Cellular Mechanisms of Supersensitivity to Acetylcholine and Potassium Ion after Ciliary Ganglionectomy in the Rat Iris Sphincter Muscle

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Abstract—The effect of parasympathectomy on the electrical and nonelectrical activation of iris smooth muscle cells was examined 7–10 days after ciliary ganglionectomy in order to explore the mechanisms involved in the nonspecific denervation supersensitivity. Resting membrane potential of rat iris sphincter muscle cells was not altered by cholinergic denervation. Although the degree of depolarization induced by raising \( [K]_o \) was little affected by denervation, K-contracture was always potentiated. Acetylcholine (ACh) never modified membrane potential in normal iris muscles even at a high concentration of 50 \( \mu \)M. After denervation, ACh produced a much larger contraction, and at higher concentrations, concomitantly produced a depolarization which was far too small to account for the enhanced contraction. There were 3 and 4.6-fold increases in sensitivity to Ca and Sr, respectively, in the contractile response in high-K, Ca-free solution after denervation. The response to Ca was greatly enhanced also in height, and a large part of the increment of the response to 2 mM Ca was suppressed by 0.1 \( \mu \)M nifedipine. In the depolarized muscle of normal irides, Mn ion had two distinct effects, a calcium blocking effect at lower concentrations (\( \leq 1.2 \) mM) and a contractile effect at higher concentrations (\( \geq 1.8 \) mM). Denervation caused a marked increase in the contractile effect of Mn (\( \geq 0.6 \) mM). ACh elicited a phasic contraction only once in the absence of external Ca. This response was much enhanced by parasympathectomy. Fluoride ion (F) also produced a distinct contraction many times in the Ca-free solution. F-induced contraction was much larger in denervated muscle than in normal muscle. These results i) indicate that denervation of the rat iris sphincter muscle does not significantly affect the electrical property of the muscle cells, ii) and suggest that denervation may increase Ca-influx of the cell membrane, iii) may increase the amount of releasable Ca from an intracellular store site, and iv) might increase the functional activity of the contractile protein.

It is generally accepted that the two mechanisms underlie the denervation supersensitivity, one is prejunctional and the other is postjunctional (1–3).

In a preceding paper (4), it was shown that postjunctional nonspecific supersensitivity occurred after denervation at sites beyond the receptor level in the rat iris sphincter. Similar conclusions have been obtained in other denervated tissues (adrenergic nerve: rat vas deferens (5), guinea-pig vas deferens (6) and rabbit iris (7); cholinergic nerve: cat iris (8); substance P-ergic nerve: rabbit iris (9)).

Studies on the mechanisms of the postjunctional supersensitivity indicate that a number of changes in intracellular events underlie this phenomenon. Kasuya et al. (5) and Hudgins and Harris (10) pointed out that changes in distribution of Ca may partly be involved in denervation supersensitivity in rat vas deferens and reserpine-induced supersensitivity in vascular smooth muscles, respectively. In some smooth muscles where electro-mechanical coupling (EMC) is
present, some changes of electrophysiological properties may be involved in this phenomenon; e.g., blood vessels (11) and vas deferens (12-14). There is, however, no extensive study on the mechanisms of nonspecific supersensitivity after cholinergic denervation.

We have previously reported that acetylcholine (ACh) contracts rat iris sphincter muscle without significant change in the membrane potential under normal conditions (typical of pharmaco-mechanical coupling: PMC), while potassium ions contract the muscle by causing depolarization (typical of EMC) (15). In this muscle, an extensive postjunctional supersensitivity develops to ACh as well as to potassium ions (K) following denervation (4). Therefore, it is expected that some changes would happen in PMC and EMC after denervation of iris sphincter muscle.

The present study was thus designed to elucidate factors responsible for the postjunctional supersensitivity to K and ACh after cholinergic denervation, ciliary ganglionectomy, in the rat iris sphincter muscle and to clarify mechanisms underlying these phenomena, with particular attention to changes in PMC and EMC.

