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Effect of Ca^{2+}/calmodulin-dependent protein kinase II inhibitors on the neurogenic cerebroarterial relaxation

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Abstract

In canine cerebral artery strips contracted with prostaglandin $F_{2\alpha}$, transmural electrical stimulation (5 Hz for 40 s) produced a relaxation which was abolished by tetrodotoxin. The neurogenic response was inhibited moderately by [*S*]-5-isoquinolinesulfonic acid,4-[2-[(5-isoquinolinyl–sulfonyl)methylamino]-3-oxo-(4-phenyl-1-piperazinyl)-propyl] phenyl ester (KN62), an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II, which however did not alter or only slightly reduced the relaxant response to electrical nerve stimulation in canine coronary arterial strips that is mediated via β-adrenoceptors stimulated by norepinephrine. Nicotine-induced relaxation, mediated by nitric oxide (NO) derived from perivascular nerves, was also attenuated by KN62, whereas the response to exogenous NO was unaffected. The nicotine-induced increase in the cyclic GMP content in cerebral arteries was depressed by KN62. The neurogenic relaxation was not influenced by phorbol 12-myristate 13-acetate, an activator of protein kinase C. 8-Bromo-cyclic GMP and 8-bromo-cyclic AMP did not significantly alter the response to nerve stimulation. It is concluded that the phosphorylation pathway involving Ca^{2+}/calmodulin-dependent protein kinase II, but not other protein kinases so far tested, appears to be involved in the function of vasodilator nerves innervating the cerebral artery. © 1997 Elsevier Science B.V.

Keywords: Ca^{2+}/calmodulin-dependent protein kinase II; Nitrergic nerve; Cerebral artery; Nitric oxide (NO) synthase; Neurogenic vasodilatation

1. Introduction

The neurogenic vasodilatation in cerebral arteries from humans, monkeys, dogs, cats, cows, pigs and sheep that is resistant to cholinoceptor and β-adrenoceptor antagonists is hypothesized to be mediated by nitric oxide (NO) formed via constitutive NO synthase from L-arginine in nerve terminals (Toda and Okamura, 1996). This hypothesis is supported by the following evidence. The response to nerve stimulation is abolished by NO synthase inhibitors, and this inhibition is reversed by L-arginine, but not by D-arginine (Toda and Okamura, 1990a,b); blockers of the non-L type Ca^{2+} channel and Ca^{2+} deprivation depress the neurogenic relaxation (Toda and Okamura, 1992; Toda et al., 1995); stimulation of perivascular nerves liberates NO from the tissue, measured as NOx, and increases the production of cyclic GMP in the tissue (Toda and Okamura, 1990a, 1992); exogenously applied NO increases the cyclic GMP content in the cerebral artery and produces vasodilatation (Toda and Okamura, 1992); and cerebral arteries are innervated by nerves containing NO synthase immunoreactivity and NADPH diaphorase activity (Yoshida et al., 1993).

NO synthase derived from rat cerebellum has recognition sites for calmodulin, NADPH, etc., and phosphorylation sites (Bredt et al., 1991). The neuronal NO synthase is phosphorylated by cyclic nucleotide-dependent protein kinase, protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II, and its activity is suggested to be regulated by phosphorylation (Bredt et al., 1992). However, little information is available concerning the involvement of the phosphorylation pathway in the physiological functions caused by endogenous NO.

The present study aimed to determine whether inhibitors and stimulators of the protein phosphorylases modulated the response to NO derived from perivascular nerves and to physiologically and pharmacologically elucidate the specificity of blockade.

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2. Materials and methods

2.1. Preparation

The study review board at our university approved the use of animal blood vessels in this study.

Thirty-two mongrel dogs of either sex, weighing 7 to 14 kg, were anesthetized with intraperitoneal injections of sodium pentobarbital (50 mg/kg) and killed by bleeding from the carotid arteries. Basilar and middle cerebral arteries (0.5 to 0.7 mm outside diameter) were isolated from the brain, and anterior interventricular branches of the left coronary artery from the heart. The arteries were helically cut into strips of approximately 20 mm long. The endothelium was removed from the cerebral arterial strips by gently rubbing the intimal surface with a cotton ball. Removal of the endothelium was verified by abolishment of the relaxation caused by substance P (10^{-8} M) (Toda et al., 1993). The specimens were vertically fixed between hooks in muscle baths (20 ml) containing the modified Ringer–Locke solution, which was aerated with a mixture of 95% O_2 and 5% CO_2 and maintained at 37 ± 0.3°C. The hook anchoring the upper end of the strips was connected to the lever of a force–displacement transducer. The resting tension was adjusted to 1.5 g, which is the optimum for contractile responses. The constituents of the solution were as follows (mM): NaCl 120, KCl 5.4, $CaCl_2$ 2.2, $MgCl_2$ 1.0, $NaHCO_3$ 25.0 and dextrose 5.6. The pH of the solution was 7.36 to 7.43. The strips were allowed to equilibrate in the bathing media for 60 to 90 min, during which time the media were replaced every 10 to 15 min.

