Supersensitivity by AF64A, a Novel Inhibitor of Neuronal Choline-Uptake, of the Rat Iris Sphincter Smooth Muscle

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Abstract

Effects of ethylcholine mustard aziridinium ion (AF64A) on contractile responses to agonists or transmural nerve stimulation (TNS) were examined in rat iris sphincter muscle. The responses to TNS of isolated sphincter muscle were abolished within 1 hr after the addition of 0.1 mM AF64A to the bathing solution, while responses to acetylcholine (ACh) and 5-hydroxytryptamine (5-HT) were not changed significantly. In sphincter muscles which were isolated 3 days after the micro-injection of 100 nmol AF64A into the anterior chamber of eyes *in vivo*, the response to TNS were decreased to about 30% of the control. The injection of AF64A at higher concentrations often resulted in serious chronic damage of the eye. When 100 nmol AF64A was injected twice with an interval of 3 days, the response to TNS was decreased to about 20% of the control. Maximum responses and sensitivities to ACh and 5-HT were markedly enhanced in sphincters from eyes which had been treated with AF64A twice. The sensitivity of depolarized sphincters to external Ca^{2+} was also increased significantly. Seven weeks after the second injection, responses to TNS recovered to more than 50% of the control and the effects of AF64A mostly disappeared. In conclusion, the acute inhibition of parasympathetic transmission without postsynaptic changes can be achieved within 1 hr after bath-application of 0.1 mM AF64A. The reduction of parasympathetic nerve activity by treatment with AF64A *in vivo* induces nonspecific supersensitivity in iris sphincter, whereas effects of AF64A were mostly reversible under the present experimental conditions.

Key words : iris sphincter muscle, AF64A, parasympathetic nerve, denervation supersensitivity, muscarinic receptor

Introduction

The iris sphincter muscle of the rat is innervated densely by parasympathetic nerves (Csillik and Koelle, 1965 ; Huhtala *et al.*, 1976 ; Narita and Watanabe, 1981) and also by sensory nerves of trigeminal origin having substance P-like immunoreactivity (Miller *et al.*, 1981 ;

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Shimizu, 1982 ; Kessler, 1985). The parasympathetic transmission in the muscle is characteristic of its electrical features, since the contraction induced by acetylcholine, which is released from nerve endings or added externally, is triggered by neither depolarization nor action potential in sphincter smooth muscle cells (Banno *et al.*, 1985, Imaizumi *et al.*, 1984). This indicates that the transmission is mediated by a typical pharmaco-mechanical coupling (Somlyo *et al.*, 1999).

Denervation is an excellent strategy to investigate the modulation of postsynaptic membranes in smooth muscle cells by the autonomic nerves (Westfall, 1981 ; Akhtar and Abdel-Latif, 1986). Since most of parasympathetic ganglia are located in the target organs, the surgical denervation is not practical except for a few organs. Surgical parasympathetic denervation of iris smooth muscle can be performed by ciliary ganglionectomy and results in specific and non-specific supersensitivities to ACh and the other agonists (5-HT and high-K⁺ solution), respectively, in the sphincter (Hasegawa *et al.*, 1987 ; Banno *et al.*, 1987) or the dilator (Hasegawa *et al.*, 1988 ; Hashimoto *et al.*, 1993). The chemical sympathetic denervation using 6-hydroxydopamine (Kostrzewa and Jacobowitz, 1975) or guanethidine (Burnstock *et al.*, 1971 ; Shibata *et al.*, 1989) and the depletion of noradrenaline in sympathetic nerve endings using reserpine (Trendelenburg and Gravenstein, 1958 ; Zaimis, 1961) have been giving advantages to clarify neuronal controls of tissue functions by both central and peripheral sympathetic nerves. These have also been widely used as standard pharmacological methods removing or decreasing the influence of sympathetic innervation in experimental preparations. Substances which effectively and selectively degenerate parasympathetic nerves or reduce acetylcholine content in nerve endings have not been developed. Although hemicholinium has been known as a choline-uptake inhibitor in cholinergic nerve endings (Gyls *et al.*, 1992), our preliminary study indicated that the compound may not be effective in the rat iris sphincter to reduce responses to nerve stimulation.

