Mechanism of prejunctional muscarinic receptor-mediated inhibition of neurogenic vasodilation in cerebral arteries

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Liu, J., and T. J. F. Lee. Mechanism of prejunctional muscarinic receptor-mediated inhibition of neurogenic vasodilation in cerebral arteries. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H194–H204, 1999.—Nitric oxide (NO) is a major transmitter in mediating cerebral neurogenic vasodilation in several species. Recent findings have suggested that acetylcholine, which is costored with NO in cerebral perivascular nerves, plays a role in modulating NO release, presumably by acting on muscarinic (M) receptors on nitric oxidergic nerve terminals. The present study was designed using an in vitro tissue bath technique to pharmacologically characterize the presynaptic muscarinic-receptor subtype(s) that mediate modulation of NO release and therefore neurogenic vasodilation and to investigate further the possible mechanisms involved in this presynaptic modulation in porcine basilar arteries. Transmural nerve stimulation (TNS) elicited a frequency-dependent, tetrodotoxin-sensitive relaxation. The relaxation was abolished by nitro-L-arginine (30 µM) and was completely reversed by L-arginine and L-dulhulline, but not by their D-enantiomers. Atropine (0.01–1 µM), pirenzepine (an M3-receptor antagonist, 0.01–1 µM), and methoctramine (an M2-receptor antagonist, 0.01–1 µM), but not 4-DAMP (an M1-receptor antagonist, 1 µM), and methoctramine (an M3-receptor antagonist) or tropicamide (an M4-receptor antagonist) at concentrations as high as 10 mM, significantly increased the TNS-elicited relaxation. This relaxation, on the other hand, was significantly attenuated by arecaidine but-2-ynyl ester tosylate (an M2-receptor agonist, 0.1 µM) but was not affected by McN-A-343 (an M1-receptor agonist, 1 µM). Double-labeling immunohistochemical study demonstrated that perivascular M2 receptor-immunoreactive fibers were completely coincident with NADPH diaphorase fibers. Furthermore, the muscarinic receptor-mediated modulation of TNS-elicited relaxation was completely prevented by w-conotoxin GVIA (0.1 µM), a specific N-type Ca2+ channel inhibitor, but was still observed in the presence of tetroethyl ammonium (1 mM), B-(−)-2-chloroaurate (100 µM), and pertussis toxin. It is concluded that the presynaptic M2 receptors on porcine cerebral perivascular nitric oxidergic nerves mediate inhibition of NO release. The inhibition is due primarily to a decreased Ca2+ influx through N-type Ca2+ channels.

Nitric oxide; presynaptic muscarinic receptor; transmural nerve stimulation; M2 receptor-immunoreactive fibers; N-type calcium channel; cerebral blood vessels; porcine

Nitric oxide (NO) has been shown to play a predominant role in mediating cerebral neurogenic vasodilation in several species (13, 34, 48). Release of NO from cerebral perivascular nerves has been demonstrated (13, 14). Because of its chemical properties, NO is not likely to be stored in the vesicles or released from neurons by the exocytotic mechanism, like classic neurotransmitters such as acetylcholine (ACh; see Ref. 37). The exact mechanism of NO release from perivascular nerves remains unclear.

NO synthase (NOS) and choline acetyltransferase (ChAT) have been found to coexist in the parasympathetic ganglion and perivascular nerves in cerebral blood vessels of several species (27, 46). Results from pharmacological studies, however, have demonstrated that NO mediates the major component of the neurogenic vasodilator response (33), whereas endogenous ACh exhibits a negligible direct effect on vascular smooth muscle tone, possibly due to its low synaptic concentration and wide synaptic distance (31). In fact, the direct effect of exogenous ACh on cerebral vascular smooth muscle is a constriction rather than a dilation (30, 31). Furthermore, the possibility that endogenous ACh, which is released from adventitial nerves, may induce an endothelium-dependent neurogenic vasodilation is very unlikely, at least in the large cerebral arteries (31). Accordingly, ACh has been proposed to act presynaptically to be more like a modulator (32). This hypothesis has recently been supported by the findings that endogenous ACh may act on presynaptic muscarinic (M) receptors on nitric oxidergic nerves to inhibit release of cotransmitter NO and therefore diminished vasodilation (4, 47). The exact mechanism of this presynaptic cholinergic modulation of NO-mediated vasodilation, however, is not clarified.

