Neurogenic Electrical Responses of Single Smooth Muscle Cells of the Dog Middle Cerebral Artery

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SUMMARY. Electrical responses induced by perivascular nerve stimulation were recorded intracellularly from the smooth muscle of dog middle cerebral artery. With nerve stimulation, the muscle membrane produced excitatory junction potential and then a slow hyperpolarization. The excitatory junction potential showed facilitation and the slow hyperpolarization showed depression phenomena, when the nerves were stimulated with twin pulses. Generation of the slow hyperpolarization was associated with an increase in the potassium conductance of the membrane and it was suppressed by tetraethylammonium, which depolarized the membrane, reduced the membrane conductance, and increased the amplitude of the excitatory junction potential. Treatment with 6-hydroxydopamine abolished the excitatory junction potential, but not the slow hyperpolarization; the latter was suppressed by tetrodotoxin. The amplitude of slow hyperpolarization was decreased by application of tetraethylammonium or ATP, but was not affected by application of atropine, neostigmine, theophylline, apamin, ouabain, norepinephrine, propranolol, or guanethidine. ATP produced transient depolarization of the membrane with associated decrease in the membrane resistance. The excitatory junction potential was attributed to activation of the noradrenergic nerves, whereas the slow hyperpolarization was not generated by activation of adrenergic, cholinergic, or purinergic receptors. Inasmuch as the electrogenic Na-K pump, cAMP, and ATP were not involved in the generation of slow hyperpolarization, the possibility of an unidentified chemical transmitter should be given attention. (Circ Res 51: 751-759, 1982)

CEREBRAL blood vessels are innervated by adrenergic nerves, and transmural nerve stimulation produces vasoconstriction in the isolated cerebral arteries of many species. However, exogenously applied norepinephrine produces a weak vasoconstriction in the cerebral arteries compared to that in the systemic blood vessels. The evidence is supported by extensive work, and has been reviewed by many authors (Owman et al., 1974; Owman and Edvinsson, 1977; Bevan et al., 1980).

Electrophysiological studies showed that—in the cerebral arteries of guinea pigs and dogs—perivascular nerve stimulation produces depolarization of the membrane (Karashima and Kuriyama, 1981; Fujiwara et al., 1981; Fujiwara et al., 1982). This excitatory junction potential (EJP) showed properties similar to those found in the other arterial smooth muscles; i.e., facilitation, depression, and summation phenomena to repetitive nerve stimulation were observed, and a low concentration of phentolamine enhanced and norepinephrine decreased the amplitude of the EJP without change in the postjunctional membrane conductances.

The present study was undertaken to investigate the mechanisms of neuromuscular transmission by recording electrical activities of the smooth muscle membrane in the middle cerebral artery of dog. Pharmacological experiments were also done to determine what transmitter substances are released from the perivascular nerves.

Methods

Mongrel dogs, weighing 10–15 kg, were anesthetized with pentobarbital sodium (40 mg/kg, iv), and then exsanguinated by cutting the femoral artery. The entire brain with blood vessels was removed and placed in Krebs solution at room temperature. The main trunk of the middle cerebral artery was taken, and the surrounding tissues were removed, under microscopic conditions. A 1.5- to 2-cm length of middle cerebral artery was mounted in a chamber made of Lucite plate. The vessel was pinned at its cut ends in order to record its smooth muscle membrane electrical activity. The chamber had a volume of about 2 ml and was superfused with warmed (35.5°C) Krebs solution. The tissue was incubated in this condition for more than 2 hours before start of the experiment. A glass capillary microelectrode filled with 3 M KCl, and with a tip resistance of 40–80 MΩ, was impaled into single cells from the outer surface of the vessel.