Materials and Methods

Male Wistar rats weighing from 350 to 500 g were used. Procedures used for sectioning cholinergic nerve were essentially the same as those described by Malmfors and Nilsson (16) and modified by Hasegawa et al. (4), except that surgical denervation was usually done at the left eye. Success of denervation was confirmed by the absence of atropine-sensitive contractile response to electrical stimulation with a constant intensity of 200 mA at 20 Hz and 0.2 msec in duration.

The iris was isolated from the eye (17) 7-10 days after denervation, when the sensitivity to agonists reached a maximum (4). The isolated ring preparation was mounted horizontally in an organ bath (4 ml) for tension recording. The bath was continuously perfused at a rate of 5 ml/min with Krebs' solution. The contractile response was measured isometrically with a strain gauge transducer (17) and was recorded on a pen recorder (B-281HS: Rika-Denki).

To obtain the electrical response, conventional glass microelectrodes were used and penetrated to the cell from the posterior side of the iris muscle. They were filled with 2 M KCl and had a resistance between 30 and 60 Mohm. Mechanical response was simultaneously recorded as described by Banno et al. (15). A 0.2 ml-organ bath was perfused with modified Krebs' solutions using a microtube pump (MP-3: Tokyo Rikakikai Co., Ltd.).

The ionic composition of the Krebs' solution was as follows (mM): NaCl, 112.0; KCl, 4.7; CaCl₂, 2.2; MgCl₂, 1.2; NaHCO₃, 25.0; KH₂PO₄, 1.2; and glucose, 14.0. The solution was bubbled with 95% O₂ and 5% CO₂, and the pH was maintained at 7.4 (37°C). To obtain the concentration-response relationships of Ca²⁺, Sr²⁺ and Mn²⁺, a Tris-buffered solution of the following ionic composition (mM) was used: NaCl, 120.3; KCl, 5.9; CaCl₂, 2.5; MgCl₂, 1.2; Tris, 16.6; and glucose, 11.5. The solution was titrated with dil. hydrochloric acid to pH 7.4 at 37°C and bubbled with 100% O₂. High-K solutions were prepared by replacing NaCl with KCl isosmotically, and 1 μM atropine and 1 μM phentolamine were usually present. For Ca-free solution, Ca was omitted and 0.1 mM EGTA was incorporated.

The following drugs were used: acetylcholine chloride, atropine sulphate, caffeine anhyd., glycoletherdiaminetetraacetic acid (EGTA), manganese chloride, sodium fluoride and tris(hydroxymethyl)aminomethane (Tris) (all from Wako Pure Chemicals, Ltd., Tokyo); phentolamine mesylate (Clia-Geigy Japan Ltd.); trifluoperazine dihydrochloride (TFP) (Sigma Chemical Co., St. Louis); chlorpromazine hydrochloride (CPZ) (Shionogi Co., Ltd., Osaka); nifedipine (gift from Kyowa Hakko Co. Ltd., Tokyo); and procaine hydrochloride (Hoei Co., Ltd., Osaka).

Values are expressed as means±S.E.M. The significance of the difference between values was evaluated using Student's t-test (P=0.05).

Results

Increased response to high-K solutions after denervation and relationship to mem-
brane potential: The resting membrane potential obtained from denervated and normal iris muscle cells were \(-57.8 \pm 0.4\) mV (n=54) and \(-58.9 \pm 0.5\) mV (n=57), respectively (P>0.05). In both preparations, increasing \([K]_o\) above 5.9 mM up to 100 mM depolarized the membrane, but neither action potentials nor slow waves were seen. When the membrane potentials of denervated and normal irides were plotted against the logarithm of \([K]_o\), linear relationships (43 mV per tenfold change in \([K]_o\)) were obtained in a range of 15-100 mM in both preparations, and only a negligible downward shift was seen after denervation (P>0.05) (Fig. 1a). These observations indicate that dominance of potassium ion permeability in deciding membrane potential was unaffected by denervation.