Four subtypes of muscarinic receptors (M1–M4) have been pharmacologically characterized, and all subtypes have been reported to be involved in the presynaptic modulation of neurotransmitter release in both central and peripheral neurons (12). In perivascular nerves, although the presynaptic M2-receptor subtype is most frequently reported to mediate muscarinic modulation of neurotransmitter release (2, 45, 47, 53), other subtypes are also involved. For example, the M3-receptor subtype has been shown to mediate inhibition of ACh and norepinephrine (NE) release in bovine cerebral arteries (19), whereas the M1-receptor subtype mediates inhibition of NE release in guinea pig carotid arteries (10). The prejunctional muscarinic receptors mediating inhibition of NE release in the rabbit ear arteries do not appear to be the M1, M2, or M3 subtype (16). Most of these studies were examining effects of presynaptic muscarinic receptors on release of classic neurotransmitters. The present study was designed to investigate, by a pharmacological approach in porcine basilar arteries, the presynaptic muscarinic-receptor subtype and its related mechanism(s) that are involved in inhibition of NO-mediated neurogenic vasodilation.
MATERIALS AND METHODS

**In Vitro Tissue Bath**

Fresh heads of adult pigs of either sex were collected from a local slaughterhouse. The entire brain was removed and placed in Krebs-bicarbonate solution equilibrated with 95% O2-5% CO2 at room temperature. The composition of the Krebs-bicarbonate solution was as follows (in mM): 122.0 NaCl, 5.2 KCl, 1.33 CaCl2, 1.2 MgSO4, 25.0 NaHCO3, 0.03 disodium EDTA, 0.01 L-ascorbic acid, and 11.0 glucose (pH 7.4).

Basilar arteries were dissected and cleaned of surrounding tissue under a dissecting microscope. The endothelium was mechanically denuded according to our previous reports (31, 34). A complete removal of endothelial cells was verified by the failure of nitro-L-arginine (L-NNA) in raising U-46619-34-induced responses in the bath. Tissues were incubated in Krebs-bicarbonate solution equilibrated with 95% O2-5% CO2 at 37°C. Tissues were equilibrated in the Krebs-bicarbonate solution for the initial 30 min and then were mechanically stretched to a resting tension of 0.75 g (34, 35). U-46619 (0.1–1 µM) was applied to induce an active muscle tone of ~0.75 g. Tissues were electrically, transmurally stimulated with a pair of platinum electrodes through which 100 biphasic square-wave pulses of 0.6 ms in duration and constant 200 mA in intensity were applied at various frequencies (31). Stimulation parameters were continually monitored on a Tektronix oscilloscope (31). The neurogenic origin of this transmural nerve stimulation (TNS)-induced response was verified by its complete blockade by tetrodotoxin (TTX; 1 µM). Papaverine (300 µM) was applied to each tissue to induce maximum relaxation at the end of the experiment. The magnitude of a vasodilator response during the experiments was expressed as a percentage of the maximum response induced by papaverine (31).

All drugs, unless otherwise stated, were dissolved in deionized water and were added directly to the tissue baths after control relaxation induced by TNS was established (35). The concentrations of drugs reported were the final concentrations in the bath. TNS was elicited 15 min after each experimental drug was added. Each tissue preparation served as its own control.

**Immunohistochemistry**

The basilar and middle cerebral arteries were dissected and immediately fixed in 4% paraformaldehyde in 0.1 phosphate-buffered saline (PBS; pH 7.4) at 4°C overnight. The fixed arterial preparations were washed in 0.01 M PBS and blocked with 1% normal goat serum (NGS) diluted in 0.05% Triton X-100-PBS for 30 min at room temperature. The specimens then were incubated with rat monoclonal anti-m2 muscarinic receptor antibody (1:100 diluted in 0.05% Triton X-100-PBS-1.5% NGS; Chemicon, Temecula, CA) at 4°C overnight. The m2 receptor antibody was raised against the third intracellular loop of m2-receptor fusion proteins. After a wash in PBS, the specimens were incubated with biotinylated goat anti-rat IgG antibody (1:200 diluted in PBS; Vector Laboratories) for 1 h at room temperature. After another wash in PBS, the specimens were incubated with FITC-labeled avidin D (1:80 diluted in PBS; Vector Laboratories) in the dark for 1 h at room temperature. The specimens were then rinsed and covered with coverslips and Vectashield mounting medium (Vector Laboratories) for photography under a fluorescence microscope fitted with proper filters (Olympus BX50 microscope). After the immunofluorescence fibers were photographed, the specimens were washed in PBS and processed for NADPH diaphorase (NADPHd) histochemistry (27, 54) as described below. For negative control, no m2 receptor- immunoreactive fibers were observed when tissues were incubated with nonimmunized serum or without m2-receptor antisemur (54).

