μ-Opioid receptor inhibits N-type Ca^{2+} channels in the calyx presynaptic terminal of the embryonic chick ciliary ganglion

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1. A study was made on the mechanisms by which enkephalins inhibit synaptic transmission at calyx-type presynaptic terminals in the ciliary ganglion of chick embryos at stages 39–40.

2. Excitatory postsynaptic currents (EPSCs) were recorded by nystatin-perforated patch clamp at low $[Ca^{2+}]_o$ and high $[Mg^{2+}]_o$. [Leu^5]enkephalin (L-ENK, 1–10 μM) reduced the quantal content (m) without changing the quantal size (q). This effect was antagonized by naloxone (1 μM). Similar results were observed under conventional whole-cell clamp of the postsynaptic neuron.

3. A specific agonist of the μ-opioid receptor, [D-Ala^2,M-Me-Phe^4,Gly^5]enkephalin-ol (DAMGO) reduced m without changing q. A specific agonist of the δ-opioid receptor, [D-Pen^2,D-Pen^5]-enkephalin (DPDPE) also reduced m without changing q.

4. Both L-ENK and [Met^5]enkephalin (M-ENK) reduced the stimulus-dependent increment of the intraterminal Ca^{2+} concentration ($\Delta[Ca^{2+}]_t$) without affecting the decay time constant of the intraterminal Ca^{2+} concentration and basal Ca^{2+} level. This effect was antagonized by naloxone. DAMGO reduced $\Delta[Ca^{2+}]_t$ more effectively than DPDPE.

5. When extracellular Ca^{2+} was replaced by Ba^{2+}, the stimulus-dependent increment of the intraterminal Ba^{2+} concentration ($\Delta[Ba^{2+}]_t$) was also reduced by L-ENK or DAMGO.

6. L-ENK reduced $\Delta[Ca^{2+}]_t$ even in the presence of 4-aminopyridine (4-AP), which blocks the transient K^+ conductance during the falling phase of the presynaptic action potential. When N-type Ca^{2+} channels were blocked by ω-conotoxin GVIA (ω-$CgTx_{GVIA}$), the $\Delta[Ca^{2+}]_t$ was no longer sensitive to L-ENK and DAMGO.

7. It is suggested that enkephalins reduce the transmitter release through presynaptic opioid receptors. The μ-opioid receptor may suppress presynaptic Ca^{2+} influx by selectively inhibiting N-type Ca^{2+} channels.

Opioid peptides and their receptors are widely distributed throughout the central and peripheral nervous systems (Dun, 1983; Hökfelt *et al.* 1984; Nicoll *et al.* 1990). In autonomic ganglia, morphine and opioid peptides suppress the cholinergic transmission by either pre- or postsynaptic mechanisms (Konishi *et al.* 1979; Cherbini & North, 1985; Margiotta & Berg, 1986; Araujo & Collier, 1987). In the avian ciliary ganglion, opioid peptides have been shown to be present in the cholinergic presynaptic terminal as co-transmitters (Erichsen *et al.* 1982*a*). The ganglion consists of two distinct populations of neurons: small choroidal cells which have bouton-type synaptic terminals and large ciliary cells which receive calyx-type presynaptic terminals (Marwitt *et al.* 1971). [Leu^5]enkephalin (L-ENK)-like immunoreactivity has been observed in both populations of presynaptic terminals and in the midbrain preganglionic neurons of the Edinger-Westphal nucleus which send their axons to the ciliary ganglion (Erichsen *et al.* 1982*a*,*b*; Reiner, 1987). Both [Met^5]enkephalin (M-ENK) and L-ENK have also been extracted from the ciliary ganglion (White *et al.* 1985). During embryonic stages 35–40 (Hamburger & Hamilton, 1951) when normal programmed neuronal death occurs in the chick ciliary ganglion (Landmesser & Pilar, 1974), the ganglionic content of M-ENK reaches its maximum (White *et al.* 1985). The presence of L-ENK immunoreactivity is also highest during the same developmental stages in bouton- and calyx-type presynaptic terminals in the chick ciliary ganglion as well as in the neurons of the Edinger-Westphal nucleus (Meriney *et al.* 1991).

Neuronal death in the ciliary ganglion of the chick embryo is retarded by exogenous administration of morphine

(Meriney *et al.* 1985) and is enhanced by naltrexone, an opioid receptor antagonist (Meriney *et al.* 1991). Enkephalins are thus assumed to be endogenous modulators of synaptic transmission in this system (Meriney *et al.* 1991; Chiappinelli *et al.* 1993). In this study, we investigated the possibility that enkephalins inhibit the release of transmitter through presynaptic opioid receptors. We show that μ-opioid receptors are present in the calyx-type presynaptic terminal in stage 39–40 chick embryos and that enkephalins reduce the quantal transmitter release. In addition, we show that the μ-opioid receptor preferentially suppresses Ca^{2+} influx through N-type Ca^{2+} channels. A preliminary report of these results has appeared elsewhere (Yawo *et al.* 1994).

METHODS

Preparation

Day 14 chick embryos (stages 39–40; Hamburger & Hamilton, 1951) were decapitated and the ciliary ganglion was removed with the oculomotor nerve. The details of the procedures have been described previously (Yawo & Chuhma, 1994; Yawo, 1999*a*). Briefly, the collagenous envelope of the ciliary ganglion was enzymatically removed by focal application of a mixture of 2000 U ml^{-1} collagenase (Type II, Sigma-Aldrich) and 100 U ml^{-1} thermolysin (Sigma-Aldrich) through a pipette with a 40–50 μm tip diameter for 30 min at room temperature (Yawo, 1999*a*). The presynaptic oculomotor nerve was attached to a stimulating glass pipette by suction. The postsynaptic ciliary cell was identified by its location and size (Gray *et al.* 1990). The ganglion was superfused with oxygenated standard saline containing (mM): NaCl, 135; KOH, 5; $CaCl_2$, 5; $MgCl_2$, 1; Hepes, 10; mannitol, ; and glucose, 11 (pH 7·4 adjusted with HCl). All experiments were carried out at room temperature (25 °C).

Recordings and analyses of the excitatory postsynaptic current (EPSC)

A conventional whole-cell patch-clamp recording (Hamill *et al.* 1981) was made from a postsynaptic ciliary neuron (Yawo & Chuhma, 1994) using an EPC-7 patch-clamp amplifier (List Electronic, Darmstadt-Eberstadt, Germany). Patch pipettes were made from thin-walled borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) and coated with silicon resin (Silpot 184W/C, Dow Corning, Midland, MI, USA). The pipettes were fire polished, reducing the tip diameter to 1–2 μm. The pipettes had a resistance of 4–6 MΩ when filled with internal solution containing (mM): CsCl, 130; CsOH, 10; Na_2EGTA, 10; Hepes, 20; and MgATP, 5 (pH 7·4 adjusted with HCl). The series resistance was usually less than 10 MΩ throughout the experiment.

To prevent the effects of intracellular dialysis under whole-cell recording conditions, we measured EPSCs by the perforated patch method using a nystatin–fluorescein mixture (Yawo & Chuhma, 1993*b*). Briefly, a stock solution was made by dissolving 5 mg nystatin (Sigma-Aldrich) and 20 mg fluorescein sodium (Uranine, Nacalai Tesque) in 1 ml methanol. Alternatively, instead of using fluorescein, 0·1 M *N*-methyl-D-glucamine (Sigma-Aldrich) was first dissolved in methanol, the pH was adjusted by methanesulphonic acid in the presence of 0·01 M phenol red (Sigma-Aldrich), and then the nystatin (5 mg ml^{-1}) was added. Immediately before use, 50 μl of the stock solution was placed in a polyethylene test tube and dried completely with a stream of N_2 gas. The tube was then filled with 1 ml of pipette solution containing (mM): KCl, 20; K_2SO_4, 60; sucrose, 60; $MgSO_4$, 1; and Hepes, 10 (pH 7·4, adjusted with NaOH), and was briefly vortexed. Thus, the pipette solution contained 250 μg ml^{-1} of nystatin and 2·7 mM fluorescein or 5 mM *N*-methyl-D-glucamine. It can be expected that bipolar molecules such as fluorescein and *N*-methyl-D-glucamine will facilitate the dispersion of nystatin in an aqueous solution. In fact, the pipette solution was filtered through a 0·22 μm syringe filter (SLGVL040S, Millipore Japan Co., Osaka, Japan) without additional pressure on the syringe. Nystatin was re-precipitated in 1 h in the fluorescein-containing solution but not in the *N*-methyl-D-glucamine-containing solution. The tip of the patch electrode was filled with filtered solution first, then backfilled with the same solution. Positive pressure was applied to the pipette as in a conventional patch-clamp technique before and while approaching the cell membrane. A tight seal was immediately formed upon switching to a slight negative pressure. The access resistance decreased rapidly to less than 40 MΩ in 5–10 min.

