

ω -Conotoxin-sensitive and -resistant transmitter release from the chick ciliary presynaptic terminal

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1. Synaptically evoked responses to stimulation of the oculomotor nerve were recorded from the ciliary nerve in chick embryos. The postsynaptic currents in response to presynaptic stimulation (EPSCs) were also recorded under whole-cell voltage clamp of the ciliary cell.
2. The ciliary nerve response was dependent on the extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_o$). ω -Conotoxin GVIA (ω -CgTX, 100 nM) increased the $[\text{Ca}^{2+}]_o$ necessary to evoke the half-maximal response by a factor of 1.7 without changing the slope of $[\text{Ca}^{2+}]_o$ dependence. Dihydropyridine (DHP) derivatives, nifedipine or Bay K 8644, did not affect the $[\text{Ca}^{2+}]_o$ sensitivity of ciliary nerve response.
3. The EPSC was usually preceded by the capacitive coupling response of the presynaptic action potential. In some records, the EPSCs were also preceded by the electrical coupling responses which were the mirror images of the presynaptic action potentials. The current–voltage relation of the EPSCs showed inward rectification.
4. The EPSC was potentiated by 4-aminopyridine (4-AP) as a result of prolongation of the falling phase of presynaptic action potential. In the presence of high $[\text{Ca}^{2+}]_o$ and 4-AP, a small fraction of EPSC was resistant to ω -CgTX.
5. The resting potential of the presynaptic terminal was changed from -69 to -57 mV by increasing $[\text{K}^+]_o$ from 1 to 10 mM. The same procedure decreased the ω -CgTX-resistant EPSC by 30 %, whereas the ω -CgTX-untreated EPSC in low- Ca^{2+} saline was not affected by the change in $[\text{K}^+]_o$.
6. The nerve-evoked increase in intracellular Ca^{2+} was recorded from the presynaptic terminal ($\Delta[\text{Ca}^{2+}]_{pre}$). The $\Delta[\text{Ca}^{2+}]_{pre}$ was larger in a solution containing 10 mM Ca^{2+} and 1 mM K^+ after treating with ω -CgTX than in a solution containing 2 mM Ca^{2+} and 16 mM Mg^{2+} before treating with ω -CgTX. The EPSC was, in contrast, smaller in the 10 mM Ca^{2+} –1 mM K^+ solution after ω -CgTX treatment than in the 2 mM Ca^{2+} –16 mM Mg^{2+} solution before ω -CgTX treatment.
7. Similarly, the EPSC was smaller in the 10 mM Ca^{2+} –1 mM K^+ solution containing $5 \mu\text{M}$ La^{3+} than in the 2 mM Ca^{2+} –16 mM Mg^{2+} solution, whereas the $\Delta[\text{Ca}^{2+}]_{pre}$ was larger in the 10 mM Ca^{2+} –1 mM K^+ solution containing $5 \mu\text{M}$ La^{3+} than in the 2 mM Ca^{2+} –16 mM Mg^{2+} solution.
8. It is concluded that the ω -CgTX-sensitive Ca^{2+} conductance of the presynaptic terminal is the principal source of Ca^{2+} involved in transmitter release. Although the ω -CgTX-resistant conductance which is inactivated at depolarized membrane potentials also participated in the transmitter release, the ability of releasing transmitters relative to $\Delta[\text{Ca}^{2+}]_{pre}$ was reduced after treating with ω -CgTX. It is suggested that the ω -CgTX-sensitive Ca^{2+} channels may cluster near the release sites. Since the ω -CgTX-sensitive channels are outnumbered, it should be rare for the ω -CgTX-resistant Ca^{2+} channels to be adjacent to each other.

One of the crucial functions of the voltage-gated Ca^{2+} channels in nerve cells is to trigger transmitter release in response to depolarization of a nerve terminal (Katz & Miledi, 1967; Augustine, Charlton & Smith, 1987). It has been demonstrated that multiple types of Ca^{2+} channel co-

exist in a given neurone (for review see Miller, 1987; Tsien, Ellinor & Horne, 1991). The role of Ca^{2+} channel subtypes in neurotransmitter release has been examined by their specific antagonists such as ω -conotoxin GVIA (ω -CgTX; Olivera *et al.* 1985) and dihydropyridines (DHPs). The high

potency of ω -CgTX in blocking noradrenaline release from sympathetic neurones (Hirning *et al.* 1988), together with the ineffectiveness of DHPs, has suggested that the ω -CgTX-sensitive N-type channel rather than the DHP-sensitive L-type channel, is responsible for transmitter release from these cells (Miller, 1987; Hirning *et al.* 1988). Multiple subtypes of Ca^{2+} channel have also been characterized in the rat neurohypophyseal secretosome (Lemos & Nowycky, 1989; Wang, Treistman & Lemos, 1992). In this preparation, the release of peptide is blocked by both ω -CgTX and DHP, but a substantial amount of the peptide release is insensitive to both Ca^{2+} channel antagonists (Dayanithi *et al.* 1988).

The calyiform presynaptic terminal of the chick ciliary ganglion is the only vertebrate presynaptic terminal so far in which the pharmacological properties of the Ca^{2+} current have been studied under voltage clamp (Stanley & Atrakchi, 1990; Stanley & Goping, 1991; Yawo & Momiyama, 1993): the major component was sensitive to ω -CgTX but insensitive to DHPs, whereas Yawo & Momiyama (1993) have shown the co-existence of the second component which is resistant to both ω -CgTX and DHPs. Since the synaptic transmission of the chick ciliary ganglion is blocked largely by ω -CgTX (Yoshikami, Bagabaldo & Olivera, 1989; Stanley & Atrakchi, 1990), the ω -CgTX-sensitive Ca^{2+} channel is undoubtedly coupled with transmitter release from the ciliary calyx. However, whether the ω -CgTX-resistant Ca^{2+} channel is also involved in releasing transmitter is not known. Is the Ca^{2+} influx through ω -CgTX-resistant Ca^{2+} channels capable of releasing the same amount of transmitter as that through ω -CgTX-sensitive channels if the amount of their Ca^{2+} influxes is comparable? The present study shows that the ω -CgTX-resistant Ca^{2+} influx can release the transmitter, but its efficacy is clearly lower than that before treating with ω -CgTX. A preliminary report of some of these results has appeared previously (Yawo & Chuhma, 1992).

METHODS

Extracellular recordings

The presynaptic oculomotor nerve together with the ciliary ganglion and postsynaptic ciliary nerve were isolated from chick embryos of days 14–15 (Yawo & Momiyama, 1993). The ganglion was treated with 1 mg ml^{-1} collagenase (Type I; Sigma, St Louis, MO, USA) for 10 min at room temperature (19–26 °C). This procedure facilitated solution exchange in the ganglion. Suction electrodes were attached to both the oculomotor and ciliary nerve as described by Landmesser & Pilar (1972). Electrical responses were differentially amplified (MEZ-7101, Nihon Kohden, Tokyo, Japan) and stored on FM magnetic tape (MR-10, TEAC, Tokyo, Japan; low-pass filtered at 2.5 kHz, 4-pole Bessel). Synaptic transmission through the ganglion was estimated using the upper area of the compound postsynaptic response divided at the 50% line of the peak amplitude (the upper-half area; Fig. 1A).