2.2. Recording of mechanical response

Most of the arterial strips were placed between stimulating electrodes. The gaps between the strip and electrodes were wide enough to allow undisturbed contraction and relaxation, and yet sufficiently narrow to allow effective stimulation of intramural nerve terminals. A train of 0.2 ms square pulses of supramaximal intensity (10 V) was transmurally applied at a frequency of 5 Hz for 40 s, unless otherwise mentioned, which produces submaximal responses (Toda and Okamura, 1990b). The stimulus pulses were delivered by an electronic stimulator (Nihon-kohden Kogyo, Tokyo).

Isometric mechanical responses were displayed on an ink-writing oscillograph. The contractile response to 30 mM K^+ was first obtained, and then the preparations were washed three times with fresh media and equilibrated for 30 to 40 min. The strips were contracted partially with prostaglandin $F_{2\alpha}$ (5 to 30×10^{-7} M); the contractions were in a range between 30 and 47% of the contraction induced by 30 mM K^+. Transmural electrical stimulation was applied repeatedly at intervals of 10 min until steady responses were obtained, and then blocking agents were applied. Nicotine in a submaximal concentration of 10^{-4} M (Toda and Okamura, 1990b) was applied directly to the bathing media. After the response was determined to be reproducible, preparations were treated for 30 min or longer with blocking agents. When the active tone was reduced by the blocking agents, supplemental doses of prostaglandin $F_{2\alpha}$ were applied to raise the tone. Nicotine and NO (acidified $NaNO_2$) in single concentrations were successively applied. At the end of each series of experiments, papaverine (10^{-4} M) was added to attain the maximal relaxation. Relaxations induced by electrical stimulation, nicotine or other vasodilator agents relative to those induced by papaverine are presented.

2.3. Cyclic GMP measurement

The content of cyclic GMP in cerebral artery strips denuded of the endothelium was measured. After 30 min of equilibration in the bathing medium, the arterial strips were exposed for 2 min to nicotine (10^{-4} M) with or without KN62 (5×10^{-6} M) and then plunged into liquid nitrogen. KN62 or vehicle was applied at the beginning of equilibration. The tissues were homogenized in 1.5 ml of 6% trichloroacetic acid at 0°C with a glass homogenizer. After centrifugation at $800 \times g$ for 10 min, an ether extraction procedure was carried out with the supernatant. An aliquot of the extract was then used for determination of cyclic GMP with a commercial radioimmunoassay kit (Yamasa, Chiba).

2.4. Statistics and drugs used

The results shown in the text, figures and table are expressed as mean values ± S.E. Statistical analyses were done using Student's paired and unpaired *t*-test and Tukey's method after one-way analysis of variance. Drugs used were nicotine (base), hexamethonium bromide (Nacalai Tesque, Kyoto), KN62 ([*S*]-5-isoquinolinesulfonic acid,4-[2-[(5-isoquinolinyl–sulfonyl)methylamino]-3-oxo-(4-phenyl-1-piperazinyl)-propyl] phenyl ester), HDBA (2-hydroxyl-5-[2,5-dihydroxybenzyl] aminobenzoic acid) (Biomol. Research, PA, USA), PMA (phorbol 12-myristate-13-acetate) (Research Biochemicals, MA, USA), 8-bromo-cyclic GMP (8-bromoguanosine 3′,5′-cyclic monophosphate), 8-bromo-cyclic AMP (8-bromoadenosine 3′,5′-cyclic monophosphate) (Sigma, MO, USA), calmidazolium (Boehringer Mannheim, Mannheim), tetrodotoxin (Sankyo, Tokyo), ω-conotoxin GVIA (Peptide Research Foundation, Minoh), prostaglandin F_{2a} (Pharmacia-Upjohn, Tokyo), and papaverine hydrochloride (Dainippon, Osaka). Responses to nitric oxide (NO) were obtained by addition of $NaNO_2$ solution, which was adjusted to pH 2 just before the application (Furchgott, 1988); concentrations of $NaNO_2$ are presented as those of NO. Oxyhemoglobin was prepared by the method described by Martin et al. (1985).