AF64A has been introduced as a selective cholinotoxin to produce a chemically induced models of central cholinergic nerve deficit including Alzheimer's disease in rats (Fisher and Hanin, 1980 ; Hanin, 1996). An injection of the compound to cerebral ventricle gives irreversible damages to central cholinergic neurons, resulting in the reduction of the biochemical markers such as choline acetyltransferase activity and choline uptake (Walsh *et al.*, 1984) and the facilitation of the activation of phosphoinositide turnover or the cAMP accumulation elicited by muscarinic receptor agonist (Eva *et al.*, 1987). Although some usefulness of AF64A as a choline-uptake inhibitor has been suggested in smooth muscle tissues such as ileum, urinary bladder (Hoyle *et al.*, 1986) and colon (Hoyle *et al.*, 1986 ; Hata *et al.*, 1990), tissue-specific application of AF64A *in vivo* is not feasible. While a decrease in activity of choline acetyltransferase in the rat iris after treatment with AF64A has been demonstrated (Kessler, 1985), functional changes in iris muscles have not been examined yet. The present study was undertaken to evaluate AF64A as a pharmacological tool to reduce or abolish ACh release from parasympathetic nerve endings and to perform chemical denervation of parasympathetic nerves in a smooth muscle tissue.

Materials and Methods

Preparation and recording of mechanical responses

Eyes were excised from male Wistar rats (300 to 500 g), which have been anesthetized and killed by bleeding. Iris sphincter preparation and tension measurement were made as described in detail in a previous paper (Narita and Watanabe, 1981). Briefly, whole sphincter was dissected as a ring preparation by cutting dilator muscle. An isolated preparation from an eye treated with AF64A (left) or contralateral eye (right) was horizontally mounted in a 4 ml organ bath and perfused with a bathing solution. One side of the sphincter was pinned on a sheet of silicone rubber which formed the bottom of the bath. The other side was fixed to a transducer lever. At the start of an experiment, resting tone was adjusted to 50 mg.

Isometric tension was measured with a strain gage transducer (Imaizumi and Watanabe, 1983) and recorded on a pen-recorder (FBR 252A, TOA Electronics Ltd., Japan). Transmural nerve stimulation (TNS) was performed by field stimulation via a pair of platinum wire electrodes which were placed at both sides of the strip. Square-wave pulses of 0.2 msec in duration were applied for 5 sec at 20 Hz in frequency. The current intensity for TNS was decided as about 10% excess of that giving the maximum response which is completely blocked by tetrodotoxin in the control sphincter muscles. A dose-response curve to a drug was determined by cumulative addition of a drug to the bathing solution under the conditions where the perfusion of the solution was stopped. A concentration-response curve to Ca^{2+} was determined in the same manner. Effects of high K^+ solution were examined by changing a perfusion solution to the high K^+ solution.

Treatment with AF64A

For application of AF64A *in vivo*, rats were anesthetized with an i.p. injection of thiopental sodium (25 mg/kg) and amobarbital sodium (50 mg/kg). Injection of AF64A into the anterior chamber of the eye was performed by slow injection of 1 mM, 10 mM or 20 mM AF64A in 10 μl saline (10, 100 or 200 nmol, respectively) using sterilized Hamilton syringe and 26 gage injection needle. After certain period from the injection, eyes were dissected and irides were used for the tension measurement. In some experiments, eyes were injected with vehicle alone in the same manner and used for control experiments.

Solution

The bathing solution was a modified Krebs' solution of the following composition (mM): NaCl 112.0, KCl 4.7, CaCl_2 2.2, MgCl_2 1.2, NaHCO_3 25.0, KH_2PO_4 1.2, and glucose 14.0. The solution was bubbled with 95% O_2 and 5% CO_2 and kept at 37°C and pH 7.4. High potassium solution was prepared by replacing NaCl in the modified Krebs' solution with equimolar KCl and by adding 1 μM atropine and 1 μM phentolamine. To prepare high K^+ , Ca^{2+} -free solution, CaCl_2 was removed from high K^+ solution and 100 μM EGTA was added.

AF64A was prepared as previously described by Fisher *et al.* (1982). AF64A was formed by dissolving ethylcholine mustard (AF64) hydrochloride in 0.9% NaCl (physiological saline) and adjusting the pH to 7.4 with solid NaHCO_3 . The solution was kept at room temperature

for at least 1 hr. The obtained AF64A solution was kept on ice and was used within 5 hrs from the pH adjustment.