Stimulation was applied to perivascular nerves with two silver wires, 0.5 mm in diameter. The cathodal electrode was coated with enamel, except for the cut end, which was allowed to gently touch the surface of the vessel wall (the point-stimulating method). Membrane responses of the smooth muscle cells were recorded from the surrounding areas of the cathodal stimulating electrode (usually within 200 μm distance). A current pulse, 0.05–0.1 msec long, was supplied from an electric stimulator (Nihonkohden SEN-7103) to stimulate the nerves. As the point-stimulating method does not allow for estimation of actual current density at the nerve fibers, the stimulus intensity was determined by the voltage at the output terminals of the stimulator. The partition-stimulating method (Abe and...
Results

Membrane Responses of Smooth Muscle Cells of Middle Cerebral Artery to Nerve Stimulation

Smooth muscle membrane of the dog middle cerebral artery was electrically quiescent, and the resting membrane potential varied from —45 to —55 mV (mean value was about —52 mV, Fujiwara et al., 1982). Perivascular nerve stimulation produced an excitatory junction potential (EJP) with an amplitude of 1–10 mV, and a following slow hyperpolarization (SHP) with an amplitude of up to 10 mV. There was no causal relationship in the amplitude between EJP and SHP. Figure 1 shows typical electrical activity of the membrane induced by nerve stimulation. The EJP had a duration of about 500 msec, whereas more than 10 seconds were required for the SHP to revert the membrane potential to the original level.

It was expected that stimulation of these nerves with different intensities could identify EJP and SHP, if each nerve has different thresholds. Perivascular nerves were stimulated at intervals of over 1 minute, and the stimulus intensity was lowered in 5 V steps from 100 V to a level 10–20 V below threshold to rule out the possible effect of the former stimulus. At each stimulus strength, nerves were stimulated five times and the mean value with so is expressed in the figure. In the cells studied, a stimulus intensity of less than 30 V (stimulus duration, 0.05 msec) produced no change in the membrane potential. In most cases, EJP and SHP were generated by a similar intensity of stimuli, and occasional differences did not exceed 10 V. In 18 cells from 10 tissues, 12 cells showed the same threshold intensity for the generation of EJP and SHP. Figure 1 shows typical electrical activity of the membrane induced by nerve stimulation. The EJP had a duration of about 500 msec, whereas more than 10 seconds were required for the SHP to revert the membrane potential to the original level.

Figure 2 shows two examples of the relationship between the intensity of current pulse and the amplitude of EJP (●) and SHP (○). Perivascular nerves were stimulated by a current pulse 0.05 msec in duration at intervals of over 1 minute. Stimulus intensity was lowered by 5 V step from 100 to 20 or 30 V, and five stimulations at each intensity were applied. The values in the figure represent the mean ± so of five observations (so is not shown in those cases in which it is smaller than the circle in the figure.) @: steps statistically significant. The values shown in panels a and b were obtained from single cells in different tissues.
intensity of 35–55 V, 60–65 V, 70–75 V, and 85–100 V. This cell also showed differences in threshold intensity of stimulus, and the threshold intensities for EJP and SHP were 45 V and 35 V, respectively.

Figure 3 shows the relationship between the interval of twin pulses and amplitudes of EJP (Fig. 3a) and SHP (Fig. 3b). The amplitude of the second EJP and SHP is expressed as a fraction of the first EJP and SHP, respectively. In the case of EJP, a facilitation phenomenon was observed, and the amplitude of the second EJP was larger than the first one, when the interval of the stimuli was less than five seconds. On the other hand, the SHP showed a depression phenomenon, and over 60 seconds were required to produce a SHP with an amplitude similar to the first one obtained.

Stimulation of the nerves with high frequency (>2 Hz) produced summation of the EJP and SHP. Figure 4 shows membrane responses produced by five pulses with different frequencies (0.2–100 Hz). With a frequency of less than 2 Hz, each EJP was generated separately and only the facilitation process was observed. The EJP showed a summation and the peak amplitude increased with a frequency of over 5 Hz. However, the action potential was not evoked even when the nerves were stimulated with five pulses at intervals as short as 10 msec. On the other hand, the amplitude of SHP increased cumulatively within this range of stimulus frequency. Figure 4b shows the relationship between the frequency of five pulses and the amplitudes of EJP and SHP, determined as potential differences from the resting membrane potential. Increase in the amplitude of EJP was divided into two phases, a slowly increasing phase (below 5 Hz frequency) and a rapidly increasing phase (over 5 Hz frequency). The amplitude of SHP increased with increase in the stimulus frequency up to 5 Hz, and over this frequency, the amplitude remained fairly constant.