EPSC recordings

The collagenous envelope of the ciliary ganglion was enzymatically removed by focal application of a mixture of 10 mg ml^{-1} collagenase (Sigma, Type I) and 100 U ml^{-1} thermolysin (Sigma) through a pipette of 40–50 μm tip diameter for 10–30 min at room temperature (Yawo, 1989). The enzymes were washed out rigorously with a Ca^{2+} -free saline solution containing 1 mM EGTA. The ciliary ganglion consists of two distinct cell populations, choroid and ciliary (Marwitt, Pilar & Weakly, 1971). The ciliary cell has a calyx-type synapse whereas the choroid cell has bouton-like synapses (Landmesser & Pilar, 1972; Cantino & Mugnaini, 1975). The postsynaptic ciliary cell was distinguished from the choroidal cell on the basis of its location and large size. After recordings, some ganglia were perfused for 3 min by a saline solution containing $10 \mu\text{M}$ 4-(4-dimethylaminostyryl)-*N*-methylpyridinium iodide (Molecular Probes Inc., Eugene, OR, USA) which selectively labelled presynaptic terminals of the mouse submandibular ganglion (Purves, Voyvodic, Magrassi & Yawo, 1987) as well as chick ciliary ganglion (H. Yawo, unpublished observation). The calyx-type nerve terminal was identified from each cell tested ($n = 8$).

The whole-cell patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) was applied to the ciliary cell as described previously (Yawo, 1989; Yawo & Momiyama, 1993). The pipette solution for recording from postsynaptic cells contained (mM): CsCl, 130; CaCl_2 , 1; EGTA, 11; Hepes, 20; MgATP, 5 (pH 7.4 adjusted with CsOH). The capacitive transient was minimized by analog circuitry. The series conductance measured by the front panel was usually larger than $0.1 \mu\text{S}$ and was compensated for by 50–70%. The whole-cell currents were low-pass filtered at 3 kHz (–3 dB, 4-pole Bessel filter), digitized at 10–20 kHz (ADX-98, Canopus, Kobe, Japan) and stored in the magneto-optical disk unit (CA-6080MO, Carabelle Data System, Tokyo, Japan) by a computer (PC-9801Vm21, NEC, Tokyo, Japan).

Whole-cell recordings from presynaptic terminals

Calyx-type nerve terminals were identified as described previously (Yawo & Momiyama, 1993). The pipette used for current clamp experiments was filled with the following solution (mM): KCl, 17.5; potassium gluconate, 122.5; NaOH, 8; EGTA, 0.2; Hepes, 10 (pH 7.2 with HCl); supplemented with (mM): MgATP, 5; GTP, 0.3; MgCl_2 , 1; mannitol, 1; 5(6)-carboxyfluorescein (Kodak, Rochester, NY, USA), 0.1. The liquid junctional potential (11 mV) was measured for the current clamp experiment and was corrected for.

Measurement of the presynaptic Ca^{2+}

The method of measuring the presynaptic Ca^{2+} is almost the same as described in Yawo & Chuhma (1993). The oculomotor nerve was cut at its exit from the orbital bone in a Ca^{2+} -free saline containing 1 mM EGTA. Crystals of a fura-2-conjugated dextran (fura-dextran, relative molecular mass 10000, Molecular Probes Inc.) were applied to the cut end of the distal stump as described previously (Yawo & Momiyama, 1993). After 30 min incubation at 10 °C, the ganglion was superfused with the oxygenated standard saline and was incubated at 36 °C for 1.5 h. Fura-dextran was transported anterogradely by axonal flow. Because of its large molecular size, fura-dextran was confined to the presynaptic axons and their

calyciform terminals. A whole ganglion was mounted in a chamber, the oculomotor nerve was sucked by the stimulating electrode and the collagenous envelope was enzymatically removed by focally applying collagenase and thermolysin (see above; Yawo, 1989). A conventional epifluorescence system equipped with a water-immersion objective ($\times 40$, numerical aperture 0.7; Olympus, Tokyo, Japan) and Xenon lamp (150 W) was used. Fluorescence was excited alternately at wavelengths of 340 and 380 nm. Because the microscope was focused on the surface of the ganglion, a fluorescence from one to three terminals was measured at a single spot with a diameter of 40–50 μm by a photomultiplier tube (OSP-3, Olympus). The signal was integrated for 50 ms and sampled at 20 Hz by a computer (PC-9801RS, NEC). The intracellular Ca^{2+} was calculated from the ratio of fluorescence intensities at wavelengths of 340 and 380 nm (Grynkiewicz, Poenie & Tsien, 1985) using the dissociation constant (K_D) of 350 nm (Haugland, 1992). The minimum ratio and the maximum fluorescence at 380 nm were measured in a Ca^{2+} -free solution containing 5 mM BAPTA and 0.1 mM ionomycin (Calbiochem, La Jolla, CA, USA). Thereafter the solution was changed to that containing 10 mM CaCl_2 , and the maximum ratio and the minimum fluorescence at 380 nm were measured. In order to reduce the signal-to-noise ratio, 12–500 records were averaged.

Solutions

The standard extracellular solution contained (mM): NaCl, 130; KOH, 5; CaCl_2 , 5; MgCl_2 , 1; Hepes, 10; glucose, 11 (pH 7.4 with HCl). Solutions with various $\text{Ca}^{2+}:\text{Mg}^{2+}$ ratios were made by mixing the following two solutions. Solution 1 contained (mM): NaCl, 129.5; KCl, 5; CaCl_2 , 10; NaOH, 5; Hepes, 10 (pH 7.4 with HCl). Solution 2 contained (mM): NaCl, 114.5; KCl, 5; MgCl_2 , 20; NaOH, 5; Hepes, 10 (pH 7.4 with HCl). The solutions containing higher concentrations of Ca^{2+} were made with or without replacing equimolar Na^+ . K^+ concentration was changed by replacing with equimolar Na^+ . Solutions were equilibrated with O_2 and superfused over the ganglion. The solution in the chamber (*ca* 1 ml) was completely replaced in less than 2 min. All the experiments were carried out at room temperature.

Drugs

Hexamethonium, *d*-tubocurarine and 4-AP were obtained from Nacalai, Kyoto, Japan. Nifedipine (Pfizer, New York, NY, USA) and Bay K 8644 (Research Biochem, Inc., Natick, MA, USA) were dissolved in ethanol and stored at -20°C . Control experiments were made by adding the same concentration of vehicle (0.03–0.1% ethanol) which showed no effect. During the experiments using DHPs, the light was dimmed in order to minimize photobleaching. The stock solution of ω -CgTX (The Peptide Institute, Osaka, Japan) was prepared at a concentration of 1 mM in a solution of 100 mg ml^{-1} cytochrome *c* or bovine serum albumin. The stock solution was added directly to the bath while the perfusion was halted. In these experiments, O_2 was applied directly to the bathing fluid.

Statistics

From a series of records of EPSCs, the characteristic failure responses were collected. Occasionally, the characteristic failure responses were elicited in the Ca^{2+} -free solution. The height of the EPSC was measured by fitting a horizontal cursor

on a graphic plane which displayed the results after subtracting the average failure response from the raw data. The bin size of the EPSC amplitude histogram was selected so that the smallest non-failure responses could be differentiated. The mean number of quanta (m) was calculated from the number of failures, based on the Poisson statistics (Martin & Pilar, 1964*b*). Alternatively, the m value was evaluated from the coefficient of variation (Martin, 1966; Kuno & Weakley, 1972). The values obtained by the two methods were almost identical when the occurrence of failure was moderate. Therefore, the m value was estimated from the coefficient of variation when the occurrence of failure was less than 10% of the trial. Otherwise, the m value was estimated from the number of failures.

The test and control values were compared using Student's two-tailed *t* test. Results are given as means \pm s.d. (number of experiments).