Drugs

The following drugs were used; ethylcholine mustard (AF64) hydrochloride (Research Biochemical International, Natick, MA, USA), acetylcholine chloride (ACh, Wako, Japan), 5-hydroxytryptamine creatine sulfate (5-HT, Wako, Japan), atropine sulfate (Atr, Wako, Japan), phentolamine mesylate (Phent, Ciba-Geigy, Japan), glycoetherdiaminetetraacetic acid (EGTA, Dojin, Japan).

Statistics

Results were expressed as mean $\pm$ S.E. Statistical significance between two or multiple groups was tested according to paired *t*-test or one-way ANOVA, respectively, ($p < 0.05$, 0.01 or 0.001) and *p* levels are represented by *, **, and ***, respectively.

Results

1) Effects of AF64A added *in vitro* on contractile responses of the rat iris sphincter muscles

Rat iris sphincter muscle exhibits contractile responses to ACh which is either released from cholinergic nerve endings by TNS or exogenously applied (Narita and Watanabe, 1981). When AF64A at the final concentration of 0.1 mM was added to the bathing solution, the perfusion was stopped. The solution was continuously oxygenated, circulated and kept at 37°C in the bath. Responses to TNS of a sphincter muscle were recorded for about 3 hrs. The amplitude of control responses to TNS in the absence of AF64A (only vehicle was added) was

Fig. 1. Effects of AF64A on the time course of relative contractile response to transmurial nerve stimulation (0.2 msec in duration, 20 Hz in frequency). The amplitude of contraction elicited by TNS was normalized with that before the application of saline (open circle) or AF64A (closed circle). Values are means $\pm$ S.E. Numbers of experiments are shown in the parentheses. S.E. were often smaller than the symbols. *, **, or *** indicates statistical significance vs. control at $p < 0.05$, 0.01 or 0.001, respectively.

consistent for 20–30 min and thereafter decreased gradually (Fig. 1). On the other hand, the responses of sphincter muscles challenged with AF64A progressively declined and almost abolished within 1 hr. The abolished response to TNS did not recover by washout for over 2 hrs (not shown, $n=3$). Three hours after the start of experiments, the amplitude of responses to TNS in the control was about 70% of the initial one (not shown). Even after the response to TNS abolished in the presence of AF64A, the response to exogenously applied ACh was not significantly affected. The maximum response to and pD_2 values of ACh in the control (1 hr after the application of vehicle) were 49.2 ± 4.9 mg and 5.21 ± 0.13 , respectively ($n=4$ for each), and those in the presence of 0.1 mM AF64A were 37.9 ± 9.9 mg and 5.31 ± 0.18 , respectively ($n=4$ for each; $p>0.05$ vs. control).

2) Effects of AF64A applied *in vivo* on contractile responses of the rat iris sphincter muscles

Effects of treatment with AF64A *in vivo* by micro-injection into the anterior chamber of the eye on contractile responses of isolated iris sphincter muscle were examined in the next series of experiments. Since it has been reported that application of 10 nmol AF64A into anterior chamber of the eye in the rat results in marked death of cholinergic neurons (Kessler, 1985), we first examined effects of 10 nmol AF64A on iris sphincter. In the preliminary experiments, however, this amount (10 nmol) of application only slightly decreased the response to TNS (by about 20%) approximately 24 hrs after the injection. Of interest was that the maximum responses to ACh and 5-HT tended to increase (ACh: $125.1 \pm 14.8\%$ of the control, $n=4$, $p>0.05$; 5-HT: $154.7 \pm 13.2\%$ of the control, $n=4$, $p<0.05$) (Fig. 2), whereas the sensitiv-

Fig. 2. Maximum responses to ACh (open columns) and 5-HT (closed columns) of iris sphincters from eyes treated with 10, 100 or 200 nmol AF64A. Eyes were isolated one day (approximately 24 hrs), three or four days or seven days after the injection of AF64A. The maximum responses to 1 mM ACh and $10 \mu\text{M}$ 5-HT of the sphincter obtained from the contralateral eye in each rat were taken as the control (100%). The injected amounts of AF64A were indicated by circles below the columns. Numbers of experiments are 4–5, 7–10 and 4 for 10, 100 and 200 nmol, respectively. *, **, *** indicate statistical significance vs. control at $p<0.05$, 0.01 and 0.001, respectively.

ities were not changed (pD_2 values of ACh: control = 5.10 ± 0.04 , AF64A = 5.11 ± 0.10 ; pD_2 values of 5-HT; control = 6.61 ± 0.05 , AF64A = 6.46 ± 0.06 , $n=4$ for each, $p>0.05$). On the 3rd day from the injection, responses to TNS recovered to a similar extent to those in the control and both the maximum responses and sensitivities to ACh and 5-HT in AF64A-pretreated sphincters were not significantly different from those in the control.