These results suggest that the dog middle cerebral artery is innervated by at least two types of nerves. Mechanical responses induced by transmural nerve stimulation indicated dual innervation of the dog cerebral arteries, sympathetic and nonsympathetic nerves (Duckles, 1979). An experiment was thus done to deplete the functional adrenergic nerves from the middle cerebral artery using 6-hydroxydopamine (6-OHDA) (Thoenen and Tranzer, 1968; Aprigliano and Hermsmeyer, 1976).

Membrane responses to nerve stimulation were recorded from different cells in the same area before and after the treatment with 6-OHDA, and mean amplitude of EJP and SHP were compared in the two conditions. With a 3-pulse stimulation at 20 Hz frequency, the EJP observed before treatment with 6-OHDA showed a mean amplitude of $5.7 \pm 1.1$ mV $(n = 17)$, and such disappeared in the 6-OHDA-treated tissue $(n = 15)$. The amplitude of SHP was $3.5 \pm 0.7$ mV $(n = 17)$ in the control condition and $3.7 \pm 0.9$ mV $(n = 15)$ in the 6-OHDA-treated tissue. Figure 5 illustrates the effect of 6-OHDA and TTX on the amplitude of EJP and SHP recorded from different cells in response to three different trains of stimuli. The disappearance of the EJP, but not the SHP, following 6-OHDA treatment is evident.

These experiments show that the dog middle cerebral artery is indeed innervated by both adrenergic and nonadrenergic nerves. Activation of the former nerves produces an EJP, whereas the SHP is produced in the latter.

**Ionic Mechanism of Generation of Slow Hyperpolarization (SHP)**

To observe changes in membrane conductance during the generation of SHP, electrotonic potentials were recorded from the smooth muscle cells of the middle cerebral artery by application of current pulse, at a constant intensity, while the perivascular nerves were stimulated for one second with five pulses (i.e., five pulses were applied with 5 Hz frequency). Figure 6a shows the membrane responses produced by this nerve stimulation, in which the summed EJP and SHP are shown in a fast-speed recording. When constant inward and outward current pulses (two seconds in duration) were applied alternatively, as shown in Figure 6b, the amplitude of electrotonic potentials decreased during the SHP. Pretreatment with atropine $(3.5 \times 10^{-6} M)$ did not modify these responses (Fig. 6c).
This observation indicates that the SHP is produced by an increase in the membrane conductance, but that the increase is not mediated through stimulation of muscarinic receptors.

The EJP and SHP were generated by perivascular nerve stimulation, in different potassium concentrations. Figure 7 shows membrane responses induced by nerve stimulation (three pulses were applied at 20 Hz in the frequency) in three different [K+], solutions (2.4, 5.9, and 20 mM). The membrane potentials in each concentration of [K+]o were 55.6 ± 1.6 mV (n = 19) in 2.4 mM, 51.8 ± 2.0 mV (n = 24) in 5.9 mM, and 43.9 ± 1.6 mV (n = 21) in 20 mM. In 2.4 mM [K+]o solution, the amplitude of EJP decreased while that of SHP increased, compared to values obtained in 5.9 mM [K+]o solution (i.e., control Krebs solution). Increase in [K+]o to 20 mM reduced the amplitudes of both EJP and SHP, and this reduction was marked in the SHP.