**NADPHd Histochemical Staining**

After immunofluorescence labeling of m2 receptor and photography as described above, the specimens were washed in PBS and then incubated in 0.1 M phosphate buffer containing 0.5 mg/ml NADPH (reduced form), 0.1 mg/ml nitro blue tetrazolium, and 0.3% Triton X-100 at 37°C for 1 h (54). The specimens were rinsed with PBS, mounted with Gel Mount (Biomedca, Foster City, CA), and examined under a microscope. The same field previously photographed for m2-receptor immunoreactivity was rephotographed for NADPHd fibers. For negative control, no NADPHd fibers were found in tissues incubated in the absence of NADPH.

**Statistical Methods and Drugs Used**

The data were computed as means ± SE and were evaluated by Student’s t-test for paired samples and ANOVA for multigroup comparisons. The following drugs were used: atropine, L-NNA, L-arginine, L-citrulline, TTX, NADPH, U-46619 (9,11-dideoxy-9α,11α-epoxymethanoprostaglandin F2α), arecaidine but-2-ynyl ester tosylate (ABET), tetraethylammonium (TEA), 8-bromo-cAMP, nitro blue tetrazolium, sodium nitroprusside (SNP), all from Sigma Chemical (St. Louis, MO); and tetrodotoxin, 4-diphenylacetoxy-N-(2-chloroethyl)-piperidine (4-DAMP) mustard hydrochloride, methoctramine hydrochloride, McN-A-343, pirenzepine, ω-conotoxin GVIA (CTX), and pertussis toxin (PTX), all from RBI (Natick, MA).

**RESULTS**

**Atropine Enhances TNS-Elicited Neurogenic Vasodilation**

In the presence of active muscle tone induced by U-46619, porcine cerebral arterial rings without endothelial cells relaxed exclusively upon TNS (Fig. 1A). The relaxation was abolished by TTX (0.1 µM) and L-NNA (30 µM), a result similar to that reported previously in the same arterial preparations (34). The relaxation induced by TNS at various frequencies (2, 4, and 8 Hz) was significantly enhanced in the presence of atropine (0.1 µM; Fig. 1A and B, and Fig. 2). The enhanced relaxation was completely inhibited by L-NNA (30 µM; Fig. 1, A and B) and nitro-L-arginine methyl ester (30 µM, n = 6, data not shown). The inhibition was fully reversed by L-arginine (1 mM) and L-citrulline (1 mM) but not by d-arginine or o-citrulline (n = 8, Fig. 1, A and B). TNS-induced relaxation, on the other hand, was significantly inhibited by exogenously applied physostigmine (0.3 µM) and ACh (1 µM; n = 6–8, Fig. 3).

**Effects of Selective Muscarinic-Receptor Antagonists and Agonists on TNS-Elicited Vasodilation**

Pirenzepine (0.1–10 µM), a selective M2-receptor antagonist, and methoctramine (0.01–10 µM), a selective M1-receptor antagonist, in a concentration-depen-
dent manner significantly increased TNS-induced relaxation in basilar arteries without endothelial cells (Figs. 4, A and B). The threshold concentration, however, was 10-fold higher for pirenzepine than methoctramine.

4-DAMP (0.01–10 µM), a selective M3-receptor antagonist, did not significantly affect TNS-induced relaxation when compared with the effect produced by its solvent, DMSO (Fig. 4C). Tropicamide (0.01–10 µM), a selective M4-receptor antagonist, did not affect TNS-induced relaxation either (Fig. 4D).