Since marked changes in contractile responses were not found after the injection of 10 nmol AF64A, effects of larger amounts of AF64A were examined. Based on the results of experiments to determine the upper limit of the amount of AF64A for one injection, it was found that 100 nmol was practically close to the limit which did not make serious chronic or subchronic damages in whole eyes in most of experiments ($>80\%$). Injection of 200 nmol AF64A resulted in opaque eyes in about 40% of treatment and the loss of stiffness of irides. Fig. 2 summarizes the maximum responses to ACh (open column) and 5-HT (closed column) of iris sphincters from eyes treated with 10, 100 or 200 nmol AF64A. The maximum contraction in a sphincter from the contralateral eye was taken as the unit in each rat. One day after the injection of 100 nmol AF64A, the maximum response was significantly smaller than the control. However, three or four days after the injection, the responses became significantly larger than the control. The responses to TNS of sphincters treated with 100 nmol AF64A were significantly smaller than the control ($34.0 \pm 17.3\%$ of the control, $n=4$, $p<0.05$), whereas the extent of decrease varied widely from preparation to preparation. When eyes were not damaged by the injection of 200 nmol AF64A, the maximum responses of sphincters were measured 7 days after the injection and tended to be larger than those in sphincters treated with 100 nmol AF64A.

The application of 100 nmol AF64A, which appeared to be the upper limit of the dose to minimize nonspecific damages, was not enough to induce chronic inhibition of parasympathetic transmission in iris sphincter. Therefore, another addition of 100 nmol AF64A was performed on the third day after the first injection. Since each application of 100 nmol AF64A induced irreversible damage at the probability of 10–20%, the third application was not examined systematically in this study. In sphincter muscles isolated on the fourth day after the second injection, the responses to TNS were significantly smaller than the control ($47.5 \pm 11.1\%$ of the control, $n=10$, $p<0.001$ vs. control), but surprisingly tended to be larger than those in sphincters treated with AF64A once ($34.0 \pm 17.3\%$ $p>0.05$). Fig. 3A shows that dose-response relationships to exogenously applied ACh in the control and AF64A-treated sphincter muscles isolated on the 4th day after the second injection. Fig. 3B and C show the time course after the second injection of the changes in maximum response to 1 mM ACh. The maximum responses of sphincter muscles isolated 4, 21 and 49 days after the second injection were 213.8 ± 16.8 , 256.8 ± 41.6 and $137.4 \pm 24.5\%$ of the control, respectively, and indicating that the change was still reversible. Fig. 3B shows changes in pD_2 values with time after the second injection, and indicates that the increased sensitivity to ACh after 4 days gradually returned to the control level. It is clear that the increase in the maximum response and sensitivity to ACh was larger than those in sphincter muscles treated once. After 3 and 7 weeks, the maximum responses of AF64A-treated sphincters were $144.5 \pm 14.4\%$ (not shown, $n=3$, $p<0.05$ vs. 100%) and $105.4 \pm 36.0\%$ of the control (not shown, $n=3$, $p>0.05$ vs. 100%), respectively, indicating that the effects of AF64A disappeared between 3 and 7 weeks.

Fig. 3. A: Dose-response relationships for exogenously applied ACh in the control (open circle) and sphincter muscles isolated on the 4th day after the second treatment with AF64A (closed circle). The number of experiments is 10. In A(b), responses were expressed as relative percentage of each maximum response in A(a). *, **, ***; $p < 0.05$, 0.01 or 0.001 vs. control, respectively. B: The time course of changes in maximum responses induced by 1 mM ACh after the second treatment. Response was normalized with each maximum response of contralateral iris sphincter (100%). The numbers in parentheses indicate the numbers of irides used. The numbers in abscissa indicate the days after the second injection. *, **, ***; $p < 0.05$ or 0.001 vs. 100%, respectively. C: The time course of changes in pD_2 values of ACh-induced responses (control; open circle, AF64A; closed circle) after the second injection. **, $p < 0.01$ vs. control. Values are means $\pm$ S.E.

