The effect of ions and second messengers on long-term potentiation of chemical transmission in avian ciliary ganglia

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- 1 The effects of tetanic stimulation of the oculomotor nerve on transmission through the avian ciliary ganglion have been determined by use of the amplitude of the compound action potential recorded in the ciliary nerve, in the presence of hexamethonium (300 μ M), as a measure of synaptic efficacy.
- 2 Tetanic stimulation for 20 s at 30 Hz potentiated the chemical phase of the compound action potential by at least 100% of its control level. This potentiation, reflecting an increase in synaptic efficacy, decayed over two distinct time courses: firstly, a rapid decay with a time constant in the order of minutes, and secondly, a slower decay, representing a smaller potentiation, with a time constant in the order of an hour. The large increase in synaptic efficacy is attributed to post-tetanic potentiation (PTP) whereas the smaller but longer lasting increase is attributed to long-term potentiation (LTP).
- 3 Higher frequencies of tetanic stimulation gave increased PTP and LTP.
- 4 In order to test whether the influx of calcium ions into the nerve terminal during the tetanus is likely to be involved in potentiation, facilitation was measured during PTP and LTP. Facilitation was reduced to approximately zero during PTP but recovered to normal values about 15 min into LTP. A requirement for the induction of LTP was shown to be the presence of calcium in the bathing solution. However, blocking synaptic transmission with a high concentration of hexamethonium (3 mM) during the tetanic stimulation did not block the induction of LTP.
- 5 Application of the muscarinic inhibitor, atropine (2 μ M), did not affect the magnitude of PTP or LTP.
- 6 The activator of protein kinase C, phorbol 12,13-dibutyrate (2 μ M) potentiated synaptic transmission and reduced the potentiation due to PTP although it did not affect that due to LTP, but the inhibitor of this kinase, staurosporine (0.5 μ M), partially blocked the appearance of LTP without affecting PTP after the tetanus.
- 7 An inhibitor of calmodulin, W-7 (5 μ M), reversibly blocked the appearance of LTP significantly after a tetanus although the size of PTP was not affected.
- 8 The results presented here suggest that the initiation of LTP in the ciliary ganglion is due to an influx of calcium ions into the calyciform nerve terminal during the tetanus and that the mechanism for LTP involves a calcium-calmodulin-dependent process.

Keywords: Synapse; long-term potentiation; avian ciliary ganglia; calcium ions

Introduction

If sympathetic preganglionic axons are stimulated at a relatively high frequency (> 5 Hz) for several seconds, the fast excitatory postsynaptic potential is increased in amplitude for periods of the order of an hour (Dunant & Dolivo, 1968; Zengel *et al.*, 1980; Brown & McAfee, 1982; Koyano *et al.*, 1985). The discovery of long-term potentiation (LTP) in the peripheral nervous system made available a relatively simple preparation for the analysis of at least one form of LTP compared with the preparations available from the central nervous system (Kuba & Kumamoto, 1990). This LTP is likely to be due to an enhanced probability of secretion of quanta from the nerve terminal as there is no change in the sensitivity of the postsynaptic membrane to applied acetylcholine (Briggs & McAfee, 1988) and an accompanying increase in quantal content (Briggs *et al.*, 1985; Koyano *et al.*, 1985). This LTP is thought to be the result of an elevated calcium concentration in the preganglionic nerve terminals of both amphibia and mammals following a tetanus, as short term facilitation is substantially decreased during LTP (Minota *et al.*, 1991); the amplitude of LTP is also dependent on the level of the extracellular calcium concentration (Briggs *et al.*, 1985).

Post-tetanic potentiation (PTP), like LTP, involves an increase in the efficacy of synaptic transmission following tetanic stimulation but unlike LTP, it has a duration in the order of minutes. PTP has been described in both peripheral

and central synapses including the ciliary ganglion (Martin & Pilar, 1964c), the hippocampus (Racine & Milgram, 1983) and the neuromuscular junction (Magleby & Zengel, 1975; Delaney *et al.*, 1989). The level of PTP has been shown to be directly related to the extracellular calcium concentration (Erulkar & Rahamimoff, 1978; but see Tanabe & Kijima, 1992). It has been suggested that PTP may be the result of the activation of calcium channels during a tetanic stimulation causing a calcium ion accumulation as a consequence of the saturation of calcium pumps or their run-down due to limited energy resources in the presence of high calcium loads (Zucker, 1989). A role in PTP for sodium accumulation in the presynaptic nerve terminal during tetanic stimulation (Rahamimoff *et al.*, 1980; Zucker, 1989) has also been suggested.

The avian ciliary ganglion is an ideal preparation for studying forms of LTP and PTP that are due to changes in the properties of presynaptic nerve terminals (Martin & Pilar, 1963; 1964c). The giant calyciform nerve terminal, the largest terminal in the vertebrate (Marwitt *et al.*, 1971), is accessible for both imaging the movements of calcium ions in the terminal after loading it with a suitable calcium indicator (Delaney *et al.*, 1989; Swandulla *et al.*, 1991; Larkum *et al.*, 1992), as well as for measuring the calcium currents in the terminal responsible for transmitter release (Stanley, 1992). Ciliary neurones receive a monosynaptic input (Martin & Pilar, 1963; Hess, 1965), thereby making investigations into their mechanisms simpler than at other synapses. The present

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work shows that LTP and PTP exist in the ciliary ganglion following a tetanus and that their initiation involves an elevation of the calcium concentration in the presynaptic calyform terminal. The involvement of a calcium-calmodulin-dependent mechanism for LTP is implied.