Furthermore, ABET (0.01–10 µM), a selective M2-receptor agonist that slightly increased the basal tone in arteries without endothelial cells, significantly and concentration dependently inhibited relaxation elicited by TNS at various frequencies (Fig. 5B, EC50 = 0.05, 0.17, and 0.44 µM for 2, 4, and 8 Hz, respectively). On the other hand, McN-A-343 (0.01–10 µM), a selective M1-receptor agonist that did not change the basal vascular tone, did not affect TNS-induced relaxation until its concentration reached 10 µM (Fig. 5A, EC50 values were at least 8 µM for all 3 stimulating frequencies).

Coincidence of m2 Receptor-Immunoreactive Fibers and NADPHd Fibers in Porcine Cerebral Arteries

In whole mount porcine basilar and middle cerebral arteries, m2 receptor-immunoreactive fibers were found to consist of bundles of various sizes and fine fibers (Fig. 6A). Most m2 receptor-immunoreactive fibers were coincident with NADPHd fibers (Fig. 6B). In negative control in which the tissue was incubated with non-immunized serum or denervated by cold storage for a week, no m2 receptor-immunoreactive or NADPHd fibers were found (data not shown).

Effect of PTX on Muscarinic Receptor-Mediated Inhibition of TNS-Induced Relaxation

After the endothelium-denuded arterial preparations were incubated with PTX (500 ng/ml-25 µg/ml) at 37°C for 4–7 h according to our previous report (26), ABET still significantly inhibited TNS-induced relaxation in the presence of active muscle tone induced by U-46619, and the inhibition was completely reversed by atropine (0.1 µM, n = 7). There was no significant
difference between the control and PTX-treated preparations on responses to ABET and atropine (Fig. 7).

Effects of Ca$^{2+}$ Channel Blocker, cAMP Analog, and Potassium Channel Blocker on Muscarinic Receptor-Mediated Inhibition of Cerebral Neurogenic Vasodilation

CTX. CTX, a selective N-type Ca$^{2+}$ channel blocker that did not affect the U-46619-induced active muscle tone of basilar arteries without endothelial cells, significantly inhibited the relaxation elicited by TNS at 2 and 4 Hz (Fig. 8). In the presence of CTX (0.1 µM), the residual relaxation induced by TNS was not affected by ABET or atropine (n = 6 for each group, Fig. 8).

8-Bromo-cAMP. 8-Bromo-cAMP (0.5 mM), a membrane-permeable cAMP analog that slightly relaxed the basal vascular tone in arteries without endothelial cells, did not significantly affect TNS-induced relaxation (Fig. 9). In the presence of 8-bromo-cAMP, ABET still inhibited TNS-induced relaxation (Fig. 9), and the inhibition was reversed by atropine (0.1 µM; data not shown). In parallel studies, ABET inhibition of vasodilation elicited by TNS at 2 and 4 Hz was partially reversed by 21.0 ± 9.2 and 30.9 ± 17.0%, respectively, after addition of 8-bromo-cAMP (0.5 mM; n = 8 for each group, Fig. 9B).

TEA. TEA (1 mM), a nonselective potassium channel blocker that significantly increased (20–50%) the basal tone of basilar arteries without endothelial cells, significantly enhanced the neurogenic vasodilation elicited by TNS at 2 and 4 Hz (Fig. 10A). TEA (1 mM), however, slightly but significantly inhibited the relaxation induced by SNP (an NO-donor; 0.1–100 µM). The EC$_{50}$ values for SNP in inducing relaxation in the control and TEA-treated tissues were 2.94 (1.59–5.43) × 10$^{-7}$ M and 8.47 (6.0–12.0) × 10$^{-7}$ M (n = 4, P < 0.05), respectively. In the presence of TEA, ABET (1 µM) significantly inhibited the relaxation induced by TNS at all frequencies examined (n = 8 for each group, Fig. 10B), and the inhibition was reversed by atropine (0.1 µM; data not shown).