Plots of the magnitude of contractile response against that of depolarization are shown in Fig. 1b. The response was greatly enhanced in a range of depolarization between 10 and 50 mV. These data are replotted in Fig. 1a in terms of % maximum response of each preparation against \([K]_o\). In both preparations, substantial tension development occurred when the membrane was depolarized by about 15 mV and attained a maximum at 40 mV-depolarization (Fig. 1b). The magnitude of K-contracture was always larger in denervated than in normal iris muscles. When the membrane was depolarized to \(-20\) mV with 60 mM-K, the amplitude of the contraction reached a maximum, \(96.0 \pm 5.4\) mg in 7 denervated and \(59.2 \pm 2.7\) mg in 7 normal iris muscles. On the other hand, the \([K]_o\) vs. relative tension curve (Fig. 1a) showed slight leftward shift (P>0.05) after denervation. \([K]_o\) and the corresponding membrane potential when half maximal contraction was elicited were \(30.1\) mM and \(-36\) mV in denervated muscles and \(33.2\) mM and \(-34\) mV in normal muscles, respectively. These results indicate that the voltage dependence of the contractile response did not change significantly after denervation, and a sequence of events beyond membrane potential change has been affected by the denervation.

Effect of ACh on membrane potential: Denervation-supersensitivity to ACh was characterized by a large leftward shift of the dose-response curve as well as an increase in maximum response (Fig. 1 in (4), Fig. 6c). It has previously been reported that ACh

![Fig. 1](image-url). Relationship between membrane potential and contractile response in normal and denervated rat iris sphincter muscle. a: Relationship between membrane potential (triangles, n=8-39) and relative contractile response (circles, percent of maximum for each preparation) induced by increasing \([K]_o\). b: Absolute value of the contraction is represented as a function of depolarization. Values in both panels were obtained from denervated (filled symbols) and contralateral control irides (open symbols). Shown are the means from 7 irides and S.E.M. larger than the symbol.
produced a dose-dependent contraction without modifying the membrane potential in the rat iris sphincter (15). Therefore, we examined the possibility that the membrane depolarizes in response to ACh in the denervated muscle.

Figure 2 shows typical recordings of the membrane potential and contractile responses during the application of various concentrations of ACh. The results are summarized in Table 1. The membrane was depolarized only to a small degree, ca. 3 mV, by 50 μM ACh in denervated iris muscles. This depolarization declined gradually during contraction (Fig. 2b). Lower concentrations of ACh generated almost no depolarization, but still produced contractions far larger than those produced by 25.9 mM-K (Fig. 2b and Table 1).

Ca- and Sr-induced contraction in depolarized muscle: The concentration-response relationships for Ca or Sr ion were compared between denervated and normal irides (Fig. 3). In these experiments, Ca-free, 60 mM-K Krebs' solution was used (see Fig. 1). The membrane was depolarized up to -20 mV in both normal and denervated preparations (Fig. 1a). Ca or Sr was added cumulatively to the bath after a 10 min incubation in the solution. Ca was more effective than Sr in producing contraction (Fig. 3). Denervation caused a significant

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### Table 1. Effects of acetylcholine on the resting membrane potential, R.M.P. (mV)

|               | R.M.P. 1 μM | Acetylcholine 10 μM | Acetylcholine 50 μM |
|---------------|------------|---------------------|---------------------|
| Denervated    | -55.5±1.4 (6) | -54.8±2.1 (4)       | -54.0±2.4 (5)       |
| Control       | -58.0±1.0 (8) | -52.3±2.0 (6)       | -57.4±1.0 (8)       |

Each value was obtained in a way similar to Fig. 2. ACh induced a depolarization, but the membrane potentials in the presence of ACh were not significantly different from R.M.P. (P>0.05). Shown are means±S.E.M. and number of observations in parentheses.
decrease in threshold concentrations of Ca and Sr. The ED50 ratios of normal and denervated irides were 3.0 for Ca and 4.6 for Sr. No difference could be detected between maximum responses produced by the two divalent cations in either preparation, i.e., 116.6±5.1 mg and 108.2±6.8 mg in denervated muscles and 57.4±2.8 mg and 59.5±5.2 mg in normal iris muscles for Ca (7 irides) and Sr (5 irides), respectively.