Methods

One to five day post-hatch white leghorn chicks (*Gallus gallus*) were used in all experiments. Animals were decapitated and a craniectomy performed to expose the right ciliary ganglion. The ciliary ganglia with the attached right eye and oculomotor nerve were then removed. The right eye was dissected to free it from the ciliary nerve from within the eye (see Pilar & Tuttle, 1982 for a further description of the dissection). Ciliary ganglia with attached oculomotor and ciliary nerves were then placed in a tissue bath. The bath was perfused at a rate of approximately 2 ml min^{-1} with Tyrode solution of the following composition (in mM): Na^+ 140, K^+ 5, Mg^{2+} 1, Ca^{2+} 3, Cl^- 153, glucose 10, HEPES 10, equilibrated by bubbling with 95% O_2 , 5% CO_2 and pH adjusted to 7.2–7.4 by adding NaOH. Stimulation was applied to the oculomotor nerve through a glass suction electrode. The application of the stimulating pulse (a square wave of height 14 V and duration 0.09 ms) was through a radio-frequency isolation unit. Experiments were conducted at room temperature (17 to 20°C).

Recordings of compound action potentials were made by applying a fine suction electrode to the postganglionic ciliary nerve. Signals obtained from this nerve were amplified by an Axoclamp 2A amplifier (Axon Instruments). These signals were digitized using a Labmaster A-D board and recorded and analysed on an IBM compatible computer using the pCLAMP (Fetchex, Fetchan) software package (Axon Instruments).

Drugs used were hexamethonium chloride, phorbol 12,13-dibutyrate (PDBu), staurosporine (SS) and N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide hydrochloride (W-7). These were purchased from Sigma Chemical Company. PDBu, SS and W7 were dissolved in dimethyl sulphoxide (DMSO) as a stock solution. The stock was then diluted with perfusing solution such that the overall concentration of DMSO used in the experiments was never greater than 0.01%.

The compound action potential in the ciliary ganglion due to nerve stimulation is biphasic (Figure 1): the first phase is due to electrical transmission through the ganglion (Figure 1) whereas the second is due to chemical transmission (Martin & Pilar, 1964a,b). Hexamethonium ($300 \mu\text{M}$), a nicotinic cholinergic antagonist, was routinely added to the bathing solution in order to reduce the size of the chemical component of the biphasic compound action potential. Supramaximal stimulation was applied to the preganglionic oculomotor nerve. The reduction in the compound action potential by hexamethonium permitted measurement of an increase in synaptic efficacy. Hexamethonium made, to a large extent, the synaptic responses within the ganglion subthreshold (the chemical component of the compound action potential reduced by at least 60% of its original size). An increase in the synaptic efficacy, such as that which might happen following tetanic stimulation during PTP and LTP, was reflected by a significant increase in the number of the post-synaptic population reaching action potential firing threshold. This was indicated by an increase in the height of the chemical component of the compound action potential.

Protocols for the measurement of LTP and PTP were followed routinely. Upon the attachment of the suction electrodes to the pre- and post-ganglionic nerves, the preganglionic oculomotor nerve was stimulated at a frequency of 0.033 Hz. Only preparations with evoked compound action potentials larger than 1 mV were used. Hexamethonium ($300 \mu\text{M}$) was then added to the bathing solution. This was

allowed to perfuse the ganglion for at least 60 min so that the chemical phase of the compound action potential could be reduced (by at least 60% of its original value) and a control measurement for its height established. The preparation was stimulated at 0.033 Hz during this period. Tetanic stimulation of 30 Hz for 20 s was applied following the establishment of the control. It has been shown that the frequency of this tetanic stimulation occurs within the normal operating range of ciliary ganglia (Fujii, 1992).

Following tetanic stimulation of the ciliary ganglion, recordings of the PTP and LTP time courses were made by measuring the heights of the chemical phase of the evoked compound action potential. Any subsequent tetanic stimulation of the ganglion or addition of a drug to the bathing solution was carried out at least 60 min after the previous tetanic stimulation. Any drug added to the bathing solution was allowed to perfuse the ganglion for at least 60 min before tetanic stimulation was applied.

Some experiments were conducted where the stimulation protocol was varied. In such an experiment, the same ganglion was stimulated tetanically at 10 Hz, then at 20 Hz and then at 30 Hz while maintaining the number of pulses applied to the oculomotor nerve during tetanic stimulation constant. Each tetanic stimulation was separated by at least 1 h in order to allow the return of the response to control levels. Between each tetanic stimulation, evoked compound action potentials were measured at a frequency of 0.033 Hz. The expression of the frequency-dependent levels of tetanic stimulation was compared between different ganglia by normalizing the increase in the chemical phase of the compound action potential. This was done by making that increase following the 30 Hz tetanic stimulation as the unit value and describing the potentiation due to lower frequency stimulation as a fraction of this normalized value.

The ordinates of many of the figures are expressed in terms of percentage of control. A control value (V_{ctl}) was obtained by averaging the heights of the chemical component of the compound action potential for the 15 min before tetanic stimulation (measured by evoking compound action potentials at a frequency of 0.033 Hz). Following tetanic stimulation, compound action potentials were again evoked at a frequency of 0.033 Hz. The amplitudes of these post-tetanic compound action potentials (V) were measured. The percentage increase was calculated as:

$$\text{Percentage of control} = 100 \times \frac{V}{V_{\text{ctl}}}$$

In the case of the measurements involving paired-pulse facilitation, the value for V , in this formula, was calculated as the difference between the heights of the chemical phase of the compound action potential evoked from the conditioning and then the test pulse. V_{ctl} represents the difference between the heights of the chemical phase of the compound action potential evoked from the conditioning and then the test pulse averaged over the 15 min prior to tetanic stimulation.