Effects of tetraethylammonium (TEA) on the EJP
Effects of Drugs on the Amplitudes of EJP and SHP

Effects of several types of blocking agents on the amplitudes of EJP and SHP produced by perivascular nerve stimulation with five pulses at 20 Hz in the frequency were observed. The effects of these drugs...
on the amplitude of EJP were much of the same as those reported previously (Fujiwara et al., 1982) i.e., the amplitude of EJP increased by application of low concentration (10^{-8} M) of phenolamine and decreased by application of high concentration (10^{-7} M) of phenolamine, guanethidine, noradrenaline, or ATP. Table 1 shows effects of these drugs on the amplitude of SHP. Associated membrane potential changes with application of these drugs (5-30 min) are shown in Table 2.

The cholinergic blocking agent, atropine (5 X 10^{-6} M), and the AChE-blocking agent, neostigmine (2 X 10^{-7} M), had no effect on the amplitude of SHP. In the case of adrenergic blocking agents, phenolamine at a concentration of 10^{-7} M had no effect, but 10^{-6} M reduced the amplitude of SHP. Propranolol (10^{-7} M) or guanethidine (10^{-6} M) had no effect on the SHP. These drugs did not modify the membrane potential of the smooth muscles.

Theophylline (10^{-5} M) had no effect on the membrane potential or the amplitudes of EJP and SHP, indicating that cAMP may not be involved in the generation of EJP and SHP. Ouabain (10^{-6} M) reduced the membrane potential by about 2 mV, with no change in the amplitude of SHP. Apamin, a blocker of the purinergic neuromuscular transmission (Shuba and Vladimirova, 1981), at a concentration of 2 X 10^{-6} M had no effect on the amplitudes of EJP and SHP. ATP, a candidate for transmitter of nonadrenergic, noncholinergic inhibitory nerves in the smooth muscles of small intestine and cerebral blood vessels (Burnstock, 1981; Muramatsu et al., 1981), at concentration of 5 X 10^{-7} M did not reduce the amplitude of SHP significantly.

The actions of ATP and TEA on the amplitudes of EJP and SHP seemed to be due in part to changes in the input resistance of the postjunctional muscle membrane, since these two drugs modified the EJP and SHP with associated depolarization of the muscle membrane. Effects of TEA and ATP on the membrane conductance were estimated by recording electrotonic potentials in the smooth muscle of dog middle cerebral artery using the partition stimulating method. Figure 9 shows effects of TEA and ATP on the membrane potential and amplitude of electrotonic potentials produced by current pulses. Application of 5 mM TEA depolarized the membrane by about 8 mV, and this depolarization was maintained at a constant level until washout of the TEA. The amplitude of electrotonic potentials produced by alternate application of inward and outward current pulses, at a constant intensity, increased during the depolarization (Fig. 9a). The current-voltage relationships observed before and during the application of 5 mM TEA (Fig. 9c) showed that the slope of the relationship was steeper in the presence of TEA than in the control, even after the depolarized membrane had shifted close to the resting membrane potential level by application of the current. ATP at a concentration of 0.1 mM produced a transient depolarization of the membrane with an associated decrease in the amplitude of electrotonic potentials, and the membrane was repolarized close to the resting membrane potential level, even under conditions of continued superfusion of ATP (Fig. 9b). In the presence of 0.1 mM ATP (2-4 minutes after application of ATP), the slope of the current-voltage relationship was not as steep as that seen in the control (Fig. 9d).

These results indicate that TEA increases and ATP decreases the membrane resistance of these smooth muscles.