Figure 4 shows the effect of nifedipine, a potent Ca-channel blocker (18, 19), on the Ca-induced contraction of depolarized muscles. A large part of the denervation-induced potentiation of the contraction caused by 2 mM Ca, a physiological concentration, was suppressed by 0.1 μM nifedipine, implying that increased contraction after denervation may have been caused via the same class of Ca-channels as that operating in normal muscles. Inhibition by nifedipine was partly prevented by increasing the concentration of Ca. Although the maximum response to Ca was 2.1 times larger in denervated than in normal muscle, it was obtained at 8 or 16 mM Ca in both preparations. It is an interesting finding that the nifedipine (0.1 μM)-sensitive component in the response to 16 mM Ca in denervated irides was not different from that in normal tissues (P>0.05, 50.4±4.6 mg for denervated irides and 47.4±2.9 mg for normal tissues).

When 0.6–1.2 mM Mn was present, higher concentrations of Ca were required for initiating a contraction in normal iris muscles (Fig. 5a). The inhibitory effect of Mn was dose-dependent, and it was overcome by raising the concentration of Ca. At these concentrations Mn may be regarded to act as a Ca-blocker, and did not affect the tone of the depolarized normal muscle (Fig. 5b). At higher concentrations Mn (1.2 mM or more) exerted an action similar to Ca and Sr and contracted the depolarized normal muscle (Fig. 5b). The threshold concentration of Mn for inducing a contraction, however, varied (1.8–3.0 mM); and in some preparations, Mn had no effects even at 3 mM, the maximum concentration tested. Otherwise, the rate of rise of Mn-induced contraction was very slow and concentration-dependent so that it took more than one hour to obtain a dose-response curve. Denervation greatly enhanced this contractile effect of Mn and markedly lowered the threshold concentration (Fig. 5b). At 2.4 mM, the tension development reached a maximum, 93.8±2.4 mg (6 irides), which corresponded to 80% of that produced by Ca shown in Fig. 4 (●). The Mn-induced contraction was largely suppressed by 0.1 μM nifedipine in both preparations (Fig. 5b).

Effect of ACh in Ca-free solution: After 15 min exposure to Ca-free solution containing 0.1 mM EGTA, high concentrations of ACh induced a contractile response which consisted of an initial transient component followed by a small tonic one in both denervated (Fig. 6b) and normal preparations (Fig. 6a). Caffeine (15 mM) did not cause tension development in the Ca-free solution (4 irides for each of both preparations, data not shown). The response could be elicited only by the first application of ACh in both preparations (Fig. 6a, b). As shown in Fig. 6c, ACh-induced contraction in the Ca-free solution, although smaller than the contraction in the presence of Ca, was much
Fig. 5. Change in the effect of Mn on depolarized muscle following denervation.  
a: Cumulative concentration-response relationship for Ca in the presence of 0.6 mM (■, 5 irides) and 1.2 mM Mn (▲, 5 irides) and in the absence of Mn (○, 10 irides) obtained from normal sphincter muscles.  
b: Mn-induced contraction in Ca-free, high-K (60 mM) solution.  
○: normal muscle (4 irides). Three other preparations did not respond to Mn up to 3 mM.  
●: Denervated muscle (6 irides). The response of denervated muscle to 0.6 mM Mn indicated by filled squares was suppressed by 0.1 μM nifedipine as indicated by ■* (4 irides). The response of normal muscle to 3 mM Mn was also suppressed as indicated by ○* (4 irides).

Fig. 6. ACh-induced contraction after 15 min exposure to Ca-free solution.  
Contralateral control (a) and denervated iris muscle (b) produced maximal contraction at the concentrations indicated, respectively. ACh was applied at the horizontal bar.  
c: Cumulative dose-response curve for ACh in the presence (circles) and in the absence (triangles) of 2.2 mM-Ca. Data were obtained from five denervated (filled symbols) and contralateral control irides (open symbols). Symbols represent means with S.E.M. as vertical bars.
larger at all concentrations of ACh in denervated than in normal iris muscles.