Similar results were obtained when changes in the response to 5-HT were examined after the second injection (Fig. 4). The increase in the maximum response to 5-HT was, however, larger than that to ACh. The sensitivity to 5-HT (pD_2 : 6.81 ± 0.09 , $n = 4$) was significantly higher than the control (6.49 ± 0.04 , $n = 4$) only on the 4th day after the injection. After 3 and 7 weeks, the maximum responses were $153.2 \pm 2.6\%$ (not shown, $n = 3$, $p < 0.01$ vs. 100%) and $129.0 \pm 19.3\%$ (not shown, $n = 3$, $p > 0.05$ vs. 100%), respectively.

Fig. 5 illustrates the time-courses of relative amplitude of responses to TNS after the first or second injection of 100 nmol AF64A. Taking the changes in muscle contractility after the treatment into account, the ordinate is expressed as a relative amplitude calculated in the following manner.

Relative amplitude (%) = $\left(\frac{\text{Response to TNS of a treated sphincter}}{\text{Response to TNS of the control sphincter}} \right) \times \left(\frac{\text{Maximum response to ACh of the control sphincter}}{\text{Maximum response to ACh of a treated sphincter}} \right) \times 100$

Even after this correction, the remaining response to TNS was about 20% of the control on the 4th day after the second injection. Since over 97% of the remaining component is blocked by 1 μM atropine, contamination of tachykinergic component or direct activation of the muscle can be negligible. These results suggest that substantial cholinergic transmission

Fig. 4. A: Dose-response relationships for exogenously applied 5-HT in the control (open circle) and sphincter muscles isolated on the 4th day after the second treatment with AF64A (closed circle). The number of experiments is 6. In A(b), responses were expressed as relative percentage of each maximum response in A(a) in the same manner as in Fig. 2. *, **, $p < 0.05$ or 0.01 vs. control, respectively. B: The time course of changes in maximum responses induced by 10 μ M 5-HT after the second treatment. Response was normalized with each maximum response of contralateral iris sphincter, taken as 100%. The numbers in parentheses indicate the numbers of irides used. The numbers in abscissa indicate the days after the second injection. *, **, $p < 0.05$ or 0.01 vs. 100%, respectively. C: The time course of pD_{50} values of 5-HT-induced responses (control; open circle, AF64A; closed circle). Values are means $\pm$ S.E.

Fig. 5. The time course of relative contractile responses induced by TNS after the first (closed circle) or second (open circle) injection of 100 nmol AF64A. The numbers in abscissa indicate the days after the first or second injection of AF64A. Relative responses were normalized as follows; Relative amplitude (%) = (Response to TNS of a treated sphincter / Response to TNS of the control sphincter) $\times$ (Maximum response to ACh of the control sphincter / Maximum response to ACh of a treated sphincter) $\times 100$. The numbers in the parentheses are numbers of irides used. Values are means $\pm$ S.E.

Fig. 6. A: Effects of AF64A on contractile response induced by 80 mM high potassium of the control sphincter muscles (open column) and those isolated on the 4th day after the second injection (solid column). Eighty millimolar NaCl was replaced with equimolar KCl to keep the osmolarity of the solutions constant. The solution contained 1 μ M atropine and phentolamine. Note that AF64A markedly increased the contraction induced by 80 mM K^+ .

B: Effects of AF64A on contractile response elicited by cumulative application of calcium to high K^+ solution which did not contain Ca^{2+} (80 mM high K^+ , Ca^{2+} -free solution). B(a) shows absolute value of contraction amplitude (mg) induced by addition of Ca^{2+} . In B(b), the response was expressed as relative percentage of maximum response in B(a) in each preparation. Open circle; control, closed circle; AF64A-treated sphincters isolated on the 4th day after the second injection. Values are means $\pm$ S.E. *, **, and, *** indicate statistical significance vs. control at $p < 0.05$, 0.01, and 0.001, respectively.

remained after the treatment with AF64A. The decreased response to TNS appeared to recover partly after 7 weeks.

3) Non-specific supersensitivity induced by AF64A

The most significant change in sphincter muscles after the treatment with AF64A was the increase in the maximum responses to ACh and 5-HT, suggesting the nonspecific increase in responses to agonists. To confirm whether the response to high K^+ which may not involve sensitization of contractile system to Ca^{2+} is changed by the treatment with AF64A or not, effects of 80 mM K^+ were examined in the control and AF64A-treated sphincters (Fig. 6A). The response to 80 mM K^+ solution was increased by about 100% in sphincters isolated 4th day after the second injection.