Data are expressed as mean $\pm$ standard error of the mean (s.e.mean) and n gives the number of experiments. The significance of the difference between n pairs of observations made before and after application of a drug was calculated by Student's paired t test. P values of 0.05 or less were considered to represent significant changes.

Results

Characteristics of potentiation of transmission in the ciliary ganglion

When the ciliary ganglion was stimulated with a single impulse through the preganglionic oculomotor nerve, the compound action potential recorded in the ciliary nerve was biphasic (Figure 1a). The first phase of this response was

Figure 1 Changes in the amplitude of the chemical and electrical phases of synaptic transmission in the ciliary ganglion following a tetanus (30 Hz, 20 s) applied to the oculomotor nerve. (a) The compound action potential recorded from the ciliary nerve; the first peak indicates the phase due to electrical transmission and the second peak indicates the phase due to chemical transmission. (b) The compound action potential following the perfusion of the ganglion in the hexamethonium ($300 \mu\text{M}$) for at least 60 min. (c) The compound action potential 20 s after the end of a tetanus, during post-tetanic potentiation (PTP), showing that the chemical component of the compound action potential is approximately 510% of the control level corresponding to (b) but also shown by the dotted line here (on a different scale from signal shown in b). (d) The compound action potential 3 min after the end of the tetanus, nearing the end of the PTP time course, with the chemical component of the compound action potential approximately 190% of the control level from (b) shown by the dotted line. (e) The compound action potential 10 min after the end of the tetanic stimulation with the chemical component of the compound action potential approximately 153% of the control level. (f) The compound action potential 35 min following the tetanic stimulation with the chemical component of the compound action potential approximately 130% of the pretetanic stimulation control level. The scaling bars refer to each of the traces a, b, c, d, e and f and represent in the vertical 0.5, 0.2, 0.5, 0.2, 0.2 and 0.2 mV respectively. The horizontal scaling bar refers to 5 ms in all traces.

usually the larger of the two whilst the second phase could be blocked with high concentrations of hexamethonium (by at least 60% of its original height using $300 \mu\text{M}$ hexamethonium; Figure 1b). The first phase is due to electrical transmission through the ganglion (Martin & Pilar, 1964a; Hess *et al.*, 1969) whereas the second phase is due to chemical transmission (Martin & Pilar, 1963; 1964b). Stimulation of the oculomotor nerve with a tetanus of 30 Hz for 20 s gave rise to a large increase in the second phase of the compound action potential due to chemical transmission without affecting any significant persistent change in the phase due to electrical transmission (Figure 1c) although often, immediately following the tetanic stimulation, there was a slight

decrease in the electrical phase of transmission of no more than 10% that lasted no more than 30 s. It is shown below that the enhancement of the chemical phase is most probably related to an increase in calcium concentration in the nerve terminal; the small but persistent change in the electrical phase may be related to the known dependence of gap junctions that mediate the electrical transmission on intracellular calcium (Rose & Loewenstein, 1975). The chemical phase was still elevated for a long time after the tetanus, returning to control levels usually in the order of an hour (Figure 1d,e and f). Evoked compound action potentials were measured by stimulating the ganglion through the oculomotor nerve at 0.033 Hz before and after the tetanic stimulation. The increase in the amplitude of the chemical phase following tetanic stimulation clearly consisted of two components with different time courses (Figure 2a). The first component consisted of a several hundred percent increase in amplitude of the chemical phase and this lasted for about 4 min; it was followed by a smaller, but more persistent, increase which generally returned to control levels in the order of 1 h (Figure 2a). The electrical phase remained about the same amplitude throughout this period (Figure 2b). The large and relatively fast declining component has the characteristics of post-tetanic potentiation (PTP) observed at other

Figure 2 Evidence for the existence of both post-tetanic (PTP) and long-term potentiation (LTP) in the ciliary ganglion following a tetanus. (a) The percentage of the control amplitude of the phase of the compound action potential due to chemical transmission following a tetanus (30 Hz, 20 s); note that the PTP component which is maximal at approximately 375% of control is over in less than 5 min whereas the LTP component, initially about 140% of the control level, lasts for over 1 h. (b) The percentage increase of the electrical phase of neurotransmission through the ciliary ganglion. No significant change in the compound action potential due to electrical transmission is noted at any time following stimulation at time zero. The control amplitude is average over the 15 min prior to tetanic stimulation.

synapses whereas the very slow declining component has the characteristics of long-term potentiation (LTP; Kuba & Kumamoto, 1990).

The time course and amplitude of PTP and LTP were measured by plotting the percentage increase in the amplitude of the chemical phase of the compound action potential every 30 s after a tetanus on log-linear axes as shown in Figure 3a. A least squares linear regression analysis of the points between 10 and 60 min results in a straight line that intercepts the ordinate scale at 50% and has a time constant of 71 min; these values correspond to the amplitude and time constants for LTP. Subtracting this line from the points in Figure 3a between 0 and 4 min produces the points shown in Figure 3b; fitting a least squares linear regression line to these gives an amplitude and time constant for PTP of 280% and 1.5 min respectively. For 50 ciliary ganglia analysed in this way the amplitude and time constant of PTP were $882 \pm 300\%$ and 1.4 ± 0.1 min (mean $\pm$ s.e.mean; $n = 50$). For LTP these values were $53 \pm 14\%$ and 50 ± 22 min respectively.