Effect of fluoride in Ca-free solution: It has been shown that application of fluoride ion (F) causes contractile response in various smooth muscles in Ca-free solution (20–22). The contraction has been considered to be due to a direct activation of contractile protein, but not to a Ca-mediated indirect mechanism. In the present study, 10 mM F caused in normal irides a contraction similar to those in other smooth muscles (Fig. 7a), but in denervated tissues, different features were observed (Fig. 7b). In both preparations, tension developed slowly after about 2 min latency and attained to a maximum within 40 min. Its absolute value obtained from denervated irides (49.7±5.3 mg, 9 irides) was markedly larger than that of normal preparations (8.1±1.1 mg, 9 irides). The amplitude was not increased by a further addition of 10 mM F in both preparations (data not shown). In normal tissues, the muscle relaxed completely after F was removed, and a similar tonic response was elicited at least two other times without exposure to solution containing Ca (Fig. 7a and 8). In denervated iris muscles, however, the second contraction was much smaller than the first one (4 irides, Fig. 7b and 8). This reduction was not due to deterioration of the muscle, since ACh-induced contraction in the presence of 2.2 mM Ca obtained after repeated application of F was 102.8±6.5% (N=4) of the control. This feature resembles that of the response to ACh in Ca-free solution (see Fig. 6).

Therefore, to examine whether the large contraction induced by the first application of F in denervated muscles was via a Ca-dependent process or not, we used calmodulin inhibitors, trifluoperazine (TFP: (23, 24)) and chlorpromazine (CPZ: (25)). As shown in Fig. 9, the first contraction induced by F in denervated muscles was suppressed by 10 to 50 µM TFP and 30 µM CPZ, but at these concentrations, TFP and CPZ had no effect on the F-induced contraction in normal muscles. However, since it has been reported that most phenothiazine derivatives have a membrane-stabilizing action (26), the effect of procaine which is well-known to have such an action (27) was also examined. Procaine, however, had little effect on the F-

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**Fig. 7.** Effect of F on denervated iris muscle (b) and contratralateral control (a) after 15 min exposure in Ca-free solution. The bar under each trace indicates the period of 10 mM-F application. Ca is omitted from the external medium throughout these experiments.

**Fig. 8.** Responses to three repeated applications of F (10 mM) at 30 min intervals in Ca-free solution. Filled symbols are obtained from denervated muscles and open symbols from four contralateral control muscles. Symbols represent means with S.E.M. as vertical bars.

**Fig. 9.** Effect of trifluoperazine (TFP) and chlorpromazine (CPZ) on F-induced contraction of denervated iris muscles (b) and contratralateral control (a) in Ca-free solution. Numbers indicate final concentrations of drugs: 1. 10 µM; 2. 30 µM; 3. 50 µM; 4. 0.1 mM; and 5. 0.3 mM for TFP; 1. 30 µM and 2. 0.15 mM for CPZ.
induced contraction even at 5 mM in both normal and denervated preparations; relaxation induced by 5 mM procaine amounted to only 5% or less of the contraction in both preparations (5 irides for each).

**Discussion**

It was found that 7–10 days after denervation, neither resting membrane potential nor the magnitude of depolarization by raising \([K]_o\) was affected, indicating that dominance of K conductance was not affected by denervation in the rat iris sphincter.

Partial membrane depolarization has been described in various tissues sensitized by denervation or drug-treatment (guinea-pig vas deferens (12), rat portal vein (11), rabbit saphenous artery (28)) and was considered to play an important role in the development of postjunctional supersensitivity. As we have previously reported, ACh contracts the muscle exclusively through nonelectrical activation (pharmaco-mechanical coupling: PMC) in normal rat iris (15). Therefore, the small depolarization induced by ACh (>10 \(\mu\)M) might account for the supersensitivity to ACh; an additional Ca influx via voltage sensitive Ca-channels which occurs during the depolarization contributes to an increase in tension. This is unlikely, however, since 1) contractile response to ACh reached a near maximum at a concentration of 10 \(\mu\)M in the denervated muscle, and 2) ACh-induced depolarization (ca. 3 mV, Table 1) was much smaller than the depolarization by which a substantial K-contracture occurred (ca. 15 mV, Fig. 1). Thus, these results suggest that the denervation supersensitivity in the iris muscle is little attributed to changes in membrane potential, but rather due predominantly to other mechanisms.