3. Results

3.1. Relaxation induced by transmural electrical stimulation

In canine cerebral artery strips denuded of the endothelium and partially contracted with prostaglandin $F_{2\alpha}$, transmural electrical stimulation at 2, 5 and 20 Hz produced frequency-related relaxations which were abolished by 3×10^{-7} M tetrodotoxin. Since the response to 5 Hz was consistent and reproducible, this frequency of stimulation was used to analyze the mechanisms of the response in the remainder of this study.

Treatment with KN62 (10^{-6} M) relaxed the prostaglandin $F_{2\alpha}$-contracted arterial strips and gradually attenuated the relaxant response to electrical stimulation. Typical recordings of the response to KN62 are illustrated in Fig. 1. The KN62-induced relaxations were not consistent. Irrespective of the magnitude of relaxation, this inhibitor elicited a consistent blockade of the response to nerve stimulation (Fig. 1). Quantitative data on the effect of KN62 20 and 40 min later are summarized in Fig. 2. After the strips were repeatedly rinsed with fresh media and equilibrated for 60 min or longer, the neurogenic response was not restored.

The relaxation induced by 5 Hz electrical stimulation of canine coronary artery strips contracted with prostaglandin $F_{2\alpha}$ was not influenced by 10^{-6} M KN62 (Fig. 3) or tended to be slightly reduced during the 40 min incubation (Fig. 4). The coronary artery response was markedly inhibited by treatment with ω-conotoxin GVIA, an N type Ca^{2+} channel inhibitor (Figs. 3 and 4), as was the response of canine cerebral arteries (Toda et al., 1995). Treatment with calmidazolium (3×10^{-6} M), a calmodulin inhibitor, significantly inhibited the response of coronary arteries to 5 Hz stimulation (24.0 ± 5.2 to $4.8 \pm 1.2\%$, $n = 4$, $P < 0.02$).

Cerebral arterial relaxations in response to transmural electrical stimulation were also reduced by treatment for

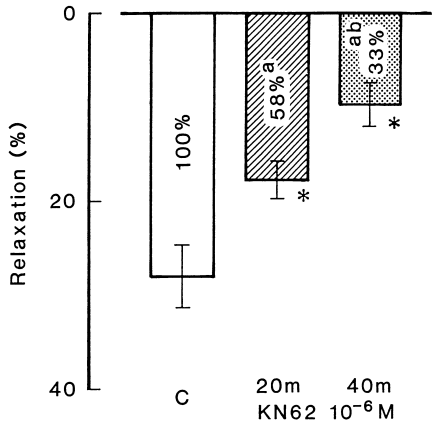

Fig. 2. Modification by KN62 (10^{-6} M treatment for 20 and 40 min) of the response to transmural electrical stimulation (5 Hz) of cerebral artery strips partially contracted with prostaglandin $F_{2\alpha}$. Significantly different from control, $^{*}P < 0.01$ (Tukey's method). The value in each column indicates the response relative to control (C; 100%), and 'a' and 'b' represent significant differences from control ($P < 0.01$) and from the value at 20 min ($P < 0.05$), respectively. The number of strips from separate dogs used in this study was 14. Vertical bars represent S.E.