DISCUSSION

The present study demonstrated that TNS-induced, NO-mediated relaxation in porcine cerebral arteries without endothelial cells was significantly enhanced by atropine and M$_1$- and M$_2$-receptor antagonists. The relaxation however was significantly inhibited by an M$_2$ (but not M$_1$-)receptor agonist, ACh, and physostigmine. These muscarinic receptor-mediated responses were completely prevented by CTX. Together with the presence of coincident m$_2$ receptor-immunoreactive fibers and NADPHd fibers in cerebral arteries, it is suggested that activation of presynaptic M$_2$ receptors on cholinergic-nitric oxidergic nerve terminals (27) by
endogenous ACh results in inhibition of NO-mediated neurogenic vasodilation. The inhibition appears to result from an M₂-receptor-mediated decrease in Ca²⁺ influx via N-type Ca²⁺ channels and therefore a diminished synthesis and release of NO.

Fig. 4. Effects of different muscarinic-receptor-subtype antagonists on TNS (4-Hz)-induced relaxation in porcine basilar arteries without endothelial cells. Pirenzepine (A) and methoctramine (B) concentration dependently increased TNS-induced relaxation. *P < 0.05 and **P < 0.01, significant difference from respective control. Effect of 4-diphenylacetoxy-N-(2-chloroethyl)-piperidene mustard hydrochloride (4-DAMP; C) on TNS-induced relaxation was not significantly different (P > 0.05) from that produced by its solvent, DMSO. Tropicamide (D) did not affect TNS-induced relaxation either (P > 0.05). Data represent means ± SE; n indicates number of experiments in each group.

Fig. 5. Effects of M₁- and M₂-receptor agonists on TNS-induced relaxation in porcine basilar arteries without endothelial cells. A: McN-A-343 did not affect TNS-induced relaxation at concentrations below 1 µM. At 10 µM, McN-A-343 significantly inhibited relaxation induced by TNS at 2, 4, and 8 Hz. B: arecaidine but-2-ynyl ester tosylate (ABET) concentration dependently inhibited TNS-induced relaxation with significantly lower EC₅₀ values than those of McN-A-343 at all stimulating frequencies. *P < 0.05 and **P < 0.01, significant difference from respective control. Data represent means ± SE; n indicates number of experiments in each group.
ation is abolished by NOS inhibitors and NO scavengers and that L-citrulline, the by-product of NO synthesis, can be recycled to form L-arginine for synthesizing NO in cerebral perivascular nerves (13, 14, 35, 54). These results provide evidence indicating that NO can be released from the perivascular nerves to induce neurogenic vasodilation. Release of ACh from perivascular nerves has also been demonstrated in isolated cerebral arteries from several species, such as the rabbit (18) and pig (our pilot studies). Therefore, it is reasonable to suggest that both NO and ACh are coreleased from the perivascular nerves upon TNS.

The relaxation of cerebral arteries from several species elicited by TNS, however, is predominantly mediated by NO (33, 34). The endogenous ACh does not exhibit any direct effect on the vascular smooth muscle. This is based on the findings that, although a direct effect of exogenous ACh on cerebral vascular smooth muscle is a constriction (30, 33), in the presence of L-NNA (34) or oxyhemoglobin (an NO scavenger; see Ref. 36) to abolish NO-mediated relaxation, TNS has never elicited a cholinergic receptor-mediated constriction in cerebral arteries without endothelial cells. These findings indicate that neurally released ACh upon TNS does not directly affect the postsynaptic smooth muscle, possibly due to a combination of long synaptic distance and a low synaptic concentration of ACh (31).

Accordingly, it has been hypothesized that ACh acts more like a presynaptic transmitter (31, 32).

In the present study, TNS-elicited neurogenic vasodilation in porcine basilar arteries was enhanced by atropine. This enhancement was most likely due to increased NO release from perivascular nerves, since it was abolished by NOS inhibitors and was fully reversed by L-arginine (the precursor of NO synthesis) and L-citrulline, which has been shown to be recycled to form L-arginine for synthesizing NO in porcine cerebral perivascular nerves (14). In addition, the exogenously applied ACh and physostigmine, a cholinesterase inhibitor, inhibited the TNS-elicited relaxation, suggesting that the endogenous ACh coreleased with NO from cholinergic-nitric oxidergic nerves (27) acts on presynaptic muscarinic receptors to inhibit further release of NO and NO-mediated relaxation. This finding is consistent with those found in the bovine and monkey cerebral arteries (4, 47).