RESULTS

Compound synaptic responses recorded from the ciliary nerve

Synaptic transmission in the ciliary ganglion was initially characterized by extracellular recordings from the postganglionic ciliary nerve in response to preganglionic oculomotor stimulation. The major synaptic response was usually preceded by a small potential as illustrated in Fig. 1*A*. The small potential elicited immediately after stimulus artifact is presumably the compound action potential of the presynaptic elements, whereas the late larger component reflects the postsynaptic response (Landmesser & Pilar, 1972). This interpretation was supported by the fact that the late component is abolished when perfused with Ca^{2+} -free (replaced with 1 mM EGTA and 6 mM Mg^{2+}) saline solution without affecting the early component (Fig. 1*B*, upper traces). Similarly, the late component was selectively suppressed by the addition of 3 mM hexamethonium, a blocker of the postsynaptic acetylcholine receptor (Fig. 1*B*, middle traces). The late component completely recovered within 15 min of washing out (Fig. 1*B*, lower traces). Similar results were obtained using *d*-tubocurarine.

The effect of extracellular calcium concentration, $[\text{Ca}^{2+}]_o$, on synaptic transmission was evaluated, taking the upper-half area of the major postsynaptic response as the magnitude of chemical transmission (Fig. 1*A*, hatched area; see Methods). Figure 1*C* shows the $[\text{Ca}^{2+}]_o$ dependence of the synaptic response. The effect of $[\text{Ca}^{2+}]_o$ was non-linear and almost saturated at about 10 mM (Fig. 1*C*, open circles). The presynaptic compound action potential was independent of $[\text{Ca}^{2+}]_o$, as indicated by the filled circles in Fig. 1*C*.

Effects of DHPs and ω -CgTX on the extracellularly recorded synaptic response

When Ca^{2+} influx into the nerve terminal is blocked, the ciliary response– $[\text{Ca}^{2+}]_o$ relation depicted in Fig. 1*C* is

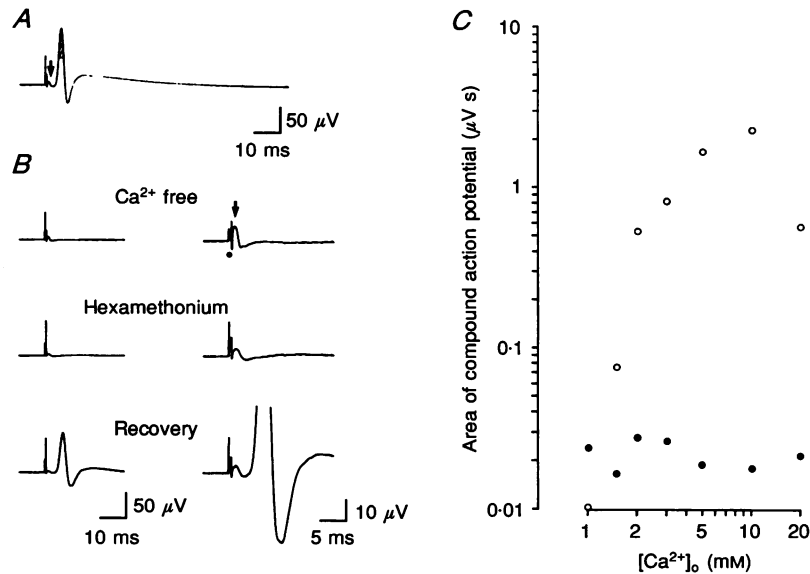


Figure 1. The ciliary nerve responses to stimulation of the oculomotor nerve

Each trace is the average of 10–20 successive records. Stimulus artifacts were truncated in this and other figures. *A*, the ciliary nerve responses recorded in control saline containing 5 mM Ca^{2+} and 1 mM Mg^{2+} . The arrow indicates the presynaptic compound action potential just after the stimulus artifact. The hatched area is the upper-half area of the postsynaptic compound action potential used as quantity of chemical transmission. *B*, effects of Ca^{2+} -free saline containing 1 mM EGTA plus 6 mM Mg^{2+} (upper traces) and 3 mM hexamethonium dissolved in the standard solution (middle traces). Records with higher magnification are shown on the right. The effects of hexamethonium were reversible as shown in the lower traces. The filled circle and the arrow indicate the stimulus artifact and the presynaptic compound action potential, respectively. *C*, log-log plots of the $[\text{Ca}^{2+}]_o$ and the ciliary nerve responses. The upper-half areas of the pre- (●) and postsynaptic (○) compound action potentials were measured as in *A*.

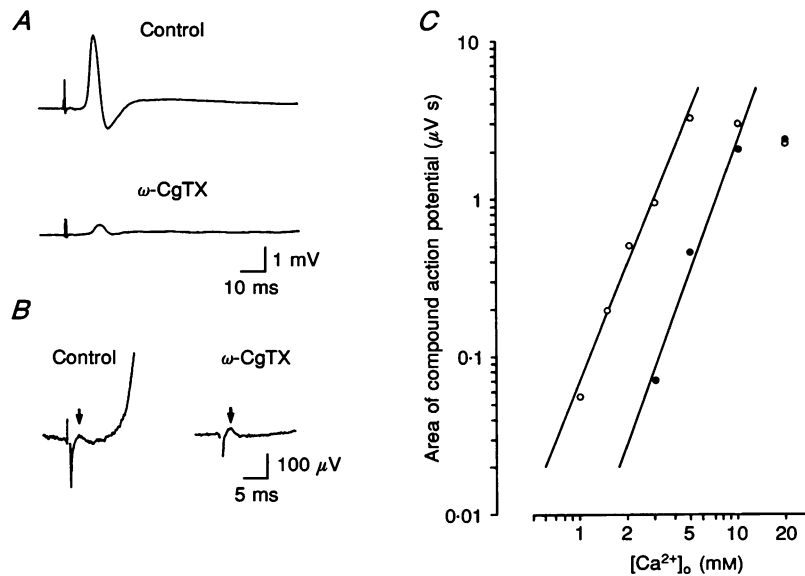


Figure 2. Effects of ω -CgTX on the ciliary nerve responses

Each trace is the average of 10–20 successive records. *A*, the postsynaptic compound action potentials recorded before (upper trace) and after (lower trace) treatment with 100 nM ω -CgTX. The external solution contained 5 mM Ca^{2+} and 10 mM Mg^{2+} . *B*, the presynaptic compound action potentials observed before (left) and after (right) treatment with 100 nM ω -CgTX. The same experiment as in *A* except that the external saline contained 3 mM Ca^{2+} and 14 mM Mg^{2+} . *C*, log-log plots of the $[\text{Ca}^{2+}]_o$ and the ciliary nerve responses. The upper-half area of the postsynaptic compound action potential was measured before (○) and after (●) treating with 100 nM ω -CgTX. The straight lines were obtained by least-squares fitting for the linear portion of the data. The slope was approximately the same before (2.6) and after (2.7) ω -CgTX treatment.

expected to shift to the right. Yawo & Momiyama (1993) have shown that the Ca^{2+} current of the calyciform presynaptic terminal of the chick ciliary ganglion is partly blocked by ω -CgTX but insensitive to DHPs. A DHP agonist, Bay K 8644 ($3 \mu M$), had no obvious effect on the ganglionic transmission. The half-maximal $[Ca^{2+}]_o$ was $4.6 \pm 0.8 \text{ mM}$ ($n=3$) in the presence of Bay K 8644, which did not differ significantly from the value in the absence of the drug, $4.2 \pm 0.6 \text{ mM}$ ($n=3$). Similarly the DHP antagonist, nifedipine ($10 \mu M$), had no effects.