In both the whole-cell and perforated patch recordings, the capacitative transient was minimized by compensating the input capacitance, then the series conductance was compensated by 50–70 %. The whole-cell currents were low pass-filtered at 3 kHz (−3 dB, eight-pole Bessel filter, P-84P; NF Electronic Instruments, Yokohama, Japan), digitized at 10–20 kHz (ADX-98E; Canopus, Kobe, Japan), and stored in a computer (PC9801Vm21; NEC, Tokyo, Japan).

The quantal content (m) was estimated from the coefficient of variation (c.v.) of EPSCs measured in a low [Ca^{2+}] (0·8–1 mM) and high [Mg^{2+}] (5 mM) solution (Kuno & Weakly, 1972) since the EPSC fluctuation approximately followed Poisson statistics under these conditions (Martin & Pilar, 1964; Yawo & Chuhma, 1994). Because of the infrequent occurrence of miniature EPSCs, the quantal size (q) was estimated as the mean EPSC divided by m.

Measurement of the intraterminal Ca^{2+} and Ba^{2+} concentrations

The method of measuring the intraterminal Ca^{2+} concentration ($[Ca^{2+}]_t$) was almost the same as that described previously (Yawo & Chuhma, 1994). The oculomotor nerve was cut at its point of exit from the orbital bone in Ca^{2+}-free saline containing 1 mM EGTA. Crystals of fura-2-conjugated dextran (fura–dextran, MW 10 000, Molecular Probes Inc.) were applied to the cut end of the distal stump as described previously (Yawo, 1999*a*). After 30 min of incubation at 10 °C, the ganglion was superfused with the oxygenated standard saline and further incubated at 36 °C for 1·5 h. Fura–dextran was anterogradely transported (Glover *et al.* 1986) and was confined to the presynaptic axons and their terminals. A whole ganglion was mounted in a chamber, the oculomotor nerve was attached by suction to the stimulating glass pipette, and the collagenous envelope was enzymatically removed by focally applying a collagenase–thermolysin mixture (see above; Yawo, 1999*a*). A conventional epifluorescence system equipped with a water-immersion objective (×40, NA 0·7, Olympus, Tokyo, Japan) and xenon lamp (150 W) was focused on calyx-type presynaptic terminals at the surface of the ciliary ganglion. Fluorescence was excited alternately at wavelengths of 340 and 380 nm through the minimum iris orifice (diameter, 100 μm). Because there is a window (diameter, 50 μm) in front of the photomultiplier tube (OSP-3, Olympus), the fluorescence from one to three terminals was measured simultaneously. The intracellular Ca^{2+} concentration was calculated from the ratio of fluorescence intensities at wavelengths of 340 and 380 nm (Grynkiewicz *et al.* 1985) using the dissociation constant (K_D) determined by the manufacturer (350 nM) and the system-dependent parameters

determined by the conventional *in situ* calibration method. The signal was integrated for 50 ms and sampled at 20 Hz by a computer (PC-9801RS, NEC) with software for measuring intracellular Ca^{2+} concentration (MiCa, provided by Drs K. Furuya & K. Enomoto, National Institute of Physiological Science, Japan). Twelve records were averaged, using the computer-generated stimulating pulse as a trigger for summation.

The intraterminal Ba^{2+} concentration ($[Ba^{2+}]_t$) was also measured as the 340 nm/380 nm ratio of fura–dextran fluorescence when extracellular Ca^{2+} was replaced with Ba^{2+} in the presence of 1 mM EGTA. The method of calibration was similar to that for Sr^{2+} (Yawo, 1999*b*), although the intracellular $[Ca^{2+}]$ was ignored. We did not directly measure the K_D of fura–dextran for Ba^{2+}; instead, the K_D of fura-2 for Ba^{2+}, 1360 nM (Kwan & Putney, 1990), was adopted. To calibrate the Ba^{2+} concentration, the minimum fluorescence ratio and the maximum fluorescence at 380 nm were measured in a divalent cation-free solution containing 5 mM BAPTA and 0·1 mM ionomycin. Thereafter the solution was changed to that containing 10 mM $BaCl_2$, and the maximum fluorescence ratio and the minimum fluorescence at 380 nm were measured.

Reagents

Pharmacological agents were usually applied in a superfusing solution over the ganglion, and were bath applied through a perfusing line. The solution in the chamber (~1 ml) was completely replaced in less than 2 min. Agents used in this study and their sources are as follows: [Met^5]enkephalin (M-ENK, Sigma-Aldrich); [Leu^5]enkephalin (L-ENK, Peptide Institute Inc., Minoh, Japan); [D-Pen^2,D-Pen^5]enkephalin (DPDPE, Peninsula Laboratory Inc., Belmont, CA, USA); [D-Ala^2,M-Me-Phe^4,Gly^5]enkephalin-ol (DAMGO, Penisula Laboratory Inc.); and naloxone (Sigma-Aldrich). ω-Conotoxin GVIA (ω-$CgTx_{GVIA}$, Peptide Institute Inc.) was prepared at a concentration of 1 mM in a solution of 100 mg ml^{-1} bovine serum albumin (Sigma-Aldrich). The stock solution of ω-$CgTx_{GVIA}$ was added directly to the bath while perfusion was halted. In these experiments, a flow of O_2 was directly applied over the bathing fluid.

All the above experiments were carried out in accordance with the guiding principles of the Physiological Society of Japan. The values in the text and figures are means ± S.E.M. (n = number of experiments). Statistically significant differences between various parameters were determined using Student's two-tailed t test for paired data. When the variability of raw data was large, normalized data were compared with unity using Student's two-tailed t test for one variable. Otherwise, the Mann-Whitney U test was used. $P < 0·05$ was considered significant.

RESULTS

Synaptic depression by enkephalins

When the nystatin-containing patch pipettes were sealed onto the ciliary cell membrane, the access resistances gradually decreased and reached a minimum (19–26 MΩ, $n = 4$) in 5–10 min. When the patch pipettes contained K^+ intracellular solution, the resting potential was in the range −57 to −61 mV ($n = 4$). EPSCs were recorded from the ciliary cell by clamping at −60 mV. Figure 1*A* shows the mean EPSCs in response to stimulation pulses applied to the presynaptic oculomotor nerve. Although the amplitude of the EPSCs fluctuated between 8 and 116 pA at 1 mM $[Ca^{2+}]_o$ and 5 mM $[Mg^{2+}]_o$, the stimulation artifact was faithfully followed by the capacitative coupling response and the EPSC before exposure to L-ENK (Fig. 1*A* and *B*). In the presence of 10 μM L-ENK, which inhibits the cholinergic synaptic transmission in autonomic ganglia (Konishi *et al.* 1979; Margiotta & Berg, 1986), the EPSC amplitude of the chick ciliary cell synapse was, on average, reduced (Fig. 1*A*), and synaptic failure was occasionally observed (Fig. 1*B*). Figure 1*C* and *D* shows the amplitude distribution of the first EPSCs before and during the application of 10 μM L-ENK, respectively. Based on Poisson statistics (Martin & Pilar, 1964; Yawo & Chuhma, 1994), m and q were calculated from the c.v. In the control experiment of Fig. 1*C*, m and q were 4·2 and 12·5 pA, respectively. In the presence of L-ENK (Fig. 1*D*), m and q were 2·5 and 10·1 pA, respectively. The expected occurrence of failure was 4 in 50 trials, which was very close to the observed occurrence (Fig. 1*D*). In all four similar experiments, L-ENK consistently decreased m to a mean of 45 ± 12% of control without changing q (111 ± 15% of control), and the difference was significant ($P < 0·05$, paired t test).