The size of PTP and LTP depended on the frequency of stimulation of the tetanus (Figure 4). PTP decreased significantly ($P < 0.05$ level, $n = 4$), as frequency of tetanic stimulation decreased from 30 Hz to 20 Hz, to $67 \pm 7\%$ of the 30 Hz level, and to $16 \pm 1\%$ of its 30 Hz level for tetanic

stimulation at 10 Hz. The magnitude of LTP also decreased as the frequency of tetanic stimulation decreased. Tetanic stimulation at 20 Hz produced LTP of a magnitude that was $40 \pm 7\%$ of that for tetanic stimulation at 30 Hz. Tetanic stimulation of 10 Hz resulted in LTP the magnitude of which was $28 \pm 7\%$ of that resulting from tetanic stimulation of 30 Hz. These results showed a significant ($P < 0.05$, $n = 4$) decrease of the magnitude of LTP as frequency decreased.

Mechanisms responsible for PTP and LTP in the ciliary ganglion

It has been suggested that potentiation following tetanic stimulation is caused to a large extent by the presence of residual calcium in the presynaptic nerve terminal (Zucker, 1989). A test that might be used to see if the slow removal of excess calcium in the nerve terminal following a tetanus is responsible for PTP or LTP in this preparation is to measure facilitation during potentiation. Facilitation is most probably due to residual calcium left in the nerve terminal after a conditioning impulse adding to the calcium influx accompanying a test impulse; if the calcium concentration is elevated by potentiation then this residual calcium will be comparatively small and so facilitation will be decreased. Figure 5 shows that facilitation is decreased during PTP and during the beginning of LTP but that it recovers to control values about 15 min after a tetanus. Facilitation was reduced to $-1.8 \pm 1.8\%$ of the control level immediately following tetanic stimulation, $52 \pm 7\%$ at 5 min into LTP and $85 \pm 9\%$ at 15 min into LTP; it returned to control levels about 20 min after tetanic stimulation ($n = 11$). These results sug-

Figure 3 The time course of post-tetanic (PTP) and long-term potentiation (LTP) following a tetanus (30 Hz, 20 s) at time zero. (a) The percentage increase, plotted on a logarithmic ordinate scale in the phase of the compound action potential due to chemical transmission relative to the average of that over 15 min prior to tetanic stimulation at different times after the tetanus; the broken line is drawn according to a least squares regression analysis of the points between 10 and 60 min. (b) The results of subtracting the regression line in (a) from the points between 0 and 3 min; this is also drawn with a logarithmic ordinate scale. The regression line in (a) gives a time constant for the LTP of 71 min and the line in (b) gives a time constant for PTP of 1.5 min. The increase of the signal, with respect to its control level, for the beginning (time zero) of LTP and PTP immediately after the tetanus were 148% and 245% of the control reading respectively in this case.

Figure 4 The dependence of post-tetanic (PTP) and long-term potentiation (LTP) on frequency. (a) The increase of the chemical component of the compound action potential during PTP shown as a fraction of the increase of that for 30 Hz stimulation as the frequency of tetanic stimulation is varied. (b) The increase of the chemical component of the compound action potential during LTP shown as the frequency of tetanic stimulation is varied. Each point represents the mean $\pm$ s.e.mean of three experiments.

gest that calcium is elevated during PTP and at the beginning of LTP but that it is not elevated during most of the LTP period.

In order to check if the induction of LTP was dependent on the binding of acetylcholine to its postsynaptic receptors, a high concentration of hexamethonium (3 mM) was used to block the receptors prior to a tetanus and then, following the tetanus, the usual low concentration of hexamethonium (300 μ M) returned. At the lower concentration of hexamethonium the ganglionic synaptic transmission recovered from the block. The control experiment (Figure 6a) shows the block of chemical synaptic transmission with 3 mM hexamethonium and its subsequent recovery to control levels following the reintroduction of the normal bathing solution at time 15 min ($n = 3$). The control reading at time zero was $108 \pm 8\%$ of the average value in the preceding 15 min; following the recovery of the ganglion it was $107 \pm 10\%$ at time 60 min. Experiments were then conducted as in the control except that immediately prior to the reintroduction of the bath solution tetanic stimulation of 20 Hz for 30 s was applied. Under these circumstances, a potentiation over the control levels of synaptic transmission was present upon recovery in 4 experiments (Figure 6b) as the level at time zero was $95 \pm 5\%$ of control and at time 60 min was $132 \pm 16\%$ of control and this increase was statistically significant ($P < 0.05$). This was in contrast to the lack of LTP observed for the control situation (Figure 6a). This indicated that nicotinic cholinergic transmission was not necessary for the induction of LTP.

In order to test further whether the initiation of LTP is likely to be dependent on the influx of calcium ions into the terminal during the tetanus, synaptic transmission through

Figure 5 Changes in facilitation during post-tetanic (PTP) and long-term potentiation (LTP). (a) Measurement of the amplitude of conditioning (●) and test (○) pulses, 30 ms apart, during PTP and LTP due to a tetanus of 30 Hz for 20 s at time zero. (b) The average facilitation as a percentage of the pretetanic stimulation control levels, averaged over the 15 min before time zero, for 11 different experiments is shown; for each point the s.e.mean is shown.