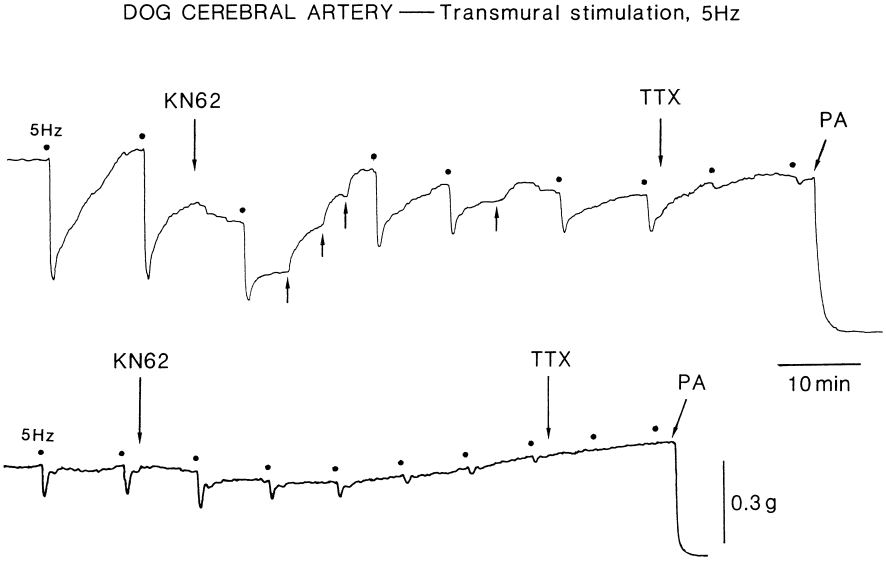

Fig. 1. Recordings of the relaxant response to transmural electrical stimulation (5 Hz) of cerebral artery strips before and after treatment with KN62 (10^{-6} M) and tetrodotoxin (TTX, 3×10^{-7} M). The strips were partially contracted with prostaglandin $F_{2\alpha}$. The upper tracing demonstrates time-dependent attenuation by KN62 of the response to nerve stimulation in association with a marked relaxation induced by this inhibitor. Upward arrows denote the application of supplemental doses of prostaglandin $F_{2\alpha}$ to raise arterial tone. The lower tracing illustrates the same, but the KN62-induced relaxation was only slight and transient. PA represents 10^{-4} M papaverine, which produced the maximal relaxation.

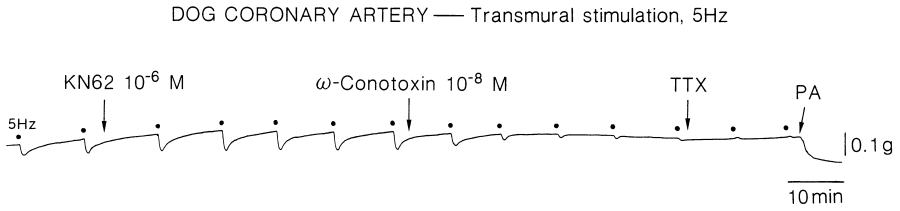

Fig. 3. Recordings of the response to transmural electrical stimulation (5 Hz) of a coronary artery strip before and after treatment with KN62 (10^{-6} M), ω-conotoxin GVIA (10^{-8} M) and tetrodotoxin (TTX, 3×10^{-7} M). The strip was partially contracted with prostaglandin $F_{2\alpha}$(10^{-6} M). Dots above the tracing represent the application of electrical stimulation. PA denotes 10^{-4} M papaverine, which produced the maximal relaxation.

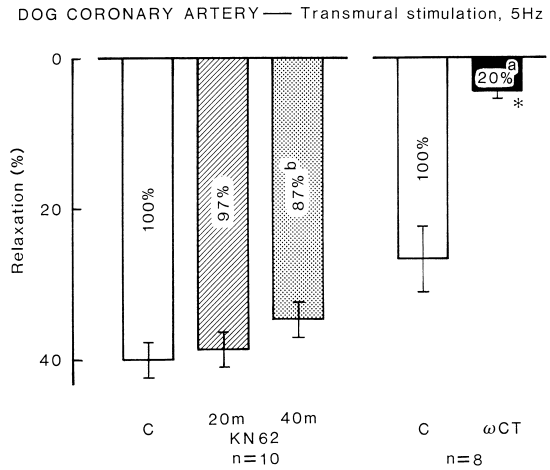

Fig. 4. Modification by KN62 (10^{-6} M treatment for 20 and 40 min) (left panel) and by ω-conotoxin GVIA (ωCT; 10^{-8} M) of the response to transmural electrical stimulation (5 Hz) of coronary artery strips partially contracted with prostaglandin $F_{2\alpha}$. Significantly different from control (C), $^{*}P < 0.01$ (unpaired *t*-test). Figures in the columns are the same as indicated in Fig. 2; '[a]' and '[b]' in the columns represent significant differences from control ($P < 0.001$ and 0.05), respectively. The '*n*' denotes the number of strips from separate dogs. Vertical bars represent S.E.