As shown in Fig. 1*B*, the inhibitory effect of L-ENK was reversed by the opioid receptor antagonist naloxone. When naloxone (1–10 μM) was added to 10 μM L-ENK, m was increased to 120 ± 9% of the control before the application of L-ENK ($n = 4$), and was significantly larger than in the presence of L-ENK alone ($P < 0·05$, Mann-Whitney U test). In another series of experiments, naloxone (1–10 μM) was applied first, and the effect of subsequent application of L-ENK (10 μM) was investigated; the mean EPSC amplitude in the presence of both naloxone and L-ENK was 91–110% that in the presence of naloxone alone ($n = 3$). These results suggest that L-ENK reduced quantal transmitter release through opioid receptors in the presynaptic terminal. Although opioid receptors are also distributed in the postsynaptic membrane and reduced both an inward Ca^{2+} current and transient and sustained K^+ currents (Polo-Parada & Pilar, 1999), the postsynaptic opioid receptors appear not to affect the synaptic transmission (see also Margiotta & Berg, 1986).

Similarly, the mean EPSC was again reversibly reduced by 10 μM L-ENK when the EPSC was measured under conventional whole-cell clamp of ciliary cells. At low $[Ca^{2+}]_o$ and high $[Mg^{2+}]_o$, the reduction was again accompanied by the increased occurrence of failure responses. For nine experiments, L-ENK (10 μM) reduced m to a mean of 42 ± 6% of control (Fig. 2*A*), i.e. to the same extent as in the nystatin-perforated patch recordings ($P > 0·4$, Mann-Whitney U test). On the other hand, q was unaffected by L-ENK (125 ± 9% of control, Fig. 2*B*), being significantly different from m ($P < 0·001$, paired t test).

In another series of experiments the effects of 1 μM L-ENK were investigated. In a summary of data from six whole-cell experiments, 1 μM L-ENK reduced m to 61 ± 5% of control (Fig. 2*A*) with a negligible change in q (99 ± 0·07% of control, Fig. 2*B*); the difference was significant ($P < 0·05$,

paired t test). The effect of 1 μM L-ENK was significantly less than that of 10 μM L-ENK (Fig. 2*A*, $P < 0{\cdot}05$, Mann-Whitney U test), indicating that the effect was dose dependent. We did not test whether L-ENK was effective at lower concentrations; instead, 1–100 μM was used in the following study for convenience.

Opioid receptor subtypes

Among opioid receptor subtypes, the μ- and δ-opioid receptors are activated by L-ENK (Reisine, 1995). To determine which receptor subtype was involved in the L-ENK-dependent reduction of transmitter release, the effects of subtype-specific agonists were investigated. DAMGO is expected to be selective for μ-opioid receptors at

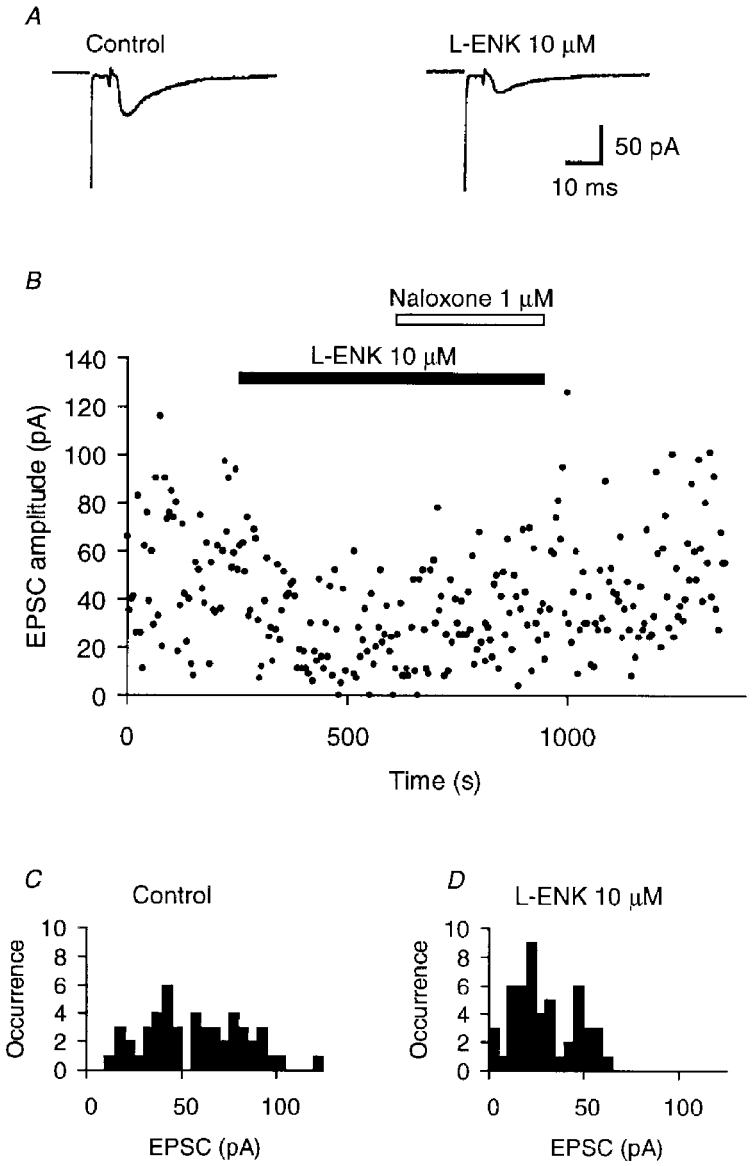

Figure 1. Inhibition of quantal transmitter release by enkephalins

A, the effect of 10 μM [Leu^5]enkephalin (L-ENK) on the mean EPSC; representative experimental data measured by the perforated-patch method using a nystatin–fluorescein mixture. The presynaptic oculomotor nerve was electrically stimulated at 0·2 Hz. The biphasic current between the stimulus artifact and the EPSC is the capacitative coupling response indicating the invasion of the action potential into the presynaptic terminal. Left, mean EPSC of 50 consecutive records in control conditions. Right, mean EPSC of 50 consecutive records during bath application of L-ENK. *B*, time-dependent plots of EPSC amplitude for the experiment shown in *A*. L-ENK was bath applied during the indicated period (filled bar). Naloxone was added to L-ENK as indicated (open bar). *C*, EPSC amplitude histogram for 50 consecutive records before the application of L-ENK, the mean of which is shown in *A* (left). The quantal content (m) and the quantal size (q) were estimated from the c.v., and were 4·2 and 12·5 pA, respectively. *D*, EPSC amplitude histogram of 50 consecutive records during L-ENK-induced depression, the mean of which is shown in *A* (right). The values of m and q were 2·5 and 10·1 pA, respectively.

1 μM, and DPDPE is expected to be selective for δ-opioid receptors at 1 μM (Reisine, 1995). Figure 2 summarizes the effects of 1 μM DAMGO and those of 1 μM DPDPE on m (Fig. 2*A*) and q (Fig. 2*B*). DAMGO reduced m to 51 ± 9% of control without changing q (104 ± 9% of control), the difference being significant ($n = 8$, $P < 0{\cdot}05$, paired t test). DPDPE reduced m to 70 ± 4% of control without changing q (122 ± 8% of control); the difference was significant ($n = 9$, $P < 0{\cdot}01$, paired t test). Therefore, the L-ENK-dependent reduction of m involves both μ- and δ-opioid receptors.