In contrast, ganglionic transmission was irreversibly suppressed by ω -CgTX. Figure 2A shows an example of the ciliary nerve response before (upper trace) and after (lower trace) the application of 100 nM ω -CgTX for 1 h. At this concentration of the toxin, the ω -CgTX-sensitive sub-population of Ca^{2+} channels is expected to be partially blocked. The toxin did not affect the presynaptic component (Fig. 2B). The relation of the ciliary nerve response to $[Ca^{2+}]_o$ was shifted to the right by ω -CgTX with little change in its saturation level (Fig. 2C). The saturation level after treating with ω -CgTX was, on average, $76 \pm 18 \%$ ($n=5$) of the control. In the ω -CgTX-treated ganglia, the half-maximal $[Ca^{2+}]_o$ was $7.5 \pm 1.4 \text{ mM}$ ($n=5$) which is significantly ($P < 0.01$) higher than that of control ganglia, $4.3 \pm 1.8 \text{ mM}$ ($n=9$). However, ω -CgTX did not change the slope of $[Ca^{2+}]_o$ dependence and shifted the relation along the $[Ca^{2+}]_o$ axis by a concentration ratio

of 1.7. The mean slope of ω -CgTX-treated ganglia was 3.8 ± 0.8 ($n=5$) which did not differ significantly from that of control ganglia, 3.4 ± 0.7 ($n=9$). The slope could reflect either co-operativity in transmitter release (Dodge & Rahamimoff, 1967), non-linearities at later stages (e.g. postsynaptic threshold), or both. The block by ω -CgTX was irreversible as reported previously (Yawo & Momiyama, 1993). The synaptic transmission was almost completely blocked by high doses ($3\text{--}10 \mu M$) of ω -CgTX. These observations indicate that ω -CgTX-sensitive Ca^{2+} influx is the major factor responsible for the release of transmitter.

Whole-cell recording of postsynaptic currents

The EPSCs evoked by presynaptic stimulation were monitored under whole-cell voltage clamp of the ciliary cell (Fig. 3A). The EPSC was usually preceded by a small biphasic current (Fig. 3B, upper trace). When the postsynaptic cell was depolarized to 0 mV , the EPSC became null (Fig. 3A, middle trace) but the early biphasic current remained unchanged (Fig. 3B, middle trace). The current-voltage relation of the EPSCs showed characteristic inward rectification (Fig. 3A, lower trace and Fig. 3C) as noted in mammalian parasympathetic ganglia (Yawo, 1989). The EPSC was completely abolished when extracellular Ca^{2+} was removed, whereas the preceding small current remained unchanged (Fig. 3B, lower trace). Thus, the early small current appears to reflect the

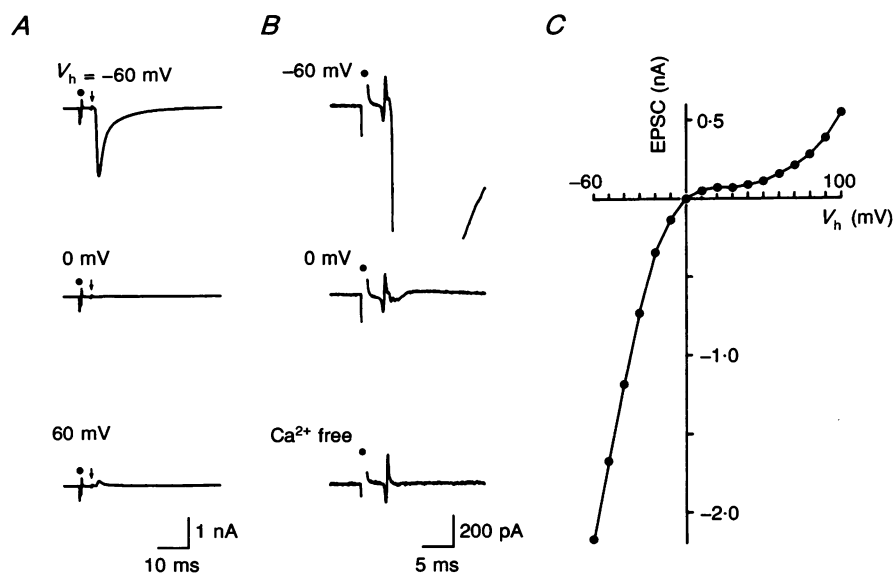


Figure 3. Whole-cell recordings of synaptic currents from the chick ciliary cell

A, the chemically mediated postsynaptic response (EPSC) was preceded by a small current (arrow). V_h , holding potential. Each trace is the average of 10 successive responses. The inward EPSC observed at -60 mV (upper trace) became null at 0 mV (middle trace) and was partly reversed at 60 mV (lower trace). The filled circles indicate stimulus artifacts. Arrows indicate capacitive coupling responses. B, the capacitive coupling response to the invasion of presynaptic action potential. The same experiment as in A. Each trace is the average of 10 successive responses. The biphasic coupling response prior to the EPSC remained unchanged at different membrane potentials (upper and middle traces). In a Ca^{2+} -free saline containing 1 mM EGTA and 6 mM Mg^{2+} , the EPSC was completely abolished at -60 mV whereas the capacitive coupling response was not affected (lower trace). C, current-voltage ($I-V$) relation of the EPSC illustrated in A.

capacitive component of the electrical coupling response of the presynaptic action potential (Martin & Pilar, 1964*a*). Since a large part of the postsynaptic membrane is covered by the presynaptic terminal in the chick ciliary ganglion (Fujiwara & Nagakuro, 1989), the rising phase of the presynaptic action potential would trigger a negative-to-positive capacitive current in the postsynaptic cell. The capacitive coupling response indicates the invasion of action potential in the presynaptic calyx.

Occasionally, the capacitive current was followed by a small inward current which is reminiscent of the presynaptic action potential (Fig. 4*A*). This current remained unaffected by depolarization to 0 mV or by removing $[Ca^{2+}]_o$. This small current might reflect the resistive component of the electrical coupling response (Martin & Pilar, 1963; Yawo & Momiyama, 1993) through gap junctions (Cantino & Mugnaini, 1975). This resistive coupling response should be a mirror image of the presynaptic action potential. The electrical coupling response was observed in fifteen of sixty-three preparations.

ω -CgTX-resistant synaptic transmission

In order to test if the ω -CgTX-resistant Ca^{2+} influx is also capable of triggering transmitter release, the Ca^{2+} influx was enhanced by increasing $[Ca^{2+}]_o$ and by broadening the action potential duration with 4-AP (Hueser, Reese, Dennis, Jan, Jan & Evans, 1979). Figure 4*B* shows the

effects of 4-AP on the presynaptic action potential. 4-AP prolonged the falling phase of the action potential without affecting the resting potential, presumably by blocking the transient K^+ conductance (H. Yawo, unpublished observation). The prolongation of activation time would enhance an increase in Ca^{2+} conductance (Jackson, Konnerth & Augustine, 1991) because of the slow activation of presynaptic Ca^{2+} conductance (Yawo & Momiyama, 1993). The EPSC was potentiated in the presence of 4-AP (Fig. 4*C*). As shown in Fig. 4*C*, the resistive coupling response was clearly prolonged by 4-AP. The prolonged resistive coupling response was not affected by 200 μ M *d*-tubocurarine (Fig. 4*D*). As shown in Fig. 4*A* and *C*, the onset of EPSC was delayed in the presence of 4-AP, indicating that Ca^{2+} influx into the presynaptic terminal was suppressed during the plateau phase of action potential because of the small driving force for Ca^{2+} . The optimum effect for potentiating EPSC was observed at 0.4 mM 4-AP. Higher doses of 4-AP (> 1 mM) partially blocked the postsynaptic acetylcholine receptor (Katz & Miledi, 1979).