30 min with HDBA (10^{-6} and 3×10^{-6} M), another Ca^{2+}/calmodulin-dependent protein kinase II inhibitor. Mean values for the inhibition were $14.7 \pm 4.0\%$ ($n = 6$, $P < 0.02$, paired *t*-test) and $60.8 \pm 8.3\%$ ($n = 6$, $P < 0.001$, paired *t*-test), respectively. This inhibitor did not alter the arterial tone elicited by prostaglandin $F_{2\alpha}$, therefore the KN62-induced relaxation is not due to the inhibition of Ca^{2+}/calmodulin-dependent protein kinase II.

Treatment with PMA (10^{-7} and 10^{-6} M), 8-bromocyclic GMP (10^{-5} M) and 8-bromo cyclic AMP (10^{-5} and 10^{-4} M) did not significantly alter the response to electrical stimulation (Table 1). The response tended to be inhibited by 10^{-4} M 8-bromocyclic GMP; however, this treatment markedly decreased the tone of strips, and it was difficult to restore the tone with supplemental doses of prostaglandin $F_{2\alpha}$ in 3 out of 5 strips. Thus, the responses before and after the treatment could not be compared.

3.2. Relaxation induced by nicotine and NO

The addition of nicotine (10^{-4} M) and NO (10^{-7} M) produced relaxations of similar magnitude. The response to nicotine was abolished by 10^{-5} M hexamethonium, and

Table 1
Modification by HDBA, PMA, 8-bromo cyclic GMP and 8-bromo cyclic AMP of the relaxation induced by transmural electrical stimulation (TES 5 Hz), nicotine (10^{-4} M) and NO (10^{-7} M) in cerebral artery strips

		Relaxant response (%) to		
		TES	nicotine	NO
Control		36.7 ± 3.5 (6) (100%)		
HDBA	10^{-6} M	31.6 ± 6.0 (6) ($85.3 \pm 4.0\%$)		
	3×10^{-6} M	17.1 ± 5.5 (6) ($39.2 \pm 8.3\%$[a,b])		
Control		26.1 ± 3.3 (12) (100%)	44.5 ± 6.5 (9) (100%)	55.2 ± 6.5 (9) (100%)
PMA	10^{-7} M	38.3 ± 4.5 (12) ($106 \pm 4.3\%$)	51.0 ± 6.3 (9) ($119 \pm 6.4\%$[a])	64.3 ± 5.2 (9) ($119 \pm 11.5\%$)
	10^{-6} M	17.7 ± 1.9 (4) ($103 \pm 7.6\%$)		
Control		20.4 ± 2.5 (5) (100%)	50.3 ± 6.9 (4) (100%)	48.0 ± 6.1 (4) (100%)
8-Br-cGMP	10^{-5} M	19.0 ± 2.3 (5) ($93.8 \pm 4.8\%$)	45.3 ± 6.5 (4) ($87.3 \pm 7.9\%$)	43.7 ± 6.8 (4) ($95.0 \pm 9.1\%$)
Control		32.0 ± 4.4 (7) (100%)	43.1 ± 6.3 (7) (100%)	52.9 ± 8.8 (6) (100%)
8-Br-cAMP	10^{-5} M	32.8 ± 5.7 (7) ($104 \pm 4.3\%$)	32.5 ± 6.1 (7) ($120 \pm 10.1\%$)	53.3 ± 7.1 (6) ($103 \pm 9.1\%$)
	10^{-4} M	28.3 ± 4.7 (6) ($98.3 \pm 8.9\%$)	27.4 ± 7.3 (5) ($84.2 \pm 8.4\%$)	39.2 ± 6.8 (5) ($88.2 \pm 8.6\%$)

Significantly different from control.
[a] $P < 0.01$; significantly different from the value with 10^{-6} M. HDBA.
[b] $P < 0.05$. Numbers in the parentheses indicate the number of strips from separate dogs. The data are presented as mean values $\pm$ S.E.