Effects of opioid peptides on $[Ca^{2+}]_t$

The $[Ca^{2+}]_t$ in a calyx-type nerve terminal was monitored by the 340 nm/380 nm ratio of fura–dextran fluorescence. The basal $[Ca^{2+}]_t$ was 40 ± 11 nM ($n = 12$) in 5 mM $[Ca^{2+}]_o$ solution, and a single nerve stimulus increased $[Ca^{2+}]_t$ by 15 ± 4 nM ($n = 12$). As shown in Fig. 3*A*, L-ENK (10 μM) reduced the stimulus-dependent peak increment of $[Ca^{2+}]_t$ ($\Delta[Ca^{2+}]_t$) by 33%. The effects of enkephalins on $\Delta[Ca^{2+}]_t$ are summarized in Fig. 3*B*. M-ENK reduced $\Delta[Ca^{2+}]_t$ to the same extent as L-ENK. The magnitude of the reduction was dependent on the dose of L-ENK although a significant $\Delta[Ca^{2+}]_t$ remained in the presence of L-ENK at concentrations as high as 100 μM.

The opioid receptor antagonist naloxone (0·1 μM) completely reversed the depressing effect of L-ENK on $\Delta[Ca^{2+}]_t$ (Fig. 3*A*). When 10 μM L-ENK was applied in the presence of 0·1 μM naloxone, the reduction of $\Delta[Ca^{2+}]_t$ was not observed (Fig. 3*B*). Therefore, naloxone-sensitive opioid receptor subtypes should be involved in the reduction of $\Delta[Ca^{2+}]_t$. To determine which opioid receptor subtype was involved, the effects of a δ-opioid receptor-selective agonist, DPDPE (0·1–1 μM), and a μ-opioid receptor-selective agonist, DAMGO (0·1–1 μM), were compared. As shown in Fig. 4*A*, the $\Delta[Ca^{2+}]_t$ was little affected by 0·1 μM DPDPE whereas it was reduced by the subsequent application of the same concentration of DAMGO. In summary, DAMGO attenuated the $\Delta[Ca^{2+}]_t$ at both 0·1 and 1 μM (Fig. 4*B*). Although the effect of 0·1 μM DPDPE was negligible, DPDPE significantly reduced the $\Delta[Ca^{2+}]_t$ at 1 μM (Fig. 4*B*,

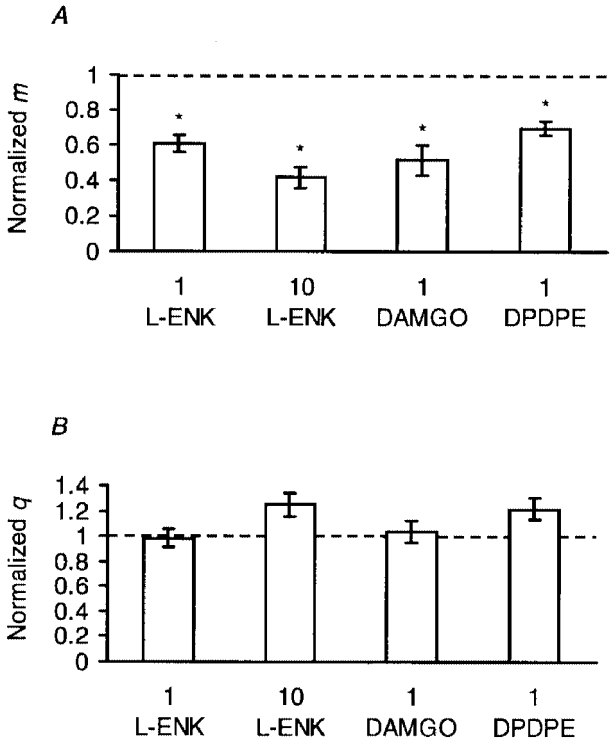

Figure 2. Summary of the effects of opioid peptides on the quantal transmitter release

A, effects of opioid peptides on the quantal content (m). In each experiment, the EPSC amplitudes were recorded in an extracellular solution containing low $[Ca^{2+}]$ and high $[Mg^{2+}]$, and m was calculated from the c.v. of the EPSC amplitude fluctuation based on Poisson statistics. Each column and bar are the mean ± S.E.M. of m normalized to the control value before the application of enkephalins. From left to right: 1 μM L-ENK ($n = 6$); 10 μM L-ENK ($n = 9$); 1 μM DAMGO, a μ-opioid receptor-selective agonist ($n = 8$); and 1 μM DPDPE, a δ-opioid receptor-selective agonist ($n = 8$). Asterisks indicate that the effect was statistically significant ($P < 0{\cdot}05$, two-tailed t test between raw data). *B*, effects of opioid peptides on the quantal size (q). q was calculated as the mean EPSC amplitude divided by m. Each column and bar are the mean ± S.E.M. of q normalized to the control value before the application of enkephalins. From left to right: 1 μM L-ENK ($n = 6$), 10 μM L-ENK ($n = 9$), 1 μM DAMGO ($n = 8$), and 1 μM DPDPE ($n = 8$). In each case the effect was not statistically significant ($P > 0{\cdot}05$, two-tailed t test between raw data).

$P < 0{\cdot}01$, two-tailed t test between raw data). Therefore, both μ- and δ-opioid receptors appear to be involved in the enkephalin-dependent suppression of $\Delta[Ca^{2+}]_t$.

Reduction of Ca^{2+} influx by enkephalins

Upon the invasion of the action potential, a focal increase of intracellular Ca^{2+} was observed in the presynaptic terminal (Zucker, 1996; Neher, 1998). This is explained by the clustering of Ca^{2+} channels around active zones (Robitaille *et al.* 1990; Cohen *et al.* 1991; Yawo & Chuhma, 1994) and the slow diffusion of intracellular Ca^{2+} (Zucker, 1996; Neher, 1998). Although $[Ca^{2+}]_t$ transients should be much larger and faster in the vicinity of Ca^{2+} channel clusters (Zucker, 1996; Neher, 1998), the activity-dependent increment of the volume-averaged fura-2 signal would be proportional to the changes in the local concentration (Sinha *et al.* 1997; Yawo, 1999*a*).

Following a single nerve stimulus, the $[Ca^{2+}]_t$ increased with a short delay, peaked within 50–150 ms and decayed slowly (Yawo & Chuhma, 1993*a*). The falling phase of $\Delta[Ca^{2+}]_t$ did not follow a simple exponential function. For convenience, the falling phase between 90 and 10% of the peak $[Ca^{2+}]_t$ was fitted to the sum of a single exponential function and constant (Fig. 5*A*). That is,

$$[Ca^{2+}]_t = \alpha \exp(-t/\tau) + \beta, \quad (1)$$

where α is the asymptote of the peak $\Delta[Ca^{2+}]_t$ and β is the basal $[Ca^{2+}]_t$. At 5 mM $[Ca^{2+}]_o$, the falling phase time constant, τ, was 417 ± 20 ms ($n = 9$). If enkephalins accelerate intracellular Ca^{2+} sequestration, a reduction of τ would be expected. However, as shown in Fig. 5*B*, the change of τ was negligible with either 10 μM L-ENK (106 ± 7% of control, $n = 5$) or 10 μM M-ENK (99 ± 4% of