Four muscarinic-receptor subtypes have been defined pharmacologically as M₁, M₂, M₃, and M₄ subtypes, corresponding to their genes of m₁, m₂, m₃, and m₄, respectively (12, 24). M₁ receptors are defined by high affinity for pirenzepine and low affinity for methoctramine and 4-DAMP. M₂ receptors are defined by high affinity for pirenzepine but low affinity for methoctramine and 4-DAMP. M₃ receptors have high affinity for 4-DAMP but not for pirenzepine (12), and M₄ receptors have high affinity for tropicamide (28, 29), although they have high affinity for pirenzepine, methoctramine,
and 4-DAMP as well (12). All four muscarinic-receptor subtypes have been reported to be involved in presynaptic modulation of transmitter release in various tissue preparations of both peripheral and central nervous systems (12). In the present study, neither 4-DAMP nor tropicamide affected TNS-induced relaxation in porcine basilar arteries, suggesting that M3 and M4 receptors are unlikely to mediate the inhibition of NO release by endogenous ACh. On the other hand, pirenzepine and methoctramine, like atropine, significantly enhanced TNS-induced relaxation, suggesting that M1 and M2 receptors may be involved in mediating the modulation of NO release. TNS-induced relaxation, however, was significantly inhibited by ABET, a selective M2-receptor agonist (42), with EC50 values ranging from 0.05 to 0.4 µM, depending on stimulating frequency, but was not inhibited by McN-A-343, a selective M1-receptor agonist (39), until its concentration was >10 µM. These results suggest that the M2 receptor is the most likely muscarinic subtype that is involved in the presynaptic inhibition by endogenous ACh of NO release. This is further supported by results from immunohistochemical studies that M2 receptor-immunoreactive fibers were coincident with NADPHd fibers in porcine cerebral arteries. Because NADPHd is a reliable marker for NOS in the porcine cerebral arteries (54), the present results suggest that M2 receptors are present on the nitric oxideergic nerve fibers. Although pirenzepine enhanced TNS-induced relaxation, the high concentration of McN-A-343 needed to affect NO-mediated relaxation may indicate a possible nonspecific effect of the M1-receptor antagonist and/or agonist. The possible involvement of presynaptic M1 receptors, however, can not be ruled out completely, since M1-receptor immunoreactivities were not performed due to inaccessibility of M1-receptor antibodies.

It is well established that neurotransmitter release in many tissues is dependent on Ca2+ influx via voltage-sensitive N-type Ca2+ channels, which are found in neurons but not in the smooth muscle (6, 38, 40). Release of NO evoked by electrical stimulation has also been shown to be Ca2+ dependent (7, 8). In the present study, the relaxation of porcine basilar arteries induced by TNS at different frequencies was significantly de-

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**References:**
due exclusively to a negative coupling of M2 receptors to bition of TNS-elicited nitric oxidergic vasodilation was significantly blocked study in porcine pial veins indicated that PTX signifi-

The exact mechanism by which activation of the M2 receptor is coupled to inhibition of N-type Ca2+ channels is not established. Activation of M2 receptors has been shown to be negatively coupled to adenylyl cyclase (51). Evidence however has also been presented to indicate that cAMP is not essential in modifying voltage-dependent Ca2+ channels after activation of G protein-coupled receptors, including the muscarinic receptor in neuronal preparations (3, 5, 15, 17, 21). This is in line with the findings of the present study that PTX failed to prevent the inhibitory effect of muscarinic (including M2)-receptor agonists or the potentiating effect of muscarinic-receptor antagonists on TNS-induced relaxation. The exact reason for the failure of PTX in modifying the muscarinic-receptor response in the present study remains to be determined. An almost identical PTX incubation condition used in our previous study in porcine pial veins indicated that PTX significantly blocked α2-adrenoceptor-mediated NE-induced vasoconstriction in these veins (26). Several reports by others also have indicated that presynaptic M2 receptor- and α2-adrenoceptor-mediated inhibition of NE release from sympathetic nerve terminals are PTX-insensitive (43, 53). Consistent with these findings is the recent suggestion of a direct interaction between voltage-dependent Ca2+ channels and G protein-coupled receptors via a membrane mechanism (23) and the observation that the beta-subunit of G protein may play an important role in this direct modification (22, 23, 25). Whether this mechanism is involved in the presynaptic inhibition of NO release from cerebral perivascular nerves remains to be examined.