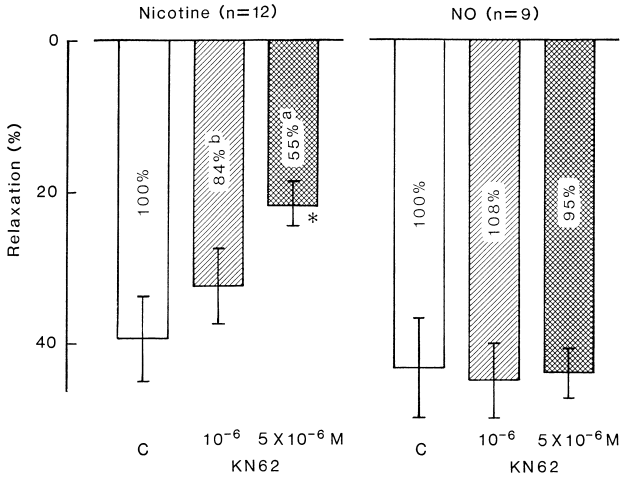

Fig. 5. Modification by KN62 (10^{-6} and 5×10^{-6} M treatment for 20 min) of the response to nicotine (10^{-4} M) or NO (10^{-7} M) of cerebral artery strips contracted with prostaglandin $F_{2\alpha}$. Significantly different from control (C), $^{*}P < 0.05$ (Tukey's method). The value in each column indicates the response relative to control; '[a]' and '[b]' in the columns represent significant differences from control ($P < 0.01$ and 0.05), respectively. The '*n*' denotes the number of strips from separate dogs. Vertical bars represent S.E.

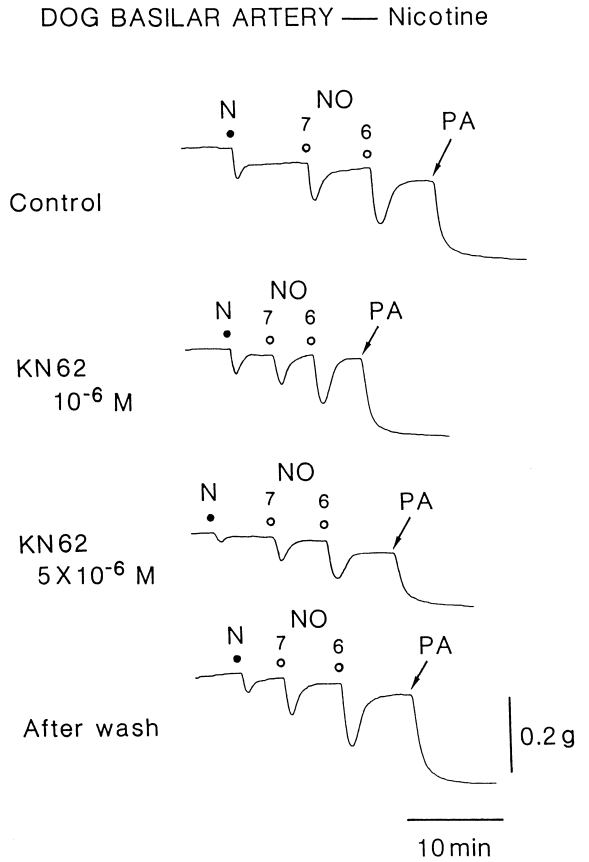

Fig. 6. Recordings of the responses to nicotine (10^{-4} M) and NO (10^{-7} and 10^{-6} M) of a cerebral artery strip before and after treatment with KN62 (10^{-6} and 3×10^{-6}M). The strip was partially contracted with 8×10^{-7} M prostaglandin $F_{2\alpha}$. The inhibition by KN62 of the nicotine-induced relaxation was not reversed by repeated wash of the strip with fresh media (bottom tracing). PA represents 10^{-4} M papaverine that produced maximal relaxation.

the NO-induced relaxation was abolished by oxyhemoglobin (1.6×10^{-5} M). Treatment of the strips for 40 min with KN62 (10^{-6} and 5×10^{-6} M) attenuated the response to nicotine dose-dependently, but did not change the NO-induced relaxation (Fig. 5). Recordings of the responses are shown in Fig. 6. As indicated in Table 1, PMA (10^{-7} M), 8-bromo cyclic GMP (10^{-5} M) and 8-bromo cyclic AMP (10^{-5} and 10^{-4} M) did not significantly influence the relaxations caused by nicotine and NO. 8-Bromo-cyclic GMP (10^{-4} M) markedly relaxed the arteries and attenuated the responses to nicotine and NO. The reduced tone could not be restored by supplemental doses of prostaglandin $F_{2\alpha}$.

3.3. Cyclic GMP content in the cerebral artery

The nucleotide content in endothelium-denuded arteries averaged 103 ± 40 fmol/mg wet tissue weight, which increased about four times following the application of 10^{-4} M nicotine (Fig. 7). Treatment with 5×10^{-6} M KN62 markedly reduced the stimulating effect of nicotine. KN62 per se did not change the basal level of cyclic GMP.