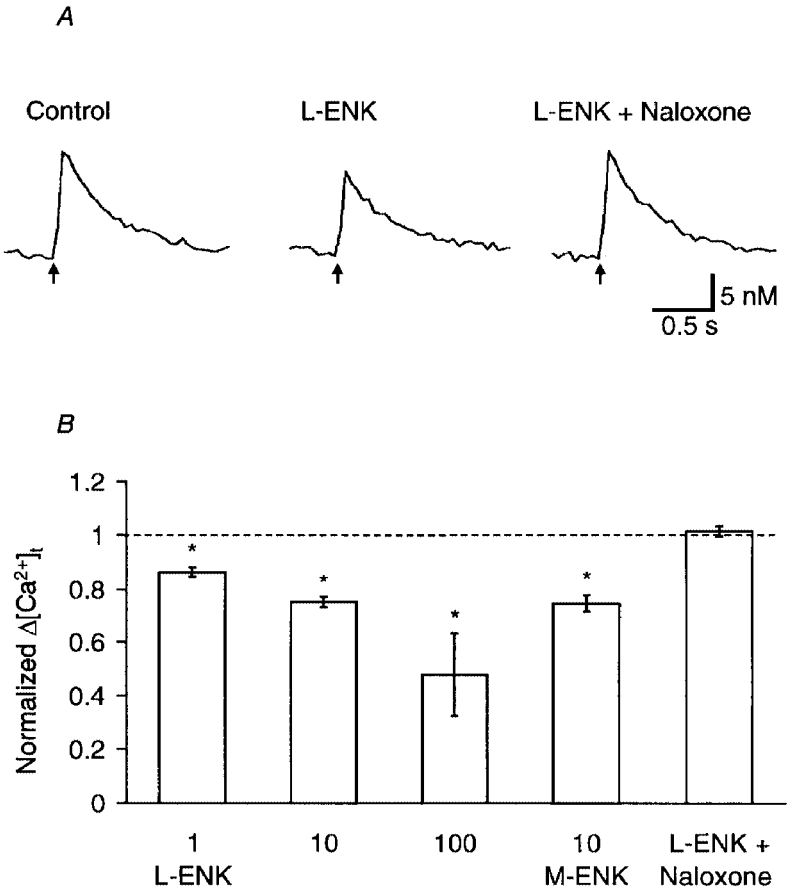

Figure 3. Effects of enkephalins on the intraterminal Ca^{2+} transient

A, sample records of the intraterminal Ca^{2+} ($[Ca^{2+}]_t$) transient in response to electrical stimulation of the oculomotor nerve at 0·5 Hz (indicated by arrows). From left to right: mean control response before the application of L-ENK, response in the presence of 10 μM L-ENK, and response in the presence of both 10 μM L-ENK and 0·1 μM naloxone. In each case, 12 records were averaged using the computer-generated stimulating pulse as a trigger for summation. *B*, summary of the effects of enkephalins on the stimulus-dependent increment of $[Ca^{2+}]_t$ ($\Delta[Ca^{2+}]_t$). Each column and bar are the mean ± S.E.M. of $\Delta[Ca^{2+}]_t$ normalized to the control value before the application of enkephalins. From left to right: 1 μM L-ENK ($n = 5$), 10 μM L-ENK ($n = 6$), 100 μM L-ENK ($n = 3$), 10 μM [Met^5]ENK (M-ENK, $n = 5$), and 10 μM L-ENK plus 0·1 μM naloxone ($n = 5$). Asterisks indicate that the effect was statistically significant ($P < 0{\cdot}05$, two-tailed t test between raw data). The difference between the 10 μM L-ENK and 10 μM L-ENK + 0·1 μM naloxone columns is also significant ($P < 0{\cdot}01$, Mann-Whitney U test).

control, $n = 4$), the effect being insignificant ($P > 0{\cdot}5$, paired t test between raw data). The basal $[Ca^{2+}]_t$, calculated as β of eqn (1), was also unaffected by the enkephalins (Fig. 5*C*).

To investigate whether enkephalins reduce Ca^{2+} influx through Ca^{2+} channels, we measured the change in $[Ba^{2+}]_t$ instead of $[Ca^{2+}]_t$. When extracellular Ca^{2+} was replaced with Ba^{2+} in the presence of 1 mM EGTA, nerve stimulation increased the 340 nm/380 nm ratio of fura–dextran fluorescence, indicating an increase of $[Ba^{2+}]_t$. The rate of rise of $[Ba^{2+}]_t$ was as fast as that of $[Ca^{2+}]_t$, whereas the decline of the action potential-dependent increase of $[Ba^{2+}]_t$ to the basal level occurred much more slowly than that of $\Delta[Ca^{2+}]_t$ (Fig. 6) with a time constant of 20–75 s ($n = 4$; Yawo & Chuhma, 1993*a*). With a stimulation interval of 120 s, the peak increment of $[Ba^{2+}]_t$ ($\Delta[Ba^{2+}]_t$) was 0·7–1·9 μM ($n = 5$), but this interval was not enough to allow full recovery to the baseline fluorescence ratio. The large $\Delta[Ba^{2+}]_t$ and the slow decline of $\Delta[Ba^{2+}]_t$ indicate that intracellular Ba^{2+} is buffered more weakly and more slowly than Ca^{2+} (Schilling *et al.* 1989; Kwan & Putney, 1990; Yawo & Chuhma, 1993*a*). Therefore, we considered that the change in $\Delta[Ba^{2+}]_t$ should be more dependent on the change of Ba^{2+} influx through Ca^{2+} channels than on that of divalent cation buffering. As shown in Fig. 6, L-ENK (10 μM) reduced the $\Delta[Ba^{2+}]_t$ to 81 ± 4% of control

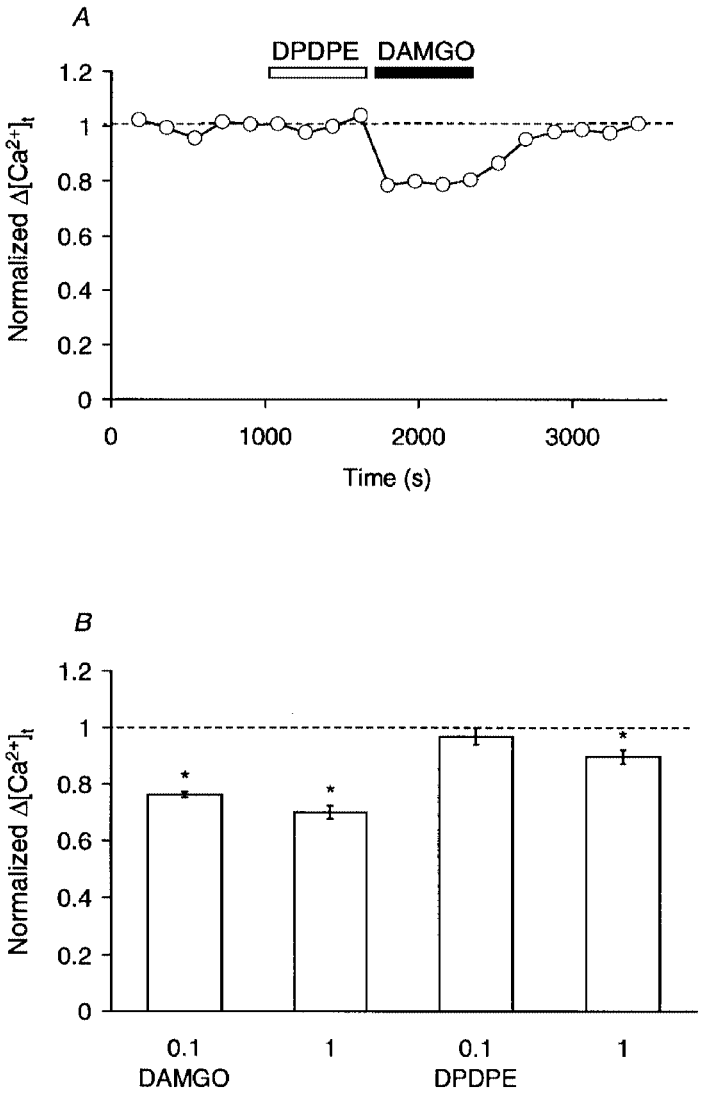

Figure 4. Reduction of $\Delta[Ca^{2+}]_t$ by μ-opioid receptor activation

A, time-dependent plots for a representative experiment comparing the effects of DPDPE and DAMGO. The $\Delta[Ca^{2+}]_t$ was normalized to the mean control value before the application of DPDPE, and was plotted against time. DPDPE (0·1 μM, open bar) and DAMGO (0·1 μM, filled bar) were bath applied during the indicated periods. *B*, summary of the effects of DPDPE and DAMGO on the $\Delta[Ca^{2+}]_t$. Each column and bar are the mean ± S.E.M. of $\Delta[Ca^{2+}]_t$ normalized to the control value before the application of the agonists. From left to right: 0·1 μM DAMGO ($n = 4$), 1 μM DAMGO ($n = 4$), 0·1 μM DPDPE ($n = 6$) and 1 μM DPDPE ($n = 5$). Asterisks indicate that the effect was statistically significant ($P < 0{\cdot}01$, two-tailed t test between raw data).