**Discussion**

The resting membrane potential of the smooth muscle of cat middle cerebral artery was found to be -70 mV (Harder, 1980), while we found this potential to be about -50 mV in the same tissue in the dog. Other cerebral arteries (basilar and pial arteries) of the cat showed a potential of -50 to -60 mV (Harder et al., 1981), whereas the potential of the basilar artery of dog and guinea pig were reportedly about -50 mV (Karashima and Kuriyama, 1981; Fujiwara et al., 1982). Therefore, these discrepancies in membrane potential probably are due to regional as well as species differences. The high membrane potential in the cat middle cerebral artery was attributed largely to the contribution of the electrogenic Na\(^+\)-K\(^+\) pump activity (Harder, 1980). A small depolarization of the membrane by ouabain observed in the dog middle cerebral artery suggests that the electrogenic pump activity (Harder, 1980). A small depolarization of the membrane by ouabain observed in the dog middle cerebral artery.
FIGURE 9. Effects of TEA and ATP on the membrane potential and membrane conductances. Panel a: effects of 5 mM TEA on the membrane potential and amplitude of electrotonic potentials produced by alternate application of inward and outward current pulses at constant intensity (pulse duration, 1.5 second). Panel b: effects of 0.1 mM ATP on the membrane potential and amplitude of electrotonic potentials. In a and b, upper, middle, and lower traces represent a time scale of 1 second per division, current monitor, and membrane potential changes, respectively. TEA or ATP was applied at the bar under each record. Panel c: effect of 5 mM TEA on the current-voltage relationship recorded from a cell in the proximity of the stimulating electrode (distance about 75 μm). The amplitude of steady level of electrotonic potential (vertical axis) is plotted against the intensity of current pulse (horizontal axis). Positive and negative values are depolarizing and hyperpolarizing potentials, respectively. The current intensity is expressed as V/cm across the partition stimulating electrodes, and positive and negative values represent outward and inward current pulses, respectively. Broken line with open circles shows the current-voltage relationship during the presence of 5 mM TEA, after the depolarized membrane was shifted close to the resting membrane potential level by applying the inward current. Panel d: current-voltage relationships obtained before and during (2-4 min) the application of 0.1 mM ATP.

of cat (Harder, 1980). This property of the cerebral artery is in contrast with findings in the pial artery of rabbits in which there was a spontaneous generation of action potentials (Lusamvuku et al., 1979). Action potentials were generated by application of norepinephrine to the cat middle cerebral artery, in the presence of TEA (Harder et al., 1981), whereas this membrane did not depolarize or generate action potentials in the dog cerebral artery. Thus, there probably are species differences in membrane properties, as related to norepinephrine. Action potentials could be generated in the dog cerebral arteries by current pulses in the presence of TEA (Fujiwara et al., 1982), thereby indicating that electrically quiescent tissues possess properties capable of generating the action potential, but under conditions of pharmacological limitations.

During the facilitation of EJP, the muscle membrane was hyperpolarized and the input resistance of the muscle membrane was decreased by the generation of SHP. With an increased driving force, amplitude of EJP would increase during the hyperpolarization, whereas a decreased membrane resistance would decrease the amplitude of EJP. Facilitation of EJP was
observed within 5 seconds, yet the SHP continued over 10 seconds from the time of stimulus. Therefore, changes in the amplitude of EJP elicited by the second stimulus might depend on at least three factors, i.e., increased release of transmitter, increased membrane potential, and decreased input resistance of the muscle membrane. The amplitudes of EJP and SHP were also determined by the stimulus intensity. Increase in the amplitude of EJP or SHP with increasing stimulus intensity may be due to the contribution of many nerves with different thresholds required to produce an EJP or a SHP, as has been observed in the case of the guinea pig mesenteric artery (Kuriyama and Suzuki, 1981).

TEA suppresses the potassium conductance in different excitable membranes (Narahashi, 1974), and also increases the transmitter release from the nerve terminals (Kirpekar et al., 1976; Holman and Suprenant, 1980). In the dog middle cerebral artery, the muscle membrane was depolarized by application of TEA, due to a concomitant decrease in the membrane conductance. Increase in the amplitude of EJP may be due both to an increased input resistance of the postjunctional membrane and to an increase in the amount of transmitter released from the nerves. On the other hand, the generation of SHP was suppressed by TEA. The SHP was associated with an increase in the membrane conductance, and the amplitude increased in low [K\(^+\)]\(_o\) and decreased in high [K\(^+\)]\(_o\) solutions. In the rabbit mesenteric artery, ACh produces a hyperpolarization of the membrane due to increase in potassium permeability, and the amplitude of the ACh-potential is increased in low [K\(^+\)]\(_o\) and decreased in high [K\(^+\)]\(_o\) solutions (Kuriyama and Suzuki, 1978). These observations suggest that the SHP is generated by an increase in the potassium conductance of the postjunctional membrane. The suppression by TEA of the generation of SHP may be related to the action of TEA which is involved in suppression of the potassium conductance of the muscle membrane and the action is sufficiently potent to inhibit the transmitter-induced increase in potassium conductance, even under conditions when the amount of the transmitter released is increased by TEA.