3.4. Relaxations induced by KN62

In order to determine whether KN62 can cause Ca^{2+} channel blockade (Lu et al., 1994; Yuan and Bers, 1994), the relaxant response was compared in the same strips of cerebral arteries contracted with 2×10^{-6} M prostaglandin $F_{2\alpha}$ and equipotent concentrations of K^{+} (13 to 18 mM). In 3 out of 6 strips from separate dogs, KN62 (5×10^{-6}

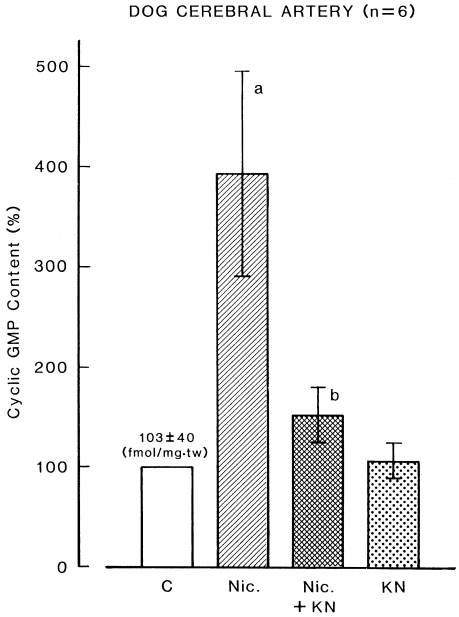

Fig. 7. Modifications by nicotine (Nic., 10^{-4} M) and KN62 (KN, 5×10^{-6} M) of the content of cyclic GMP in cerebral arterial strips denuded of the endothelium. The nucleotide content under control conditions (C) was taken as 100%. Significantly different from control, $^{a}P < 0.01$; significantly different from the value with nicotine, $^{b}P < 0.05$ (Tukey's method). The results were obtained in the arteries obtained from 6 separate dogs.

M) was applied to the strips contracted with prostaglandin $F_{2\alpha}$ and the remaining 3 strips were contracted first with K^+. Mean values for the relaxation in prostaglandin $F_{2\alpha}$ and K^+-contracted strips were 70.2 ± 4.0 and $70.8 \pm 5.2\%$ ($n = 6$ from separate dogs) relative to the relaxations induced by 10^{-4} M papaverine.

4. Discussion

The relaxation of canine cerebral arteries induced by perivascular nerve stimulation is mediated by NO synthesized from L-arginine via constitutive NO synthase (Toda and Okamura, 1996). The response was markedly attenuated by treatment with 10^{-6} M KN62, an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II (inhibition constant $= 0.9 \times 10^{-6}$ M) (Hidaka and Kobayashi, 1992), whereas the relaxation caused by electrical neural stimulation of canine coronary arteries was not or was only slightly inhibited. The coronary artery response was abolished by β-adrenoceptor antagonists as well as tetrodotoxin, suggesting the involvement of norepinephrine from adrenergic nerves, which then stimulates β-adrenoceptors (Toda and Hayashi, 1982). Therefore, the inhibition by KN62 appears to be exerted selectively on processes relating to the production of NO from L-arginine. The slow onset of the inhibition by KN62 may not support the idea of a direct inhibition of nNOS or interference with cofactor binding by KN62. However, the possibility of delayed access of the inhibitor to the binding sites can not be excluded. Although less evident than its effect on the response to electrical stimulation, KN62 also inhibited the relaxant response to nicotine that is mediated by NO produced in nerve terminals (Toda, 1995). In addition, the nicotine-induced increase in cyclic GMP in the arterial tissue was markedly reduced by KN62, which by itself did not alter the nucleotide content. The increase in the cyclic GMP content elicited by nicotine is postulated to result from guanylate cyclase activation by neurogenic NO (Toda and Okamura, 1991). HDBA, another Ca^{2+}/calmodulin-dependent protein kinase II inhibitor (O'Dell et al., 1991), also suppressed the response to vasodilator nerve stimulation. These findings suggest that the suppression of the cerebroarterial response elicited by these inhibitors is associated with the inhibition of NO synthesis, possibly due to inhibition of the phosphorylation of NO synthase by Ca^{2+}/calmodulin-dependent protein kinase II.