($P < 0{\cdot}05$, single value t test). The effect of L-ENK was reversible within 10 min and the subsequent application of DAMGO (1 μM) again reduced the $\Delta[Ba^{2+}]_t$ (Fig. 6). When the declining phase of $[Ba^{2+}]_t$ was fitted to eqn (1), τ was 37 ± 7 s in control and 33 ± 8 s in the presence of L-ENK, the difference being insignificant ($n = 4$, $P > 0{\cdot}4$, paired t test). These results indicate that the μ-opioid receptor reduces Ba^{2+} influx through Ca^{2+} channels.

Preferential inhibition of N-type Ca^{2+} channels by enkephalins

Since the presynaptic action potential has been shown to be prolonged by 4-aminopyridine (4-AP; 0·4 mM), the transient K^+ current would be involved in the falling phase of the action potential in the calyx-type presynaptic terminal of chick ciliary ganglion (Yawo & Chuhma, 1994). The $\Delta[Ca^{2+}]_t$ was enhanced by 4-AP (Yawo & Chuhma, 1993*a*) because of the slow activation of the presynaptic Ca^{2+} conductance (Yawo & Momiyama, 1993). As shown in Fig. 7*A*, the $\Delta[Ca^{2+}]_t$ was reversibly reduced by L-ENK in the presence of 4-AP. In five experiments in the presence of 0·4 mM 4-AP, L-ENK (10 μM) reduced $\Delta[Ca^{2+}]_t$ to $79 \pm 5\%$ of control (Fig. 7*B*, $P < 0{\cdot}05$, paired t test between raw data), a magnitude comparable to that without 4-AP ($P > 0{\cdot}3$, Mann-Whitney U test). Therefore, the 4-AP-sensitive transient K^+ channels appear not to be involved in the enkephalin-dependent reduction of Ca^{2+} influx in the calyx-type presynaptic terminal. It is possible that enkephalins directly inhibit Ca^{2+} channel activity (see Discussion).

At least two Ca^{2+} channel subtypes have been identified in the calyx-type presynaptic terminal; one is sensitive to ω-$CgTx_{GVIA}$ but insensitive to dihydropyridines (N-type); the other is resistant to both ω-$CgTx_{GVIA}$ and dihydropyridines (Yawo & Momiyama, 1993). To determine which subclass of Ca^{2+} channels is involved in the modulation through opioid receptors, the $\Delta[Ca^{2+}]_t$ was compared before

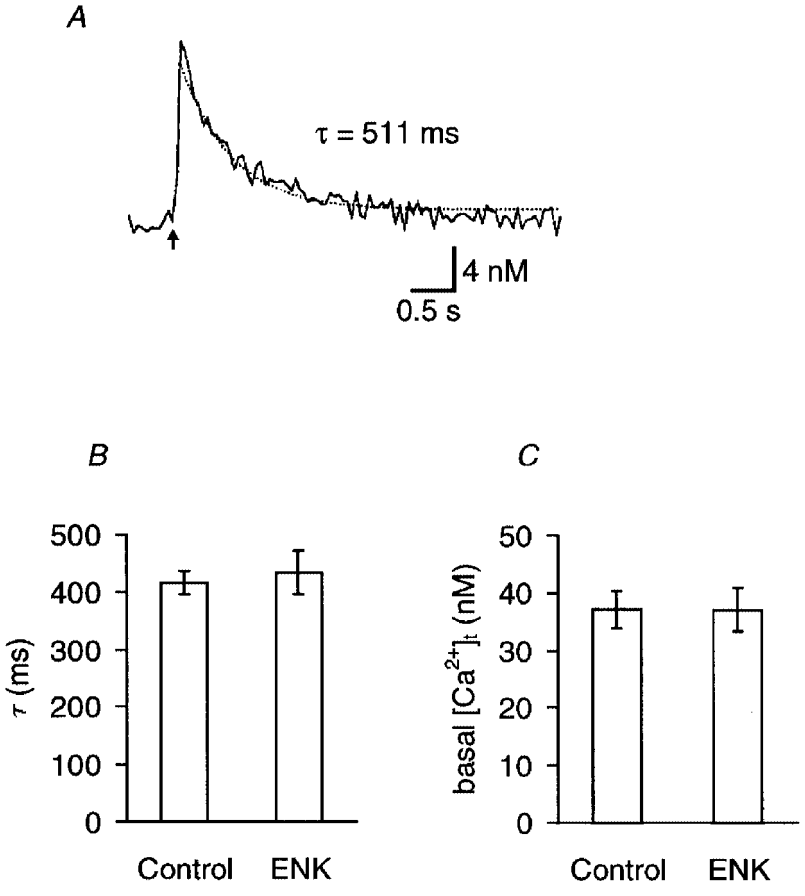

Figure 5. Effects of enkephalins on the falling phase of $\Delta[Ca^{2+}]_t$

A, a sample record of $[Ca^{2+}]_t$ in response to an electrical stimulus to the oculomotor nerve (indicated by the arrow). The falling phase of $\Delta[Ca^{2+}]_t$ between 90 and 10% of its peak was fitted to eqn (1) in the text: $[Ca^{2+}]_t = \alpha \exp(-t/\tau) + \beta$, where α is the asymptote of the peak $\Delta[Ca^{2+}]_t$ and β is the basal $[Ca^{2+}]_t$. In this example, $\tau = 511$ ms, $\alpha = 13{\cdot}0$ nM and $\beta = 42{\cdot}2$ nM. *B*, the decay time constant (τ) of $[Ca^{2+}]_t$. Summary of nine experiments in control and in the presence of 10 μM enkephalin (5 experiments with L-ENK and 4 experiments with M-ENK). The difference was not significant ($P > 0{\cdot}5$, two-tailed t test). *C*, basal $[Ca^{2+}]_t$ (β). Summary of nine similar experiments in control and in the presence of 10 μM enkephalin (5 experiments with L-ENK and 4 experiments with M-ENK). The difference was not significant ($P > 0{\cdot}8$, two-tailed t test).

and after treatment with 10 μM ω-CgTx$_{GVIA}$ for 30 min, which fully blocks the N-type Ca^{2+} channel subpopulation (Yawo & Momiyama, 1993; Yawo & Chuhma, 1994). As a result, the ω-CgTx$_{GVIA}$ treatment reduced $\Delta[Ca^{2+}]_t$ (Fig. 7*A*) on average to 22 ± 4 % of control ($n = 6$, $P < 0{\cdot}005$, paired t test between raw data). After treatment with ω-CgTx$_{GVIA}$, the addition of L-ENK (10 μM) was no longer effective (Fig. 7*A* and *B*). Figure 7*C* shows another example in which DAMGO (0·1 μM) was used instead of L-ENK. Before treatment with ω-CgTx$_{GVIA}$, the $\Delta[Ca^{2+}]_t$ was reversibly reduced by DAMGO whereas the ω-CgTx$_{GVIA}$-resistant component was completely insensitive to DAMGO. It is therefore suggested that the μ-opioid receptor preferentially inhibits the ω-CgTx$_{GVIA}$-sensitive Ca^{2+} influx through N-type channels.

DISCUSSION

Inhibition of transmitter release by enkephalins

The present study was focused on the effects of enkephalins on the synaptic transmission between the calyx-type presynaptic terminal and the ciliary cell in the ciliary ganglion of the chick embryo. Since the calyx-type terminal contains L-ENK as a co-transmitter (Erichsen *et al.* 1982*a*; Meriney *et al.* 1991), the endogenous enkephalins would modulate the cholinergic synaptic transmission at the presynaptic terminal, the postsynaptic membrane, or both. In the present work, we studied the effects of exogenous L-ENK on the quantal transmitter release under a nystatin-perforated patch clamp. Since L-ENK reduced m with negligible changes in q and the inhibitory effect of L-ENK was antagonized by naloxone (Fig. 1), it is suggested that opioid receptors are present in the presynaptic terminal and inhibit transmitter release. On the other hand, the postsynaptic junctional acetylcholine receptors appear not to be sensitive to L-ENK (Margiotta & Berg, 1986).

In sympathetic ganglia conditioning tetanic stimulation applied to the presynaptic nerve releases the endogenous enkephalins and inhibits acetylcholine release from the presynaptic terminal (Konishi *et al.* 1981). Were the endogenous enkephalins released following stimulation of the presynaptic nerve in our preparation? Did they inhibit transmitter release? Further experiments are necessary to address these issues.