Histochemical studies showed that the cerebral artery of many species are richly innervated by catecholamine-containing nerves, and ultrastructural and biochemical studies also suggested the presence of cholinergic and noncholinergic, nonadrenergic nerves in the cerebral blood vessels (see review of Owman et al., 1974; Owman and Edvinsson, 1977; Bevan et al., 1980). In the smooth muscle of dog middle cerebral artery, stimulation of perivascular nerves produced biphasic electrical responses, EJP and SHP. The amplitude of EJP was increased by application of low concentration of phenolamine and was decreased by norepinephrine (Fujiwara et al., 1982). This suggests involvement of a junctional feedback regulation of transmitter release at the adrenergic nerve terminals (Westfall, 1977). Treatment with 6-OHDA abolished the EJP, but the TTX-sensitive SHP remained intact. Thus, the dog middle cerebral artery probably is innervated by both excitatory and inhibitory nerves, the former—possibly—noradrenergic nerve, activation of which produces an EJP in the smooth muscle. However, the SHP was not suppressed by atropine; neither was it augmented by neostigmine. Exogenously applied ACh had no effect on the membrane potential and membrane conductance in the dog cerebral arteries (Fujiwara et al., 1982). These observations indicate that SHP is not generated by activation of cholinergic receptors in the middle cerebral artery of dog.

Harder et al. (1981) showed regional differences in populations of adrenergic receptors in the cat cerebral arteries, and the pial artery possesses both \(\alpha\)- and \(\beta\)-adrenoceptors. Exogenously applied norepinephrine hyperpolarized the muscle membrane through activation of propranolol-sensitive \(\beta\)-receptors. As the SHP observed in the dog middle cerebral artery was not affected by propranolol, \(\beta\)-receptors for generation of SHP probably are not involved. Within 30 minutes of application of ouabain, the amplitude of SHP was not changed, thereby suggesting that the SHP is not generated by activation of the electrogenic Na\(^+\)-K\(^+\) pump. The nerves which produce SHP were not purinergic, since inhibitors of P1 or P2 purinergic receptors, namely, theophylline or apamin (Burnstock, 1981), had no effect on the SHP. In the present experiment, pharmacological agents did not modify the SHP; therefore, the possible transmitter substance related to the SHP could not be identified.

Recently, peptidergic nerves have been given increasing attention, and some polypeptides were shown to have vasodilator actions (Fahrenkrug, 1979). In preliminary work, we found that vasoactive intestinal polypeptide (VIP) did not hyperpolarize the smooth muscle membrane in the dog middle cerebral artery (H. Suzuki, unpublished observation). However, it has frequently been observed that endogenous but not exogenous norepinephrine produces a membrane depolarization in different arterial smooth muscles (Holman and Suprenant, 1979; Hirsh and Neild, 1980; Fujiwara et al., 1982). Therefore, differences in membrane responses between exogenous and endogenous substances have to be given attention when evaluating neuromuscular transmission mechanisms.

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References

Abe Y, Tomita T (1968) Cable properties of smooth muscle. J Physiol (Lond) 196: 87-100

Aprigliano O, Hermmsmeyer K (1976) In vitro denervation of the...
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Holman ME, Surprenant AM (1979) Some properties of the excitatory junction potentials recorded from saphenous arteries of rabbits. J Physiol (Lond) 287: 337–351


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