Figure 5 shows the effects of 10 μ M ω -CgTX on the EPSCs recorded in the presence of 4-AP and 20 mM $[Ca^{2+}]_o$. Although the EPSC was markedly suppressed by ω -CgTX, a small fraction of EPSC remained over 10 min after application of the toxin (Fig. 5, inset *b*). In another experiment, the application of 10 μ M ω -CgTX over 1 h did

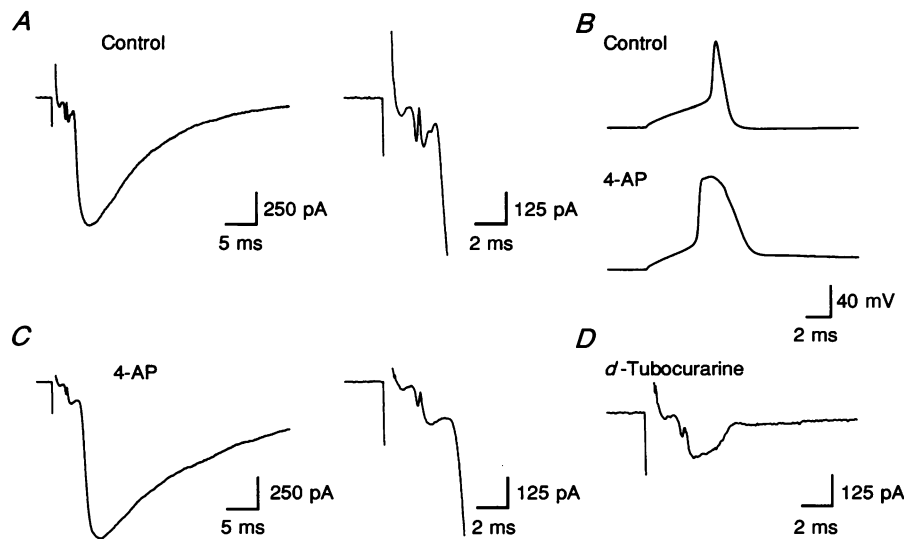


Figure 4. Effects of 4-aminopyridine (4-AP) on the EPSC and the presynaptic action potential in the ciliary ganglion

A, average of 10 successive whole-cell recordings of EPSCs in a saline containing 3 mM Ca^{2+} and 14 mM Mg^{2+} . The electrical coupling response is shown in the right panel with expanded scales. *B*, whole-cell current clamp recordings of action potentials from the presynaptic terminal. Each trace is the average of 10 successive records. The control action potential was elicited by a 10 ms depolarizing pulse from a resting potential of -63 mV after correction of the liquid junction potential (upper trace). 4-AP (4 mM) prolonged the falling phase of the action potential (lower trace). *C*, the EPSC in the presence of 4-AP (0.4 mM). The same cell as in *A*. The prolongation of the electrical coupling response is illustrated in the right panel with expanded scales. Note that the onset of EPSC was delayed in the presence of 4-AP. *D*, the electrical coupling response in the presence of 4-AP and *d*-tubocurarine (200 μ M). The same series of experiments as in *C*.

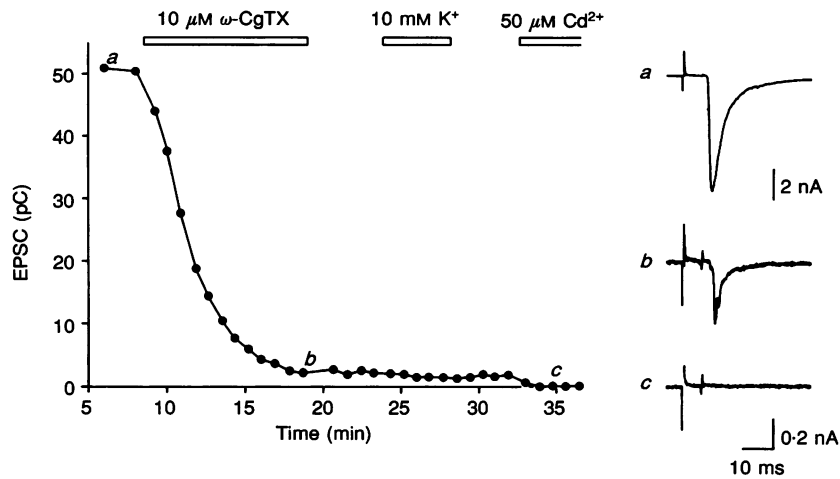


Figure 5. Effects of ω-CgTX on the 4-AP-enhanced EPSCs of the ciliary cell

The EPSC was recorded at a holding potential of -60 mV in a saline containing 20 mM Ca²⁺, 1 mM K⁺ and 0.4 mM 4-AP. The size of EPSC was expressed by the area underneath the baseline and expressed in picocoulombs. Each point represents the average of 10 successive records at 0.2 Hz. Abscissa, the time after establishing the whole-cell configuration. Note that the suppression of EPSC by ω-CgTX was irreversible and that the residual EPSC was reversibly suppressed by increasing K⁺ to 10 mM (see also Fig. 7B). Insets are: *a*, a sample record of the control EPSC before the application of ω-CgTX; *b*, the EPSC recorded in the presence of 10 μM ω-CgTX; *c*, the application of 50 μM Cd²⁺ completely blocked the EPSC, whereas the capacitive coupling remained.

not completely abolish the EPSC. The effects of ω-CgTX was irreversible, and the remaining EPSC was completely blocked by Cd²⁺ (Fig. 5, inset *c*).

Since a high concentration of Ca²⁺ facilitates the unbinding of ω-CgTX (Abe, Koyano, Saisu, Nishiuchi & Sakakibara, 1986), the ω-CgTX-resistant EPSC as shown in Fig. 5 might be evoked by the Ca²⁺ influx through ω-CgTX-sensitive Ca²⁺ channels which were not bound by ω-CgTX. In order to test this possibility, 10 μM ω-CgTX was first

applied in a solution containing 2 mM Ca²⁺ and 4 mM Mg²⁺ (Fig. 6). After incubating for 30 min, the synaptic response was completely blocked. When the ω-CgTX-containing solution was washed by a solution containing 20 mM Ca²⁺, 1 mM K⁺ and 0.4 mM 4-AP, a small EPSC reappeared with a time constant of 126 s which is comparable to that of solution exchange (94 s in the same synapse).

As shown in Fig. 6, the size of EPSC was not reduced by the addition of ω-CgTX indicating that the remaining EPSC

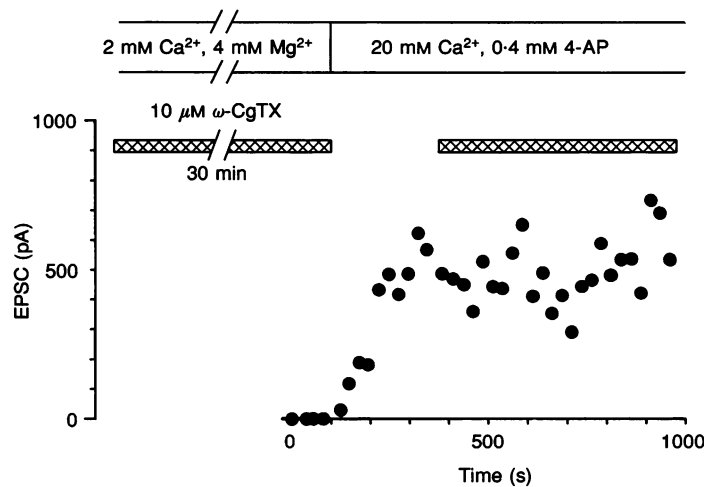


Figure 6. The resistivity of EPSC to ω-CgTX

The ciliary ganglion was first treated with 10 μM ω-CgTX for 30 min (cross-hatched bar) in a solution containing 2 mM Ca²⁺, 4 mM Mg²⁺ and 5 mM K⁺. When the solution was switched to a toxin-free solution containing 20 mM Ca²⁺, 1 mM K⁺ and 0.4 mM 4-AP, the EPSCs reappeared with a time constant of 126 s. Reapplication of 10 μM ω-CgTX (cross-hatched bar) did not further suppress the EPSCs.

was evoked by the Ca^{2+} influx through the ω -CgTX-resistant Ca^{2+} channels.