Opoid receptor subtypes

In the present study, both DAMGO and DPDPE reduced transmitter release from calyx-type presynaptic terminals (Fig. 2). Therefore, it is suggested that L-ENK would activate both μ- and δ-opioid receptors in the presynaptic terminal. Although the endogenous agonist of the κ-opioid receptor has not been identified in the chick ciliary ganglion, the excitability of the calyx-type presynaptic terminal is modulated by a κ-opioid receptor-selective agonist, U-50,488 (Fletcher & Chiappinelli, 1993). U-50,488 inactivates at least three membrane conductances: an inward rectifier permeable to Na^+ and K^+, a K^+ conductance which is active at the resting potential and is blocked by Ba^{2+}, and a Ca^{2+}-dependent K^+ conductance (Fletcher & Chiappinelli, 1993). How these modulations of membrane conductances influence the transmitter release should be examined experimentally.

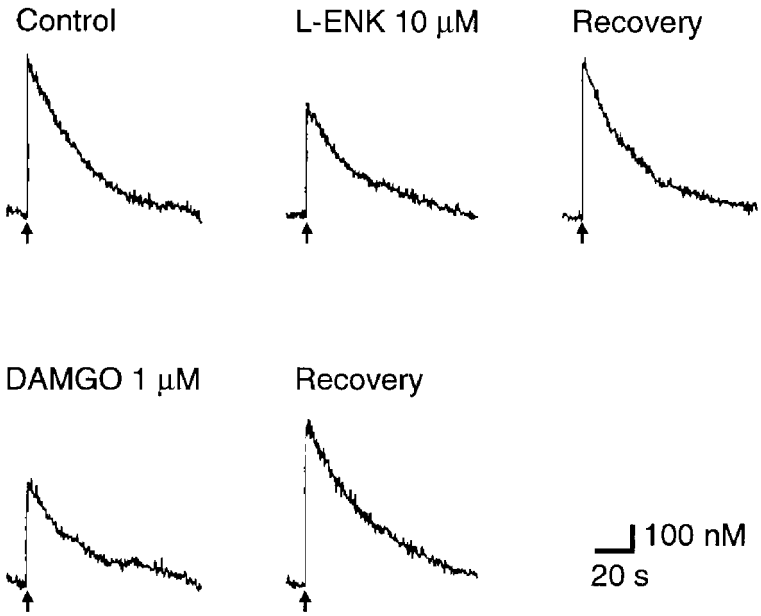

Figure 6. Effects of enkephalins on the intraterminal Ba^{2+} transient

Records of the intraterminal Ba^{2+} ($[Ba^{2+}]_t$) transient in response to electrical stimulation of the oculomotor nerve (indicated by arrows). Top, a representative experiment. From left to right: control record before the application of L-ENK, after bath application of 10 μM L-ENK, and after 9 min recovery. Bottom, the same series of experiments as above, after bath application of 1 μM DAMGO (left) and after 9 min recovery (right).

The reduction of Ca^{2+} influx

The enkephalin-dependent reduction of $\Delta[Ca^{2+}]_t$ (Figs 3 and 4) can be attributed to the inhibition of Ca^{2+} influx, the enhancement of Ca^{2+} sequestration or both. However, the modulation of Ca^{2+} sequestration by opioid peptides is unlikely for the following three reasons. First, the rate of the falling phase of $\Delta[Ca^{2+}]_t$ was not accelerated by the enkephalins (Fig. 5) although the early transient of local $[Ca^{2+}]_t$ in the vicinity of Ca^{2+} channel clusters was not detectable in our system. Second, the $\Delta[Ba^{2+}]_t$, which may

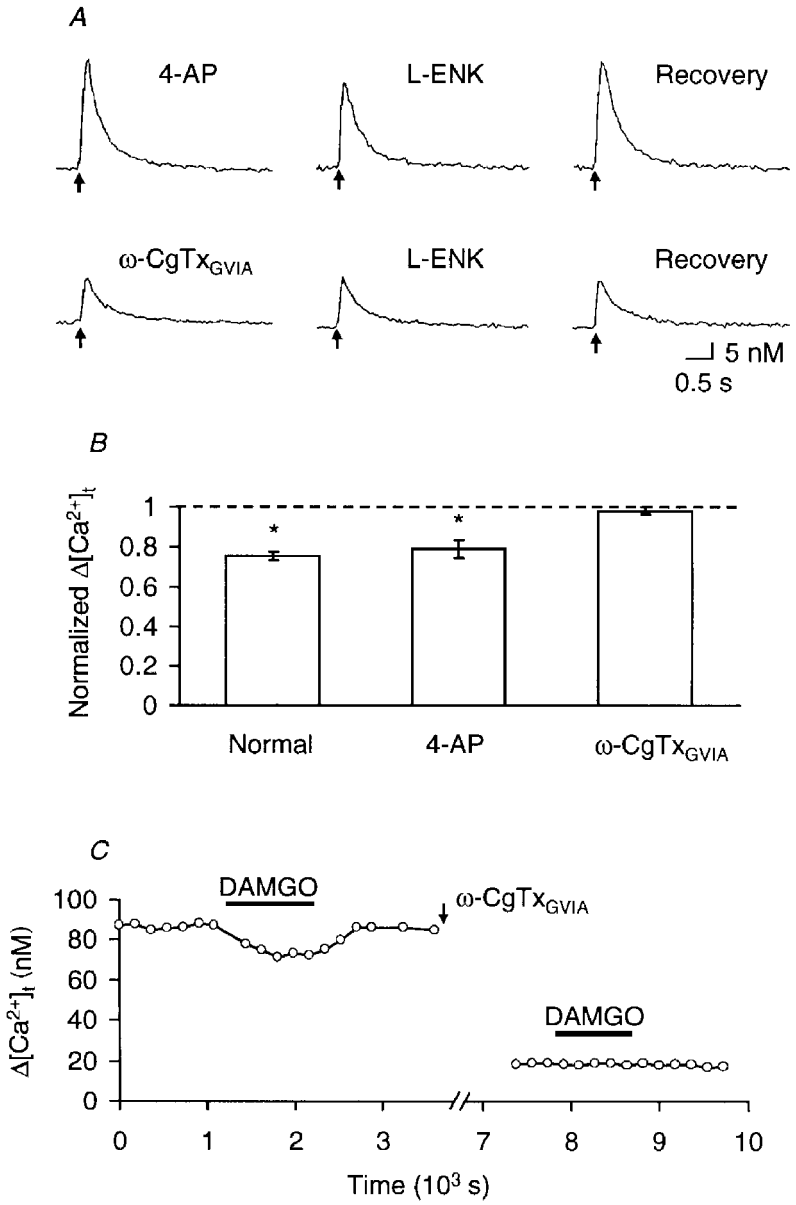

Figure 7. Inhibition of ω-$CgTx_{GVIA}$-sensitive Ca^{2+} influx by enkephalins

A, a series of sample records of $[Ca^{2+}]_t$ in response to a single presynaptic stimulus (indicated by arrows) in a solution containing 0·4 mM 4-AP. To remove the voltage-dependent inactivation of ω-$CgTx_{GVIA}$-resistant Ca^{2+} conductance, the external $[K^+]$ was reduced to 1 mM. Top, control experiment before the application of ω-$CgTx_{GVIA}$. From left to right: control record before the application of L-ENK, the effect of 10 μM L-ENK and recovery. Bottom, the effect of L-ENK (10 μM) on the same presynaptic terminal after 30 min treatment with 10 μM ω-$CgTx_{GVIA}$. *B*, summary of the effects of 10 μM L-ENK on $\Delta[Ca^{2+}]_t$, normalized to the control value before the application of L-ENK. Each column and bar are the mean ± S.E.M. From left to right: normal extracellular solution ($n = 6$, the same as that shown in Fig. 3*B*, 10 μM L-ENK column), in the presence of 0·4 mM 4-AP ($n = 5$), and in the presence of 4-AP after treatment with 10 μM ω-$CgTx_{GVIA}$ ($n = 6$). Asterisks indicate that the effect was statistically significant ($P < 0{\cdot}05$, two-tailed t test between raw data). The difference between the 4-AP and ω-$CgTx_{GVIA}$ columns is also significant ($P < 0{\cdot}01$, Mann-Whitney U test). *C*, effects of DAMGO on $\Delta[Ca^{2+}]_t$ before and after pre-treatment with ω-$CgTx_{GVIA}$ (10 μM, arrow). DAMGO (0·1 μM) was bath applied during the indicated period (filled bars).

not be influenced by the Ca^{2+}-buffering mechanisms, was also reduced by the enkephalins to the same extent as $\Delta[Ca^{2+}]_t$ (Fig. 6). Finally, the $\Delta[Ca^{2+}]_t$ was not reduced by the enkephalins after treatment with ω-$CgTx_{GVIA}$ (Fig. 7). Because DAMGO and DPDPE reduced $\Delta[Ca^{2+}]_t$ (Fig. 4), it is suggested that the enkephalins inhibit the Ca^{2+} influx during the presynaptic action potential through both μ- and δ-opioid receptors.