Evidence for change in distribution of intracellular Ca after denervation was obtained in the present study. High concentrations of ACh could elicit a phasic contraction only once in the absence of external Ca in the rat iris sphincter, implying that ACh releases and depletes Ca available for this response from pool(s) of limited capacity, e.g., Ca tightly bound at membrane components. Denervation potentiated the first response to ACh in the Ca-free solution (Fig. 6). This finding may be comparable with that of Carrier and Jurevics (29) that supersensitive rabbit aortae were able to retain a larger amount of Ca available for the contraction than normal aortae.

In this study, several observations may suggest that qualitative and probably also quantitative changes occur following denervation in Ca-channels activated by depolarization in high-K solution. 1) Both sensitivity and the maximum in contractile response of depolarized muscle to Ca and Sr were significantly increased after denervation. 2) Mn elicited a nifedipine-sensitive large contraction in depolarized muscle from denervated iris, while the ion blocked Ca-induced contraction in normal iris at concentrations of 0.6–1.2 mM. 3) Most of the enhanced contraction induced by 2 mM Ca in depolarized muscle from denervated iris was inhibited by nifedipine.

These observations suggest that Ca influx through channels sensitive to nifedipine and also to voltage may increase after denervation. They seemed to be comparable to the phenomena of nonspecific supersensitivity in arterial tissues which has been explained as the increase in Ca mobilization (29, 30). It has been reported that there is a significant increase in the number of nitrendipine binding sites, which is probably the Ca-channel, in denervated rat and chick skeletal muscles (31). This might be the case in denervated iris sphincter.

From the observations, it can be also assumed that Ca-channel activated in high-K solution is more permeable to Mn and probably also Ca and Sr after denervation. Although detailed mechanisms underlying the contraction induced by Mn were not investigated in this study, Mn may partially activate contractile protein, as has been suggested in the skinned antral smooth muscle of the guinea-pig stomach (32). Of interest is that, in spite of these alterations, the voltage sensitivity of Ca-channel activated in high-K solutions was unaffected significantly after denervation.

Fermum et al. (20) reported that F ions can cause contractions independent of extracellular Ca. The possibility has recently
been raised that F ions activate contractile protein of various smooth muscles via Ca-independent process without elevating intracellular Ca concentration (21, 33). These responses can be induced many times in Ca-free solution by repetitive applications of the ions. In contrast to these findings, the contractile response to ACh in Ca-free solution was elicited only once in both normal and denervated rat iris sphincter. Therefore, a Ca-independent contractile mechanism does not seem to take part in the response of iris muscle to ACh. The F contraction in the iris sphincter muscle was, however, elicited repetitively in Ca-free solution. Since F can permeate the cell membrane and the solubility of CaF₂ is very low, it is assumed that during F-induced contraction, intracellular Ca concentration can not be elevated enough to activate a Ca-dependent process such as Ca-calmodulin interaction (21). It has, however, been shown that the first F-induced contraction in Ca-free solution still contains a Ca-dependent component which is due to the release of stored Ca (21). It seems to be actually the case in denervated iris sphincter. The first F-induced contraction was much larger and more sensitive to calmodulin inhibitors in denervated than in normal muscles. Therefore, this finding may be further evidence suggesting an increase in the amount of releasable Ca after denervation.

In addition to the first, the second and third contractions induced by F in Ca-free solution were significantly larger in denervated than in normal muscles. This fact may imply that some Ca-independent process in the contraction, presumably the activity of contractile machinery could be enhanced after denervation. As the contractile protein activated by F may be the same as that activated via Ca-dependent process by ACh and K, the enhancement of the response to F can be taken into account as one of the mechanisms of nonspecific supersensitivity to ACh and K. This idea might be supported by the finding using denervated vas deferens under skinning condition that the non-electrical component of supersensitivity may be related to some alteration of contractile protein (34).

In the present study, it was suggested that nonspecific denervation supersensitivity to ACh and K in the rat iris sphincter was ascribed predominantly to some changes in nonelectrical events, including 1) increase in Ca-influx, 2) increase in amount of releasable Ca from intracellular store sites, and 3) increase in functional activity of contractile protein. There may, however, remain other possible mechanisms which could not be elucidated in the present study, for example, some changes in Ca-calmodulin interaction.

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