Figure 7 tested the above problem in an alternative way. The control EPSC was recorded in a saline solution containing 1 mM Ca^{2+} , 18 mM Mg^{2+} and 0.4 mM 4-AP (Fig. 7A), so that the charge of the synaptic response was comparable with that after treating with ω -CgTX in a saline solution containing 20 mM Ca^{2+} and 0.4 mM 4-AP (Fig. 7B). The time course of EPSC was slower in a low- Ca^{2+} -high- Mg^{2+} saline (Figs 4A and 7A) than in a high- Ca^{2+} saline (Figs 3A, 5 and 7B). Although we did not rule out the possibility that the high Ca^{2+} affects the presynaptic release mechanism, divalent cations possibly interact with the nicotinic acetylcholine receptor (nAChR) channel (Mathie, Cull-Candy & Colquhoun, 1987) and may modify the open-close kinetics (Marchais & Marty, 1979; Vernino, Amador, Luetje, Patrick & Dani, 1992). Since the EPSC falling phase may represent the mean lifetime of the postsynaptic nAChR channels (Anderson & Stevens, 1973; Yawo, 1989), the rapid EPSC decay observed in a high- Ca^{2+} saline could be attributed to the properties of the postsynaptic nAChRs.

Yawo & Momiya (1993) have shown that the ω -CgTX-resistant Ca^{2+} conductance manifests steady-state inactivation, whereas the ω -CgTX-sensitive conductance is non-inactivating. Therefore, the effects of presynaptic membrane potentials on the ω -CgTX-resistant EPSC were investigated. The resting potential of presynaptic terminals shifted from -69 ± 1.8 to -57 ± 2.9 mV ($n = 5$) when $[\text{K}^+]_o$ was changed from 1 to 10 mM. In one experiment, action potentials were elicited by 10 ms depolarizing pulses with amplitudes just above the threshold. In a solution containing 1 mM K^+ , the mean

resting potential and the mean action potential were -67 and 115 mV, respectively, whereas they were -59 and 107 mV in a solution containing 10 mM K^+ . Thus, the overshoot potential was unchanged, at 48 mV, by the 10-fold change in $[\text{K}^+]_o$. Changing $[\text{K}^+]_o$ from 1 to 10 mM did not affect the control EPSC (Fig. 7A, lower trace). On the other hand, the ω -CgTX-resistant EPSC (Fig. 7B, top trace) was markedly decreased by changing $[\text{K}^+]_o$ from 1 to 10 mM (Fig. 7B, lower trace). The effects of $[\text{K}^+]_o$ were reversible (Fig. 5). These results are summarized in Fig. 7C. The size of EPSC was not affected by the change in $[\text{K}^+]_o$ in the absence of ω -CgTX (range 98–104%, $n = 6$). In contrast, ω -CgTX-resistant EPSCs in 10 mM K^+ saline was about 70% (range 57–79%, $n = 5$) of those in 1 mM K^+ saline. This difference was highly significant ($P < 0.001$). It is concluded that the ω -CgTX-resistant Ca^{2+} channel is capable of releasing the transmitter in the presence of high Ca^{2+} and 4-AP concentrations.

Efficacy of transmitter release

In Fig. 8, the efficacy in triggering transmitter release was compared between ω -CgTX-sensitive and -resistant Ca^{2+} channels. When the presynaptic nerve was stimulated in a solution containing 2 mM Ca^{2+} and 16 mM Mg^{2+} , the spatial average of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_{\text{pre}}$) was increased by 3.1 nM (Fig. 8A). After treating with ω -CgTX (10 μM for 30 min), the nerve-evoked increase of $[\text{Ca}^{2+}]_{\text{pre}}$ ($\Delta[\text{Ca}^{2+}]_{\text{pre}}$) was 5.5 nM in a solution containing 10 mM Ca^{2+} and 1 mM K^+ (Fig. 8B). On average, the $\Delta[\text{Ca}^{2+}]_{\text{pre}}$ was 2.6 ± 1.4 nM ($n = 11$) in a 2 mM Ca^{2+} -16 mM Mg^{2+} solution; and 28.0 ± 2.7 nM ($n = 4$) in a 10 mM Ca^{2+} -1 mM K^+ solution before treating with ω -CgTX; the latter value was reduced to 7.8 ± 2.2 nM ($n = 16$) after treating with ω -CgTX (10 μM

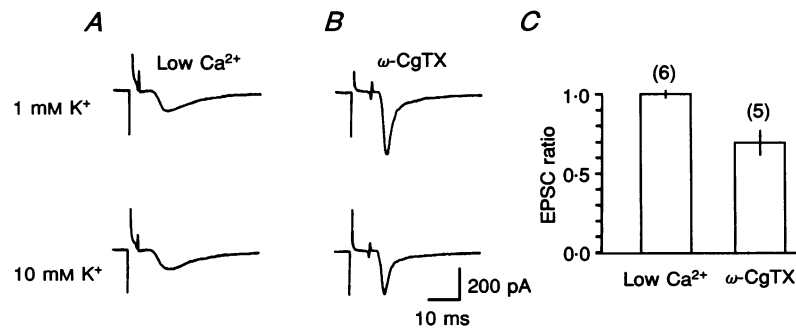


Figure 7. Effects of $[\text{K}^+]_o$ on EPSCs in the ciliary cell

Each trace is the average of 10 successive recordings. A, the EPSCs were recorded in a saline containing 1 mM K^+ , 1 mM Ca^{2+} , 18 mM Mg^{2+} and 0.4 mM 4-AP (upper trace). The size of EPSCs was not affected by increasing $[\text{K}^+]_o$ to 10 mM (lower trace). B, after treatment with 10 μM ω -CgTX. The same experiment as in Fig. 5. The size of EPSC was reduced by increasing $[\text{K}^+]_o$ from 1 mM (upper trace) to 10 mM (lower trace). C, the effect of $[\text{K}^+]_o$ is expressed by the ratio of the EPSC size at 10 mM $[\text{K}^+]_o$ to that at 1 mM $[\text{K}^+]_o$. Low Ca^{2+} , the EPSCs recorded in a saline solution containing 1 mM Ca^{2+} , 18 mM Mg^{2+} and 0.4 mM 4-AP. ω -CgTX, the ω -CgTX-treated EPSC in a saline solution containing 20 mM Ca^{2+} and 0.4 mM 4-AP. Each column and bar represent the mean and s.d. respectively. Numbers in parentheses indicate the number of experiments.