In addition, the contraction induced by adding Ca^{2+} to a high K^+ , Ca^{2+} -free solution was markedly increased in sphincters pretreated with AF64A twice (Fig. 6Ba). It is notable that the sensitivity to extracellular Ca^{2+} as well as the maximum response is significantly increased in irides pretreated with AF64A (Fig. 6Bb).

Discussion

The results obtained from experiments applying 0.1 mM AF64A *in vitro* clearly indicate that parasympathetic transmission was suppressed by the treatment within 1 hr. Of importance is that the effect was not removed by washout for over 2 hrs and that the responses to externally applied ACh and 5-HT were not affected. Acute treatment with AF64A *in vitro* may possibly be a useful pharmacological method to suppress parasympathetic transmission without causing significant postsynaptic influence in isolated tissues.

In preceded works, it has been shown that intraperitoneal application of AF64A at doses close to or over LD₅₀ to the guinea-pig results in partial decrease in contractile responses in skeletal muscle to motornerve stimulation and in smooth muscles of ileum, urinary bladder and colon to TNS (Hoyle *et al.*, 1986). Although the increase in sensitivities to ACh and carbachol has been found in ileum, the increase in maximum responses by AF64A has not been reported in any smooth muscle preparation. From these results, it has been concluded that i.p. application of AF64A is not useful for the selective degeneration of parasympathetic nerves (Hoyle *et al.*, 1986). In contrast, it has been reported that the actions of AF64A are irreversible in the mouse hippocampus (Fisher and Hanin, 1980) and result in cholinergic hypofunction (Hanin, 1990). In rat iris, it has been reported that the cholinergic nerve degeneration as decrease in choline acetyltransferase has been induced by an injection of 10 nmol AF64A into the anterior chamber (Kessler, 1985). The local application of AF64A performed in the present study may have, therefore, a great advantage similar to that in central nervous system. This research is the first report showing marked functional changes of smooth muscle induced by AF64A.

The volume of anterior chamber of human eye is approximately 210 μ l (Johnson *et al.*, 1978). Supposing that the ratio between the size of eye and the volume of anterior chamber is similar in various animals, the volume in the rat may be approximately 11 μ l. The concentration of AF64A in the anterior chamber just after the injection of 10 nmol/10 μ l AF64A can be, therefore, assumed as ~ 480 μ M. The aqueous humor in the anterior chamber of the human eye is exchanged in approximately 2 hrs, while the exchange rate depends upon the intraocular pressure. If it is also the case in the rat, the concentration of AF64A progressively declined to about 180 μ M after 2 hrs. Although 180 μ M AF64A *in vitro* is almost enough to inhibit parasympathetic transmission, 10 nmol AF64A added *in vivo* did not affect significantly the transmission. Moreover, the treatment with 100 nmol AF64A decreased the transmission significantly but not abolished it. Even when eyes were injected with 100 nmol AF64A twice with interval of 3 days, the transmission was neither abolished. The increase in maximum contractility and sensitivity to ACh by the injection of AF64A partly masks the pronounced decrease in amount of stored ACh in parasympathetic nerve endings. Moreover, the decreased transmission by injection of AF64A *in vivo* recovered significantly after several weeks. Since 100 nmol AF64A was close to the upper limit to inject into the chamber, complete parasympathetic denervation in iris sphincter muscle by AF64A injection *in vivo* appeared to be difficult to perform.

The most striking finding in the present study is that the intraocular injection of AF64A induced a typical postsynaptic supersensitivity, which is characterized by extraordinary non-

specific features. It is notable that not only the maximum response to high K^+ but also the sensitivity to extracellular Ca^{2+} was markedly increased. These are exactly the same as the results obtained by parasympathetic denervation by ciliary ganglionectomy (Hasegawa *et al.*, 1987). The mechanisms, however, underlying the nonspecific supersensitivity by the AF64A injection were not further examined in the present study. The increase in availability of Ca^{2+} has been reported in vas deferens of the guinea-pig after sympathetic denervation (Kasuya *et al.*, 1969). An increase in the sensitivity of the contractile proteins by a change in the activity or amount of calmodulin has been proposed to explain the supersensitivity induced by the sympathetic denervation (Ramos *et al.*, 1986). Similar possibility has been suggested in parasympathectomized iris sphincter or dilator (Hasegawa *et al.*, 1987; Banno *et al.*, 1987; Hashimoto *et al.*, 1993). Further experiments are required, especially using skinning techniques, to elucidate the mechanisms for AF64A-induced nonspecific supersensitivity.

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