The insignificant role of cAMP in negative coupling of M2 receptor to N-type Ca2+ channels is further supported by the present findings that the M2-receptor agonist-produced inhibition of TNS-induced relaxation was still observed in the presence of a high concentration (0.5 mM) of 8-bromo-cAMP, a membrane-permeable cAMP analog. When it was administered after ABET inhibition of neurogenic vasodilation, however, 8-bromo-cAMP slightly but significantly reversed the neurogenic effect of ABET. Although the exact mechanism of this partial reversal by 8-bromo-cAMP remains unknown, this finding suggests that, should it be involved in M2 receptor-mediated inhibition of neurogenic vasodilation, the intracellular cAMP is not a major factor in causing M2-receptor-mediated downregulation of N-type Ca2+ channels in cerebral perivascular nerves.

Activation of muscarinic receptors has also been shown to increase potassium conductance, resulting in membrane hyperpolarization and a decrease in voltage-dependent Ca2+ channel openings in neurons (6, 9). In the present study, however, TEA (1 mM), a nonspecific potassium channel blocker, significantly enhanced TNS-elicited neurogenic vasodilation. This enhancement was most

![Figure 9](http://www.ajpheart.physiology.org/)

**Fig. 9. Effect of 8-bromo-cAMP (8-Br-cAMP) on M2-receptor agonist-induced inhibition of TNS-induced relaxation in porcine basilar arteries without endothelial cells.** A: 8-Br-cAMP (0.5 mM) alone did not affect TNS-induced relaxation. In the presence of 8-Br-cAMP (0.5 mM), ABET (1 µM) significantly inhibited TNS-induced relaxation at both frequencies. *P < 0.01, significant difference from respective control and 8-Br-cAMP group. B: neurogenic vasodilation induced by TNS at 2 and 4 Hz was inhibited by ABET. Inhibition was partially reversed by 8-bromo-cAMP (0.5 mM). #P < 0.01, significant difference from ABET group. Data represent means ± SE; n indicates number of experiments in each group.
likely due to increased release of NO from perivascular nerves, since TEA did not increase but rather decreased relaxation induced by SNP (an NO-donor; 0.1–100 µM). Furthermore, in the presence of TEA, relaxation was decreased by ABET and reversed by atropine. These effects of TEA and ABET on relaxation elicited by TNS at different frequencies are summarized in B, showing that TEA (1 mM) significantly enhanced relaxation elicited by TNS at 2 and 4 Hz. *P < 0.01, significant difference from respective control. In the presence of TEA (1 mM), ABET (1 µM) significantly inhibited relaxation induced by TNS at both frequencies. #P < 0.01, significant difference from respective TEA group. Data represent means ± SE; n indicates number of experiments in each group.

In conclusion, the present study demonstrated that endogenous ACh, by acting on presynaptic M2 receptors on perivascular nitric oxideergic-cholinergic nerves in porcine basilar arteries, inhibited NO release and therefore diminished NO-mediated neurogenic vasodilation. This M2 receptor-mediated inhibition of NO release and NO-mediated vasodilation appear to be mainly due to a negative coupling of M2 receptors to N-type Ca2+ channels. This results in diminished Ca2+ influx, leading to a decreased NOS activity, NO synthesis, and neurogenic vasodilation. The exact mechanism underlying this negative coupling remains undetermined. However, it is not likely mediated by a decrease in cAMP synthesis and/or potassium channel function. The present finding on the role of muscarinic cholinergic receptors in mediating inhibition of neurogenic vasodilation in large cerebral arteries at the base of the brain is different from that found in the cortical microvascular circulation. Electrical stimulation of a central cholinergic system originating in the nucleus basalis of Meynert and substantia innominata has been shown to contribute to the cortical vasodilator response via activation of muscarinic cholinergic receptors (44), although nicotinic cholinergic receptors have been shown to mediate the vasodilator response in both cortical circulation and large arteries at the base of the brain (44, 50, 55). This finding on the regional difference in cholinergic receptor mechanisms can be important for a complete elucidation of the physiological role of cholinergic innervation in regulating cerebral vascular function.
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