At 5 mM $[Ca^{2+}]_o$, 10 μM L-ENK reduced $\Delta[Ca^{2+}]_t$ to 75 ± 2% of control. Therefore, the Ca^{2+} influx should be reduced to 75% of control or less by L-ENK because of the non-linear relationship between $[Ca^{2+}]_o$ and $\Delta[Ca^{2+}]_t$ (Yawo, 1999*a*). If L-ENK (10 μM) could reduce the Ca^{2+} influx to a maximum of 75% at 1 mM $[Ca^{2+}]_o$, the EPSC would be expected to be reduced to a maximum of 55% of control based on the non-linear relationship between $[Ca^{2+}]_o$ and the EPSC (Yawo, 1996). Since this value is comparable to the experimental observation at 1 mM $[Ca^{2+}]_o$ (51 ± 10% of control, $n = 7$, data not shown), most of the effects of L-ENK could be explained by the reduction of Ca^{2+} influx. We cannot, however, exclude the possibility that the opioid receptors downregulate an exocytotic mechanism other than Ca^{2+} influx (Neher, 1998; Yawo, 1999*a*).

Preferential inhibition of N-type Ca^{2+} channels

The reduction of Ca^{2+} influx could be attributed to the inactivation of Ca^{2+} channels, the activation of K^+ channels, or both. One candidate among such K^+ channels is the 4-AP-sensitive transient K^+ channel activated during the falling phase of the presynaptic action potential (Yawo & Chuhma, 1994), as suggested for the GABAergic synaptic transmission in the midbrain (Vaughan *et al.* 1997). However, L-ENK reduced $\Delta[Ca^{2+}]_t$ to the same extent in the presence of 4-AP as in its absence (Fig. 7). Alternatively, enkephalins might inhibit the Ca^{2+}-dependent K^+ channels (Twitchell & Rane, 1993) co-distributing with the N-type Ca^{2+} channels (Robitaille *et al.* 1993). However, this notion conflicts with the fact that enkephalins reduced the $\Delta[Ba^{2+}]_t$ to the same extent as the $\Delta[Ca^{2+}]_t$ when extracellular Ca^{2+} was replaced by Ba^{2+} (Fig. 6), which does not, in general, activate Ca^{2+}-dependent K^+ conductances (Hille, 1992).

It has been reported that the activation of a certain subclass of K^+ channels is the major function of μ-opioid receptors in some neuronal somata (North, 1986; Williams *et al.* 1988) as well as in the presynaptic terminal of myenteric plexus. (Cherubini & North, 1985). This K^+ conductance is active around the resting potential and is blocked by Ba^{2+}. As a result, opioid peptides would hyperpolarize the membrane potential and reduce the excitability of the membrane, thereby effectively suppressing neuronal activity. On the other hand, enkephalins depolarize the resting membrane potential of the ciliary presynaptic terminal of the hatched chick, although they hyperpolarize the Edinger-Westphal neuronal soma which sends its axon to the ciliary ganglion (Chiappinelli *et al.* 1993). When the resting membrane potential of the calyx-type presynaptic terminal was monitored by the nystatin-perforated patch method, it was actually unaffected by DAMGO (1 μM, $n = 2$, data not shown). We also tested the effects of membrane hyperpolarization by reducing $[K^+]_o$ from 5 to 1 mM. However, the hyperpolarization *per se* did not reduce the EPSC (Yawo & Chuhma, 1994) as has been suggested in some other synapses (Cohen *et al.* 1992; Capogna *et al.* 1993). Therefore, the hyperpolarization of the presynaptic membrane potential appears not to be the principal mechanism of the μ-opioid receptor-dependent inhibition of transmitter release in the chick ciliary presynaptic terminal. Because of the prominent reduction of $\Delta[Ba^{2+}]_t$ by L-ENK and DAMGO (Fig. 6), a modulation of the Ba^{2+}-sensitive K^+ conductance would be unlikely (Hori *et al.* 1992; Vaughan & Christie, 1997). All the above observations strongly suggest that the μ-opioid receptor reduces the Ca^{2+} influx directly through the inhibition of Ca^{2+} channels rather than through the activation of K^+ channels.

We have previously shown that two distinct subtypes of Ca^{2+} channel coexist in the calyx-type presynaptic terminal of embryonic chick ciliary ganglion (Yawo & Momiyama, 1993). Because the major one was sensitive to ω-$CgTx_{GVIA}$ but insensitive to dihydropyridines, it was classified as the N-type Ca^{2+} channel (Stanley, 1991). The other subtype gave rise to a high-voltage activated current resistant to ω-$CgTx_{GVIA}$, dihydropyridines and ω-agatoxin IVA (Yawo & Momiyama, 1993; Yawo, 1994), and was classified as the R-type Ca^{2+} channel. The N-type Ca^{2+} channels are closely coupled with exocytosis (Stanley, 1993, 1997) and release transmitters more efficiently than the R-type (Yawo & Chuhma, 1994). In the present study, the L-ENK- and DAMGO-dependent reduction of $\Delta[Ca^{2+}]_t$ completely disappeared after ω-$CgTx_{GVIA}$ treatment (Fig. 7). This conflicts with the notion that enkephalin-sensitive Ca^{2+} influx could occur through R-type channels. It is suggested that the μ-opioid receptor would preferentially inhibit N-type Ca^{2+} channels.

Pivotal role of N-type Ca^{2+} channels in the regulation of transmitter release

Accumulating evidence indicates that the N-type Ca^{2+} channel is one of the common sites of presynaptic modulation (Wu & Saggau, 1997). At least four inhibitory receptors, A_1-adenosine (Yawo & Chuhma, 1993*a*), α_2-adrenergic (Yawo, 1996), and μ- and δ-opioid receptors, appear to converge at the N-type Ca^{2+} channel in the calyx-type presynaptic terminal of the chick ciliary ganglion. In fact, the presynaptic N-type Ca^{2+} channels are the targets of G-protein-mediated modulation in this system (Stanley & Mirotznik, 1997). N-type Ca^{2+} channels are suggested to cluster near the release sites (Miller, 1987; Robitaille *et al.* 1990; Cohen *et al.* 1991; Yawo & Chuhma, 1994; Stanley, 1997) and are the principal pathways of Ca^{2+} influx during the presynaptic depolarization (Miller, 1987; Yawo & Momiyama, 1993). The transmitter release appears to be tightly coupled with Ca^{2+} influx through the N-type Ca^{2+} conductance (Miller, 1987; Yawo & Chuhma, 1994; Stanley,

1997), although in some mammalian peripheral and central synapses a significant part of transmitter release is resistant to ω-CgTx$_{GVIA}$ (Dunlap *et al.* 1995; Takahashi *et al.* 1998). Since the N-type Ca^{2+} channels are rapidly and reversibly modulated by G-proteins (Kasai, 1992; Taussig *et al.* 1992; Wilding *et al.* 1995; Dolphin, 1998), the manipulation of these channels would be the most reliable mechanism for the rapid and reversible modulation of transmitter release.

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