Figure 6 The induction of long-term potentiation (LTP) in the absence of nicotinic cholinergic synaptic transmission. (a) Chemical synaptic transmission through the ganglion was blocked by the application of 3 mM hexamethonium chloride (C6) at time zero for 15 min after which, normal solution was returned to the bath and normal transmission allowed to recover. The percentage of control refers to the size of the chemical component of synaptic transmission relative to its average size over the 15 min prior to time zero. (b) The same protocol as (a), except that tetanic stimulation was applied to the ganglion at time 15 min, immediately before the reintroduction of normal solution to the bath. Upon the recovery of the transmission, the signal was potentiated above control levels indicating that LTP had been induced by the tetanic stimulation during the block of transmission. Each point represents the mean $\pm$ s.e.mean for 3 experiments.

the ganglion was blocked with a high magnesium concentration (8 mM) and zero calcium concentrations (0 mM). In a control experiment, synaptic transmission was blocked with 8 mM $[Mg^{2+}]$ and 0 mM $[Ca^{2+}]$. Normal bathing solution was then reapplied to the ganglion at time 15 min and chemical synaptic transmission allowed to recover to control levels (Figure 7a; $n = 3$). In other experiments tetanic stimulation was applied immediately before the reintroduction of normal bath solution (Figure 7b). The results showed that potentiation of the chemical synaptic transmission was not observed following the recovery of the ganglion from the block if calcium was absent during the tetanus in 3 experiments (Figure 7b). This showed that the induction of LTP required the presence of calcium.

To ascertain whether the LTP mechanism shown to occur in ciliary neurones involved muscarinic transmission, a muscarinic antagonist, atropine was used. A control experiment was carried out, initially, involving the usual elicitation of PTP and LTP at time zero (Figure 8a). At least 60 min following the tetanic stimulation, after the completion of the majority of the LTP time course, the ganglion was perfused for at least 60 min with 2 μ M atropine sulphate. Following this perfusion, tetanic stimulation of the ganglion was repeated. Again PTP and LTP were observed and these were

not significantly different from those obtained in atropine-free conditions (Figure 8b).

Given that calcium has been shown to play an important role in LTP in the ciliary ganglion, the question arose as to whether the calcium-activated signal transduction mechanisms of protein kinase C or calcium-calmodulin kinase were involved in the initiation or maintenance of LTP. Tetanic stimulation was applied to the ganglion under control conditions (Figure 9a), then, at least 1 h after this stimulation, the protein kinase C activator, phorbol 12,13-dibutyrate ($2 \mu\text{M}$), was added to the bathing solution and allowed to perfuse the ganglion for at least 60 min. The activator elevated transmission by $158 \pm 103\%$ ($n = 5$) but, following a second tetanic stimulation, no significant effect on the levels of LTP ($P > 0.10$; $n = 5$; Figure 9b). PTP was reduced from $356 \pm 69\%$ to $221 \pm 12\%$ ($n = 5$) indicating that the potentiation produced by PDBu used a similar mechanism to that for PTP. For four separate ganglia, following their tetanic stimulation under control conditions (Figure 10a), the protein kinase C blocker, staurosporine ($0.5 \mu\text{M}$) was used to perfuse the ganglion. This perfusion did not significantly change the magnitude of PTP but did decrease the magnitude of LTP from $135 \pm 12\%$ to $115 \pm 2\%$ ($P < 0.05$; $n = 4$; Figure 10b). It is surprising that while

Figure 7 The dependence on calcium for induction of long-term potentiation (LTP). (a) Measurements were made of the chemical component of the compound action potential as a percentage of the average of that 15 min prior to time zero. At time zero a solution containing high magnesium (8 mM) and low calcium (0 mM) was added for 15 min only in order to block chemical transmission before the reintroduction of the normal bathing solution and the subsequent recovery of synaptic transmission. (b) The same protocol as (a) except immediately before the reintroduction of normal bathing solution at time zero, tetanic stimulation was applied to the ganglion. After the recovery of the ganglion no potentiation of synaptic transmission was seen as occurred in Figure 6 indicating a dependence on calcium for the induction of LTP. Each point represents the mean $\pm$ s.e.mean for 3 experiments.

Figure 8 The effect of atropine on long-term (LTP) and post-tetanic potentiation (PTP). (a) Ganglia were stimulated at 30 Hz for 20 s to elicit PTP of $390 \pm 52\%$ of the control and LTP of $131 \pm 5\%$ of the control (mean $\pm$ s.e.mean; $n = 4$). (b) The same ganglia were stimulated after their perfusion with $2 \mu\text{M}$ atropine sulphate for at least 60 min to give PTP of $362 \pm 37\%$ and LTP of $127 \pm 6\%$ (mean $\pm$ s.e.mean; $n = 4$).

Figure 9 The effect of activating protein kinase C with phorbol 12,13-dibutyrate (PDBu) on post-tetanic (PTP) and long-term potentiation (LTP). (a) The ganglia were stimulated tetanically in the control period ($n = 5$). (b) The same ganglia, after perfusion of the system with PDBu for at least 1 h, were stimulated tetanically.

Figure 10 The effect of blocking protein kinase C with staurosporine (SS) on post-tetanic (PTP) and long-term potentiation (LTP). (a) The ganglia were stimulated tetanically in the control period ($n = 4$). (b) The same ganglia, after perfusion of the system with SS for at least 1 h, were stimulated tetanically.