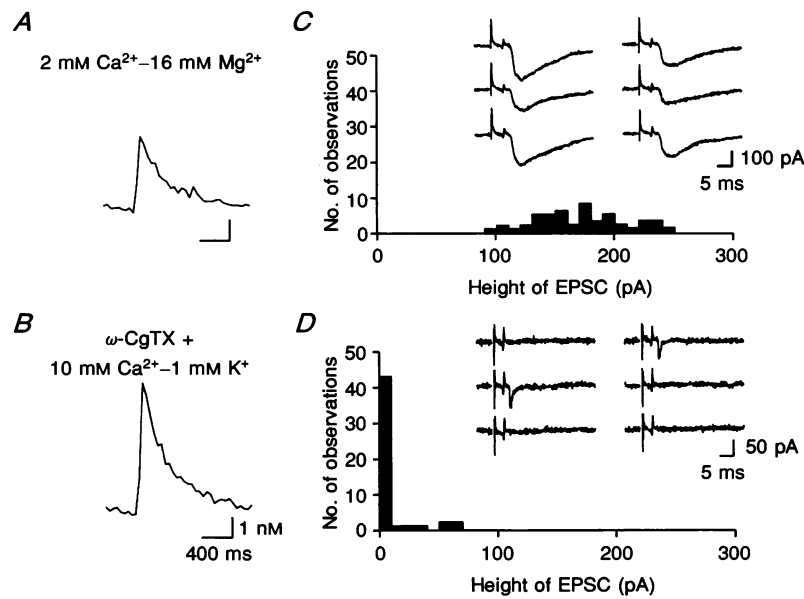


Figure 8. Comparison of the presynaptic Ca^{2+} ($[\text{Ca}^{2+}]_{\text{pre}}$) and the EPSC before and after treating with $\omega\text{-CgTX}$

A, the change of $[\text{Ca}^{2+}]_{\text{pre}}$ in response to a single presynaptic stimulus in a solution containing 2 mM Ca^{2+} and 16 mM Mg^{2+} . Average of 500 records. *B*, the same calyx as in *A* was treated with 10 μM $\omega\text{-CgTX}$ for 30 min and the $[\text{Ca}^{2+}]_{\text{pre}}$ transient was measured in a solution containing 10 mM Ca^{2+} and 1 mM K^{+} . The same calibrations for *A* and *B*. *C*, the amplitude histogram of the EPSCs in a 2 mM Ca^{2+} -16 mM Mg^{2+} solution. Inset (from left top to bottom, right top to bottom) shows consecutive records of representative EPSCs. *D*, amplitude histogram of the EPSCs in a 10 mM Ca^{2+} -1 mM K^{+} solution. The same calyx as in *C* was treated with 10 μM $\omega\text{-CgTX}$. Inset (from left top to bottom, right top to bottom) shows the consecutive records of representative EPSCs. The biphasic current following the stimulus artifact was the capacitive coupling response which indicates the invasion of presynaptic action potential into the calyx.

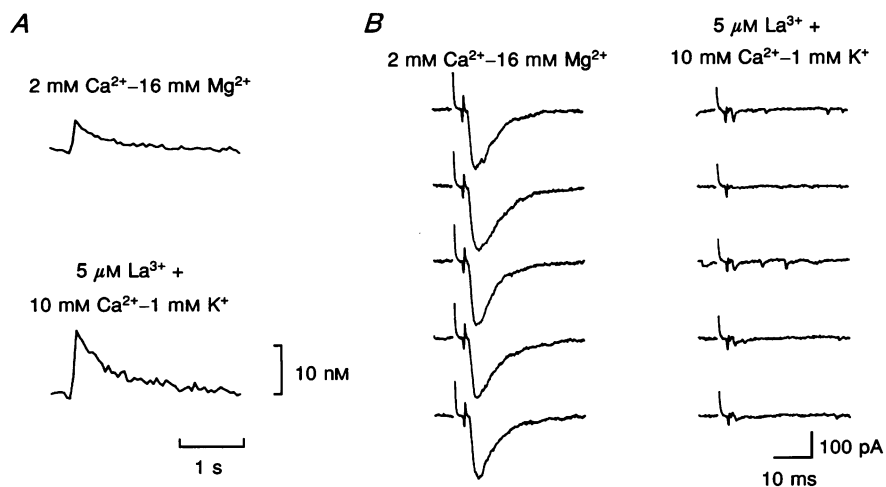


Figure 9. Effects of La^{3+} on the $[\text{Ca}^{2+}]_{\text{pre}}$ and the EPSCs

A, the change of $[\text{Ca}^{2+}]_{\text{pre}}$ in response to a single presynaptic stimulus in a solution (upper trace) and in a 10 mM Ca^{2+} -1 mM K^{+} solution containing 5 μM La^{3+} (lower trace). Both traces were obtained from the same 3 calyces and were the average of 120–200 records. *B*, the EPSCs recorded from another ciliary cell. Left, 5 consecutive records in a 2 mM Ca^{2+} -16 mM Mg^{2+} solution. Right, consecutive records of representative EPSCs in a 10 mM Ca^{2+} -1 mM K^{+} solution containing 5 μM La^{3+} . Note the increased occurrence of the miniature EPSCs.

for 30 min). No failure of synaptic transmission was observed in all seven experiments in 2 mM Ca^{2+} -16 mM Mg^{2+} solutions as exemplified in Fig. 8C. In the same preparation, the EPSCs mostly failed in a 10 mM Ca^{2+} -1 mM K^+ solution after treating with ω -CgTX (10 μM for 10 min; Fig. 8D). Similar transmission failure was observed in eight of nine ω -CgTX-treated cells. It should be noted that the capacitive coupling response is still present, despite the failure of the synaptic response (Fig. 8D, inset). The m value was 25.3 ± 23.5 ($n = 12$) in 2 mM Ca^{2+} -16 mM Mg^{2+} solutions, whereas it was 1.42 ± 1.22 ($n = 9$) in 10 mM Ca^{2+} -1 mM K^+ solutions after treatment with ω -CgTX. Therefore, the ability to release transmitter relative to the $\Delta[\text{Ca}^{2+}]_{\text{pre}}$ appears to be reduced after treating with ω -CgTX.

Similar reduction of efficacy was, however, observed when a substantial number of Ca^{2+} channels were blocked by La^{3+} which is less specific than ω -CgTX; the ω -CgTX-resistant EPSC was completely blocked by 1 μM La^{3+} even in a solution containing 20 mM Ca^{2+} , 1 mM K^+ and 0.4 mM 4-AP. As shown in Fig. 9A, the $\Delta[\text{Ca}^{2+}]_{\text{pre}}$ was usually larger in a 10 mM Ca^{2+} -1 mM K^+ solution containing 5 μM La^{3+} than in a 2 mM Ca^{2+} -16 mM Mg^{2+} solution containing no La^{3+} . In contrast, the EPSCs were much smaller and more frequently failed in a 10 mM Ca^{2+} -1 mM K^+ solution containing 5 μM La^{3+} than in a 2 mM Ca^{2+} -16 mM Mg^{2+} solution containing no La^{3+} (Fig. 9B). On average, the $\Delta[\text{Ca}^{2+}]_{\text{pre}}$ and the m value were 9.3 ± 2.8 nM ($n = 4$) and 1.85 ± 0.61 ($n = 4$), respectively, in a 10 mM Ca^{2+} -1 mM K^+ solution containing 5 μM La^{3+} . The time course of EPSC in a 10 mM Ca^{2+} -1 mM K^+ solution containing La^{3+} was as fast as the ω -CgTX-resistant EPSC in a 10 mM Ca^{2+} -1 mM K^+ solution. Although La^{3+} changes the fluorescence ratio with higher affinity than Ca^{2+} (Kwan & Putney, 1990; Haugland, 1992), no change in the fluorescence was observed when the oculomotor nerve was stimulated in a solution containing no Ca^{2+} and 5 μM La^{3+} .

