood vessels could be observed using a CR system. Although neogenetic fine blood vessels in recovery can be observed, the image resolution of the CR system should be improved as much as possible, and a dynamic CR system such as a flat panel system is useful to observe blood flows.

In this research, we developed a low-dose-rate X-ray tube in order to perform preliminary study on angiography using iodine-based contrast mediums. Because we are designing a high-dose-rate tube to decrease the exposure time, the K-series characteristic X-rays from cerium target can be employed to perform angiography for cases of cardiovascular disease.

6. Summary

In summary, we developed, new portable X-ray generator with a cerium-target tube and succeeded in producing K-series characteristic X-rays of cerium, which can be absorbed easily by iodine-based contrast mediums. Both the characteristic and bremsstrahlung X-ray intensities increased with corresponding increases in the tube voltage, and quasi-monochromatic X-rays were produced by a cerium oxide filter. In this preliminary experiment, although the maximum tube voltage and current were 65 kV

and 0.4 mA, respectively, the voltage and current could be increased. Subsequently, we observed a 50 μm tungsten wire easily, and high-contrast angiography was performed using a CR system with an imaging plate.

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