PDBu effects on PTP implicated a role for protein kinase C, blocking this with staurosporine did not affect PTP.

In a separate experiment, at least 60 min following tetanic stimulation under control conditions (Figure 11a), the calcium-calmodulin kinase blocker W-7 ($0.5 \mu\text{M}$) was introduced into the bath and allowed to perfuse the ganglion for 60 min. After this time, a decrease in the chemical component of the compound action potential occurred ($-23.4 \pm 6.9\%$; $n = 6$). PTP was not significantly reduced by W-7 whereas LTP was completely inhibited (Figure 11b). Indeed, W-7 reversed the LTP to a long-term depression ($92 \pm 3\%$ in W-7 compared to $150 \pm 32\%$ in control; $n = 6$). This surprising result was maintained for a period of at least 60 min. In six experiments, PTP was $440 \pm 146\%$ during the control period and $526 \pm 86\%$ after the introduction of W-7 ($P < 0.05$). Four experiments continued with a wash of the W-7 from the ganglion with normal bathing solution for at least 60 min. The chemical component of the compound action potential increased slightly ($6.3 \pm 1.5\%$; $n = 4$) although not completely back to control levels. Tetanic stimulation of the washed ganglion gave the usual PTP, with LTP being partially restored to a new level of $113 \pm 3\%$ ($n = 4$; Figure 11c) which was smaller than the control measurement of LTP for these experiments ($166 \pm 47\%$; $n = 4$). In four experiments the PTP for the control was $481 \pm 262\%$; in the presence of W-7 it was $413 \pm 70\%$ and after the wash, it was $290 \pm 27\%$. Although the mean value reduced considerably, this change was not significant.

Discussion

The induction and amplitude of LTP and PTP in the ciliary ganglion showed a dependence on the frequency of impulses during the tetanus and its duration in much the same way as at other preganglionic nerve terminals (Briggs *et al.*, 1985). It has been proposed (Dolphin, 1985) that activity-dependent LTP in sympathetic ganglia is expressed from the presynaptic terminal as the result of increased presynaptic calcium accumulation or sensitivity. As the frequency of tetanic

Figure 11 The effect of blocking Ca-calmodulin with N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide hydrochloride (W-7) on post-tetanic (PTP) and long-term potentiation (LTP). (a) The ganglion was stimulated tetanically in the control period ($n = 6$). (b) The same ganglia, after perfusion of the system with W-7 for at least 1 h, were stimulated tetanically. (c) Four of the six ganglia analysed in (a) and (b) were washed with normal bathing solution to test the reversibility of the W-7 effect; following washing of at least 1 h, tetanic stimulation was applied.

stimulation increases, the inward calcium signals at the terminal overlap and summate, indicating that the calcium level here is dependent on the frequency of stimulation up to the level of saturation. This high calcium level in the terminal accounts for PTP which is a product of residual calcium (Erulkar & Rahamimoff, 1978; see however Tanabe & Kijima, 1992). Also, it is proposed (Kuba & Kumamoto, 1990) that this enlarged calcium influx persistently affects the calcium-dependent second messenger systems in the presynaptic terminal during ganglionic LTP. Therefore, the maximum amplitude of LTP and PTP is directly proportional to the frequency of stimulation as this affects the amount of overlap and summation of the inward presynaptic calcium signal. The PTP following the tetanus of 30 Hz for 20 s declined with a time constant of about 1.5 min, which is similar to that at other ganglionic synapses (Waziri *et al.*, 1969; Zengel *et al.*, 1980), as is the time constant of decline of the LTP of about 50 min (Dunant & Dolivo, 1968; Brown & McAfee, 1982; Briggs *et al.*, 1985; Briggs & McAfee, 1988; Minota *et al.*, 1991).

During potentiation, elevated calcium levels in the presynaptic nerve terminal (Zucker, 1989; Kuba & Kumamoto, 1990; Swandulla *et al.*, 1991) cause impulses to add an equal increment of residual calcium to the presynaptic nerve terminal so that the time course of removal of this residual

calcium following tetanic stimulation described the separate time courses of decay of the different components of potentiation (Zengel & Magleby, 1982; Zucker, 1989). Facilitation, while having a high control level following a tetanus, is reduced to almost zero during PTP and remains depressed for about 15 min during LTP. This suggests that during PTP the presynaptic terminal has a high concentration of calcium and that this is reduced to control levels following the establishment of LTP. This implies that calcium concentration in the presynaptic nerve terminal is raised for the induction but not the maintenance of LTP in the ciliary ganglion.

Short term facilitation is also reduced during LTP at bullfrog sympathetic preganglionic nerve terminals but this lasts for the entire period of LTP (Minota *et al.*, 1991), and therefore suggests that intraterminal calcium is elevated during LTP here. However at mossy fibre terminals on CA3 pyramidal neurones the intraterminal calcium concentration is only elevated during the first few minutes of LTP (Zalutsky & Nicholl, 1990). Certainly the induction of LTP in the calyciform terminal is dependent on calcium ions, as withdrawing calcium ions from the extracellular medium during a tetanus blocks LTP as it does at bullfrog sympathetic ganglia (Koyano *et al.*, 1985; Minota *et al.*, 1991) and rabbit sympathetic ganglia (Briggs *et al.*, 1985). The action of acetylcholine released from the calyciform terminals during a tetanus on either presynaptic or postsynaptic nicotinic receptors does not seem to be involved in the induction of LTP. Blocking these receptors with high concentrations of hexamethonium during a tetanus did not block the subsequent appearance of LTP when the high concentration was washed out (see also Briggs *et al.*, 1985). These results suggest that the chemical activation of the postsynaptic neurone is not required for the induction of LTP. However, it is possible that the activations of the postganglionic neurones through electrical transmission together with the accumulation of intraterminal Ca^{2+} act cooperatively to activate the LTP mechanism in the terminals. Muscarinic transmission is not required for the expression of LTP here as the application of atropine, a muscarinic receptor antagonist, had no significant effect on LTP.