DISCUSSION

Transmitter release triggered by the ω -CgTX-sensitive Ca^{2+} channel

The present study shows that the nerve-evoked release of transmitters from the ciliary calyx was largely blocked by ω -CgTX, as reported previously (Yoshikami *et al.* 1989; Stanley & Atrakchi, 1990). Low doses of ω -CgTX decreased the $[\text{Ca}^{2+}]_0$ sensitivity of the ganglionic transmission. Therefore the ω -CgTX-sensitive Ca^{2+} channel is undoubtedly the major source of Ca^{2+} in transmitter release from the ciliary calyx. There is general agreement that transmitter release at the vertebrate peripheral synapse including the mammal is triggered mainly by ω -CgTX-sensitive Ca^{2+} channels (Perney, Hirning, Leeman & Miller, 1986; Miller, 1987; Hirning *et al.* 1988; Holz, Dunlap & Kream, 1988; Lindgren & Moore, 1989; Yoshikami *et al.* 1989; Rittenhouse & Zigmond, 1991), although in some mammalian peripheral

and central synapses, a significant part of transmitter release is resistant to ω -CgTX (Sano, Enomoto & Maeno, 1987; Takahashi & Momiyama, 1993). This might be due to one or both of the following two mechanisms. First, the density of the ω -CgTX-sensitive Ca^{2+} channel subpopulation is higher than that of other subpopulations in the presynaptic terminal (Yawo & Momiyama, 1993). Second, the ω -CgTX-sensitive Ca^{2+} channels are preferentially related to the transmitter release mechanism (Miller, 1987).

In the chick ciliary ganglion, some synaptic transmission still remained in the presence of high doses of ω -CgTX. This ω -CgTX-resistant transmission was enhanced by an increase in $[\text{Ca}^{2+}]_0$ or by 4-AP, and was blocked by Cd^{2+} . The residual transmission, thus, might be evoked by the Ca^{2+} influx through the ω -CgTX- and DHP-resistant Ca^{2+} channel that co-exists with the ω -CgTX-sensitive channel in this nerve terminal (Yawo & Momiyama, 1993). This implication was supported by the observation that the ω -CgTX-resistant EPSC is more susceptible to the membrane potential of the presynaptic calyx than the EPSC in the absence of the toxin. The ω -CgTX-resistant EPSC decreased by 30 % in response to an approximately 10 mV depolarization effected by raising $[\text{K}^+]_0$. In contrast to the ω -CgTX-sensitive Ca^{2+} conductance, the ω -CgTX-resistant conductance is susceptible to steady-state inactivation at depolarized membrane potentials (Yawo & Momiyama, 1993).

Efficacy of transmitter release

The coupling of ω -CgTX-resistant Ca^{2+} influx to the transmitter release, however, appears to be much less efficient than that of ω -CgTX-sensitive Ca^{2+} influx. Although we have no information on the spatial distribution of intracellular Ca^{2+} in the presynaptic terminal, the $[\text{Ca}^{2+}]_{\text{pre}}$ which is the change of spatial average of the presynaptic $[\text{Ca}^{2+}]_i$ should be dependent on the Ca^{2+} influx into the whole nerve terminal during an action potential. The m value of ω -CgTX-resistant EPSC was small relative to the $\Delta[\text{Ca}^{2+}]_{\text{pre}}$. If the efficacy of transmitter release was tentatively described as $m/\Delta[\text{Ca}^{2+}]_{\text{pre}}$, it was 0.18 nM^{-1} for the ω -CgTX-resistant Ca^{2+} channel, whereas it was 10 nM^{-1} before treating with ω -CgTX. Thus, the efficacy of transmitter release of the ω -CgTX-sensitive Ca^{2+} channel was more than 50 times higher than that of the ω -CgTX-resistant Ca^{2+} channel. The ineffectiveness of the ω -CgTX-resistant Ca^{2+} influx for triggering transmitter release could not, however, be attributable to the molecular differences in Ca^{2+} channel subtypes. Similar ineffectiveness of triggering transmitter release was observed when the number of functional Ca^{2+} channels of the ω -CgTX-sensitive subpopulation was reduced by La^{3+} .

The ω -CgTX-sensitive Ca^{2+} channels are presumed to distribute in a cluster at the active zone as shown in the frog motor nerve terminal (Robitaille, Adler & Charlton, 1990; Cohen, Jones & Angelides, 1991). This should be also true for the chick ciliary calyx; the 14 pS Ca^{2+} channels

were distributed in a cluster on the synaptic face of the presynaptic terminal (Stanley, 1991). When [Ca²⁺]_o was lowered and [Mg²⁺]_o was raised, the Ca²⁺ influx through a single Ca²⁺ channel should be small, but the elevation of [Ca²⁺]_i would range throughout the active zone. On the other hand, when [Ca²⁺]_o was raised in the presence of La³⁺, the Ca²⁺ influx through the unblocked Ca²⁺ channel should be high. However, the elevation of [Ca²⁺]_i would be restricted to the very near vicinity of the inner mouth of the unblocked channel and the [Ca²⁺]_i level of the other part of the active zone would remain low, because the diffusion of Ca²⁺ should be delimited around the mouth of the Ca²⁺ channel (Smith & Augustine, 1988). If the molecule responsible for the final step of transmitter release has a low affinity for Ca²⁺, the Ca²⁺ influx through a single Ca²⁺ channel could only trigger transmitter release in the close vicinity of the channel even if the number of Ca²⁺ ions is large. Therefore, the synchronized elevation of [Ca²⁺]_i throughout the active zone may be important for the efficient release of transmitters (Smith & Augustine, 1988).

Unlike the ω -CgTX-sensitive Ca²⁺ channels, the ω -CgTX-resistant Ca²⁺ channels do not appear to form a cluster in the active zone as considered from the ineffectiveness of transmitter release after treating with ω -CgTX. When the Ca²⁺ influx through the ω -CgTX-resistant Ca²⁺ channel was increased in a high-Ca²⁺ solution containing 4-AP, the probability of transmitter release could be increased in a region remote from the site of Ca²⁺ influx. This may explain the unsynchronous EPSCs frequently observed after treating with ω -CgTX (Yawo & Chuhma, 1992).

It has been suggested that La³⁺ could enter the nerve terminal and modify transmitter release (Curtis, Quastel & Saint, 1986). Although we did not detect any change of fluorescence in a solution containing 5 μ M La³⁺ and no Ca²⁺, the frequency of miniature EPSCs appeared to increase in a 10 mM Ca²⁺-1 mM K⁺ solution containing 5 μ M La³⁺. Therefore, it remains possible that La³⁺ may modify the nerve-evoked release of transmitter and reduce the efficacy of transmitter release.

Functional implication of ω -CTX-resistant Ca²⁺ channels

Although the contribution of the ω -CgTX-resistant Ca²⁺ influx to triggering transmitter release was small, it would increase cytoplasmic Ca²⁺ concentration in concert with the ω -CgTX-sensitive Ca²⁺ influx. It is not known if the ω -CgTX-resistant Ca²⁺ influx has some specific function in the nerve terminal. The ω -CgTX-resistant conductance is susceptible to steady-state inactivation at depolarized membrane potentials (Yawo & Momiyama, 1993). During repetitive stimulation of the nerve terminal, the membrane potential could be hyperpolarized by the activation of Ca²⁺-activated K⁺ conductance (Bennett & Ho, 1992). The hyperpolarization may remove the inactivation of ω -CgTX-resistant Ca²⁺ conductance, then further increase Ca²⁺ influx during action potentials.

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