KN62 blocks T- and L-type Ca^{2+} channels in isolated calf adrenal cells (Lu et al., 1994) and rabbit and ferret ventricular myocytes (Yuan and Bers, 1994). The response to nerve stimulation of canine cerebral arteries is markedly inhibited or abolished by treatment with a non-selective Ca^{2+} channel inhibitor (Toda and Okamura, 1992), ω-conotoxin (only for the response to electrical stimulation but not the response to nicotine), Ca^{2+} deprivation (Toda et al., 1995) and calmodulin inhibitors (Okamura and Toda, 1994). The possible involvement of Ca^{2+} channel inhibition by this Ca^{2+}/calmodulin-dependent protein kinase II inhibitor should thus be excluded. An N-type Ca^{2+} channel inhibitor, ω-conotoxin, depressed the neurogenic relaxation of coronary arteries, as seen with cerebral arteries (Toda et al., 1995). However, cerebral artery responses were evidently more susceptible to KN62 than those of coronary arteries. In addition, the relaxant responses to KN62 were identical in the cerebral artery strips contracted with prostaglandin $F_{2\alpha}$ and with K^+. It is widely recognized that drugs that have a Ca^{2+} entry-blocking action preferentially relax vascular smooth muscle contracted with membrane-depolarizing agents, including K^+ (Godfraind, 1994). Therefore, as far as the concentrations used (1 and 5×10^{-6} M) are concerned, there was no evidence showing that KN62 interferes with the influx of Ca^{2+} across the cell membrane of perivascular nerves innervating the canine cerebral artery. Calmidazolium, a calmodulin inhibitor, suppressed the response to nerve stimulation of coronary arteries, as observed with dog cerebral arteries (Okamura and Toda, 1994), whereas KN62 selectively attenuated the cerebral artery response. The calmodulin-inhibitory action of KN62 would therefore be ruled out. As far as the results obtained from the present study are concerned, phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase II of NO synthase is expected to participate in the activation of NO synthase in nerve terminals. In contrast, biochemical studies with NO synthase purified from the rat brain indicates that phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase II reduces the enzyme's activity (Nakane et al., 1991). Whether the discrepancy is due to different materials (dog cerebral artery vs. rat brain) or different experimental conditions (integrated tissue vs. isolated enzyme) remains to be clarified.

Phosphorylation by protein kinase C increases the activity of NO synthase isolated from the rat brain (Nakane et al., 1991) but, in contrast, decreases its activity in NO synthase-transfected cells (Bredt et al., 1992). In the present study, PMA in concentrations sufficient to activate the kinase (Castagna et al., 1982; Tanaka et al., 1984) did not significantly alter the responses of canine cerebral arteries to nerve stimulation by electrical pulses or nicotine and to exogenous NO. Evidence for the activation of NO synthase associated with phosphorylation by protein kinase C was not obtained with the materials used in this study. The exocytotic release of norepinephrine from adrenergic nerves is suggested to be regulated by protein kinase C (Wakade et al., 1985). In contrast to the classic type of neurotransmission, nitrergic nerves are expected to liberate NO that is newly synthesized from L-arginine but which has not been stored in synaptic vesicles for exocytotic release (Toda and Okamura, 1991).

8-Bromo-cyclic AMP, which easily permeates through the cell membrane, did not influence the neurogenic relaxation. Phosphorylation by cyclic AMP-dependent protein

kinase is unlikely to be involved in the activation and deactivation of NO synthase. The same conclusion was reached in a study of NO synthase purified from the rat and porcine cerebellum (Brune and Lapetina, 1991). However, Dinerman et al. (1994) have reported the inhibitory effect of dibutyryl cyclic AMP on NO synthase in transfected cells.

8-Bromo-cyclic GMP did not alter the neurogenic response at 10^{-5} M, but tended to reduce the response to electrical stimulation at 10^{-4} M and the relaxations induced by nicotine and NO. Because of the intense vasodilator and non-selective inhibitory actions of this compound, modification by cyclic GMP-dependent protein kinase of NO synthase activity could not be proven in the present study. In contrast, 8-bromo-cyclic GMP reduces the nitrite release in response to Ca^{2+} ionophore in neuronal NO synthase-transfected cells (Dinerman et al., 1994).

This report provides pharmacological evidence to suggest the possible involvement of the phosphorylation pathway involving Ca^{2+}/calmodulin-dependent protein kinase II in the production of NO as a neurotransmitter in vasodilator nerves innervating the cerebral artery. It is important to clarify the physiological roles of NO synthase phosphorylation by other protein kinases, which could not be determined in the present study with isolated canine cerebral arteries with a nitrergic innervation.

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