Given that calcium has been shown to be a necessary component in the induction of LTP, an investigation was made into some of the calcium-activated biochemical processes which may be involved in the formation of LTP. The two calcium-activated intracellular transduction mechanisms, both of which have been implicated in the expression of LTP, are protein-kinase C (Bar *et al.*, 1984) and calcium-

calmodulin (Minota *et al.*, 1991). Activation of protein kinase C by application of PDBu caused a potentiation of synaptic transmission. Following tetanic stimulation LTP was unaffected, simply adding to the protein-kinase C induced potentiation. It is unlikely that protein-kinase C is involved in the expression of LTP here as the potentiations were independent of each other. Application of the protein-kinase C inhibitor, staurosporine, reduced LTP significantly without any significant effect on PTP. In view of the effect of PDBu application, this was most likely to be due to the non-specific effects of staurosporine (Tamaoki *et al.*, 1986; Minota *et al.*, 1991) as it has been shown to block the activity of other protein kinases such as those involving cyclic AMP and cyclic GMP.

The calcium-calmodulin inhibitor, W-7, produced a significant reduction in LTP with little significant change in the level of PTP. This result indicates that calmodulin is likely to be involved in LTP in the avian ciliary ganglion. Two possible roles which calmodulin might play in the mechanism for LTP here are via the modulation of the passive and active calcium transport at the presynaptic nerve terminals or through a possible conformational change in the interaction of the calmodulin-binding protein, calspectin, and a cytoskeletal protein, F-actin (Sobue *et al.*, 1983). Calcium transport might be modulated by the phosphorylation of calcium-calmodulin-dependent protein kinase II (CaMKII) which inhibits active calcium transports or accelerates passive calcium transports at the presynaptic nerve terminal (Minota *et al.*, 1991). Alternatively, the effectiveness of the protein, calspectin, in its binding to F-actin, in the presence of the calmodulin-binding protein, protein 4.1, is inversely proportional to the terminal calcium concentration; it has been suggested that this process modulates terminal membrane calcium-transporting molecules (Minota *et al.*, 1991). Thus, a very large calcium transient, such as that which follows tetanic stimulation, might cause a persistent change in the calcium-transporting molecules at the presynaptic membrane, so altering the availability of transmitter for secretion from the terminal or the actual excitation-secretion mechanism itself.

LTP in the avian ciliary ganglion has been shown here to be highly dependent on the modulation of the calcium concentration and calcium-dependent mechanisms in the presynaptic nerve terminal. It is likely, here, that the activity-dependent modulation of synaptic efficacy involves calmodulin and, possibly, the phosphorylation of its protein kinase, CaMKII.

References

- BAR, P.R., WIEGANT, F., LOPES DE SILVA, F.H. & GISPEN, W.H. (1984). Tetanic stimulation affects the metabolism of phosphonitides in hippocampal slices. *Brain Res.*, **321**, 381–385.
- BRIGGS, C.A., BROWN, T.H. & MCAFEE, D.A. (1985). Neurophysiology and pharmacology of long-term potentiation in the rat sympathetic ganglion. *J. Physiol.*, **359**, 503–521.
- BRIGGS, C.A. & MCAFEE, D.A. (1988). Long-term potentiation at nicotinic synapses in the rat superior cervical ganglion. *J. Physiol.*, **404**, 129–144.
- BROWN, T.H. & MCAFEE, D.A. (1982). Long-term synaptic potentiation in the superior cervical ganglion. *Science*, **215**, 1411–1413.
- DELANEY, K.R., ZUCKER, R.S. & TANK, D.W. (1989). Calcium in motor nerve terminals associated with posttetanic potentiation. *J. Neurosci.*, **9**, 3558–3567.
- DOLPHIN, A.C. (1985). Long-term potentiation at peripheral synapses. *Trends Neurosci.*, **8**, 376–378.
- DUNANT, Y. & DOLIVO, M. (1968). Plasticity of synaptic function in the excised sympathetic ganglion of the rat. *Brain Res.*, **10**, 271–273.
- ERULKAR, S.D. & RAHAMIMOFF, R. (1978). The role of calcium ions in tetanic and posttetanic increase of miniature end-plate potential frequency. *J. Physiol.*, **278**, 501–511.
- FUJII, J.T. (1992). Repetitive firing properties in subpopulations of the chick edinger westphal nucleus. *J. Comp. Neurology*, **316**, 279–286.
- HESS, A. (1965). Developmental changes in the structure of the synapse on the myelinated cell bodies of the chicken ciliary ganglion. *J. Cell Biol.*, **25**, 1–19.
- HESS, A., PILAR, G. & WEAKLY, J.N. (1969). Correlation between transmission and structure in avian ciliary ganglion synapses. *J. Physiol.*, **202**, 339–354.
- KOYANO, K., KUBA, K. & MINOTA, S. (1985). Long-term potentiation of transmitter release reduced by repetitive presynaptic activities in bullfrog sympathetic ganglia. *J. Physiol.*, **359**, 219–233.
- KUBA, K. & KUMAMOTO, E. (1990). Long-term potentiations in vertebrate synapses: a variety of cascades with common sub-processes. *Prog. Neurobiol.*, **34**, 197–269.
- LARKUM, M.E., SCOTT, T.R.D. & BENNETT, M.R. (1992). Calcium concentration changes in avian ciliary ganglia during post-tetanic potentiation and long-term potentiation of transmitter secretion. *Proc. Aust. Neurosci. Soc.*, **3**, 73.

- MAGLEBY, K.L. & ZENGEL, J.E. (1975). A quantitative description of tetanic and posttetanic potentiation of transmitter release at the frog neuromuscular junction. *J. Physiol.*, **245**, 183–208.
- MARTIN, A.R. & PILAR, G. (1963). Dual mode of synaptic transmission in the avian ciliary ganglion. *J. Physiol.*, **168**, 443–463.
- MARTIN, A.R. & PILAR, G. (1964a). An analysis of electrical coupling at synapses in the avian ciliary ganglion. *J. Physiol.*, **171**, 454–475.
- MARTIN, A.R. & PILAR, G. (1964b). Quantal components of the synaptic potential in the ciliary ganglion of the chick. *J. Physiol.*, **175**, 1–16.
- MARTIN, A.R. & PILAR, G. (1964c). Presynaptic and postsynaptic events during post-tetanic potentiation and facilitation in the avian ciliary ganglion. *J. Physiol.*, **175**, 17–30.
- MARWITT, R., PILAR, G. & WEAKLEY, J.N. (1971). Characteristics of two ganglion cell populations in avian ciliary ganglia. *Brain Res.*, **25**, 317–334.
- MINOTA, S., KUMAMOTO, E., KITAKOGA, O. & KUBA, K. (1991). Long-term potentiation induced by a sustained rise in the intraterminal Ca^{2+} in bull-frog sympathetic ganglia. *J. Physiol.*, **435**, 421–438.
- PILAR, G. & TUTTLE, J.B. (1982). A simple neuronal system with a range of uses: the avian ciliary ganglion. In *Progress in Cholinergic Biology: Model Cholinergic Synapses*. ed. Hanin, I. & Goldberg, A.M. pp. 213–247. New York: Raven Press.
- RACINE, R.J. & MILGRAM, N.W. (1983). Short-term potentiation phenomena in the rat limbic forebrain. *Brain Res.*, **260**, 201–216.
- RAHAMIMOFF, R., LEV-TOV, A. & MEIRI, H. (1980). Primary and secondary regulation of quantal transmitter release: calcium and sodium. *J. Exp. Biol.*, **89**, 5–18.
- ROSE, B. & LOEWENSTEIN, W.R. (1975). Permeability of cell junction depends on local cytoplasmic calcium activity. *Nature*, **254**, 250–252.
- SOBUE, K., KANDA, K., ADACHI, J. & KAKEUCHI, S. (1983). Calmodulin-binding proteins that interact with actin filaments in a Ca^{2+} -dependent flip-flop manner: survey in brain and secretory tissues. *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 6868–6871.
- STANLEY, E.F. (1992). Single calcium channels on a cholinergic presynaptic nerve terminal. *Neuron*, **7**, 585–591.
- SWANDULLA, D., HANS, M., ZIPSER, K. & AUGUSTINE, G.J. (1991). role of residual calcium in synaptic depression and posttetanic potentiation: fast and slow calcium signalling in nerve terminals. *Neuron*, **7**, 915–926.
- TAMAOKI, T., NOMOTO, H., TAKAHASHIO, I., KATO, Y., MORIMOTO, M. & TOMITA, F. (1986). Staurosporine, a potent inhibitor of phospholipid/ Ca^{++} dependent protein kinase. *Biochem. Biophys. Res. Commun.*, **135**, 397–402.
- TANABE, N. & KIJIMA, H. (1992). Ca^{2+} -dependent and -independent components of transmitter release at the frog neuromuscular junction. *J. Physiol.*, **455**, 271–290.
- WAZIRI, R., KANDEL, E.R. & FRAZIER, W.T. (1969). Organization and inhibition in abdominal ganglion of *Aplysia*. II. Posttetanic potentiation, heterosynaptic depression, and increments in frequency of inhibitory postsynaptic potentials. *J. Neurophysiol.*, **32**, 509–515.
- ZALUTSKY, R.A. & NICOLL, R.A. (1990). Comparison of two forms of long-term potentiation in single hippocampal neurons. *Science*, **248**, 1619–1624.
- ZENGEL, J.E. & MAGLEBY, K.L. (1982). Augmentation and facilitation of transmitter release. A quantitative description at the frog neuromuscular junction. *J. Gen. Physiol.*, **80**, 583–611.
- ZENGEL, J.E., MAGLEBY, K.L., HORN, J.P., MCAFEE, D.A. & YAROWSKY, P.J. (1980). Facilitation, augmentation and potentiation of synaptic transmission at the superior cervical ganglion of the rabbit. *J. Gen. Physiol.*, **76**, 213–231.
- ZUCKER, R.S. (1989). Short-term synaptic plasticity. *Annu. Rev. Neurosci.*, **12**, 13–31.

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