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Presynaptic inhibition of synaptic transmission in the rat hippocampus by activation of muscarinic receptors: involvement of presynaptic calcium influx

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1 Modulation of presynaptic voltage-dependent calcium channels (VDCCs) by muscarinic receptors at the CA3–CA1 synapse of rat hippocampal slices was investigated by using the calcium indicator fura-2. Stimulation-evoked presynaptic calcium transients ($[Ca_{pre}]_t$) and field excitatory postsynaptic potentials (fe.p.s.ps) were simultaneously recorded. The relationship between presynaptic calcium influx and synaptic transmission was studied.

2 Activation of muscarinic receptors inhibited $[Ca_{pre}]_t$, thereby reducing synaptic transmission. Carbachol (CCh, 10 μM) inhibited $[Ca_{pre}]_t$ by 35% and reduced fe.p.s.p. by 85%. The inhibition was completely antagonized by 1 μM atropine. An approximate 4th power relationship was found between presynaptic calcium influx and postsynaptic responses.

3 Application of the N-type VDCC-blocking peptide toxin ω-conotoxin GVIA (ω-CTx GVIA, 1 μM) inhibited $[Ca_{pre}]_t$ and fe.p.s.ps by 21% and 49%, respectively, while the P/Q-type VDCC blocker ω-agatoxin IVA (ω-Aga IVA, 1 μM) reduced $[Ca_{pre}]_t$ and fe.p.s.ps by 35% and 85%, respectively.

4 Muscarinic receptor activation differentially inhibited distinct presynaptic VDCCs. ω-CTx GVIA-sensitive calcium channels were inhibited by muscarinic receptors, while ω-Aga IVA-sensitive channels were not. The percentage inhibition of ω-CTx GVIA-sensitive $[Ca_{pre}]_t$ was about 63%.

5 Muscarinic receptors inhibited presynaptic VDCCs in a way similar to adenosine (Ad) receptors. The percentage inhibition of ω-CTx GVIA-sensitive $[Ca_{pre}]_t$ by Ad (100 μM) was about 59%. There was no significant inhibition of ω-Aga IVA-sensitive channels by Ad. The inhibitions of $[Ca_{pre}]_t$ by CCh and Ad were mutually occlusive.

6 These results indicate that inhibition of synaptic transmission by muscarinic receptors is mainly the consequence of a reduction of the $[Ca_{pre}]_t$ due to inhibition of presynaptic VDCCs.

Keywords: Synaptic transmission; presynaptic inhibition; voltage-dependent calcium channel; presynaptic muscarinic receptor; modulation of synaptic transmission

Introduction

Immunocytochemical studies have identified cholinergic innervation of the hippocampus from the medial septum (Frotscher & Leranth, 1985). Presynaptic inhibition mediated by muscarinic receptors, has been found in the hippocampus (Hounsgaard, 1978; Valentino & Dingledine, 1981). A muscarinic receptor subtype was shown to be involved in the inhibition of glutamate release evoked by high K^+ concentration in synaptosomes prepared from the hippocampus (Marchi & Raiteri, 1989). A direct action on voltage-dependent calcium channels (VDCCs) was shown in rat sympathetic neurones, where activation of muscarinic receptors inhibited both N-type and L-type calcium currents (Mathie *et al*., 1992). This modulation was controlled by two different mechanisms. The modulation of L-type VDCCs by muscarinic receptors was via a Ca-mediated, BAPTA-sensitive slow pathway, while the inhibition of N-type channels was fast, membrane-delimited and voltage-dependent. A pertussis toxin-sensitive G-protein was involved in this fast pathway. Such parallel pathways were also found in the central nervous system. Agonists of muscarinic receptors inhibit N-, P- and L-type calcium channels in rat acutely isolated striatal neurones (Howe & Surmeier, 1995). In hippocampal neurones, muscarinic receptor activation also reduced both ω-conotoxin (ω-CTx)GVIA-sensitive and -insensitive somatic high threshold VDCCs (Toselli & Taglietti, 1994; 1995). These studies of the modulation of somatic Ca channels by muscarinic receptors suggest that the inhibition of synaptic transmission by muscarinic receptors in hippocampus may be due to the same mechanism, i.e., the inhibition of presynaptic VDCCs (for review see Wu & Saggau, 1997). However, recently, inhibition of the frequency of miniature excitatory postsynaptic currents (me.p.s.cs) recorded from CA3 pyramidal cells in cultured hippocampal slices by muscarinic receptors was demonstrated (Scanziani *et al*., 1995). A direct interference in the neurotransmitter release process at some point subsequent to Ca^{2+} influx was proposed to underlie this kind of inhibition. Whether or not Ca channels are involved in the presynaptic inhibition by muscarinic receptors is still not known. In the present study, we tested the hypothesis of muscarinic inhibition of Ca channels by simultaneously recording action potential-evoked presynaptic calcium influx and postsynaptic responses. We found that muscarinic receptor activation inhibits presynaptic VDCCs in a way similar to the by activation of adenosine receptors.

Methods

Recording of fe.p.s.ps and the presynaptic calcium transients in hippocampal slices

Transverse hippocampal slices (300–400 μm) were prepared from Sprague-Dawley rats (four weeks of age) and incubated in artificial cerebrospinal fluid containing (in mM): NaCl 124, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 22, NaH_2PO_4 1.25 and D-glucose 10; gassed with 95% O_2/5% CO_2 to maintain a constant

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pH of 7.4. The bath temperature was 30°C. The procedure for loading calcium indicator into presynaptic terminals of CA3–CA1 synapses has been described elsewhere in detail (Wu & Saggau, 1994a). Briefly, a small amount of 1 mM Fura-2 AM (Molecular Probes) dissolved in dimethylsulphoxide (DMSO) (80% DMSO + 20% pluronic acid) was pressure-injected into the brain slice in the *stratum radiatum* (SR) of area CA1, where it was locally taken up into CA3 axons (Figure 1). About 2 h after injection, an area with a diameter of 150 μm in SR, about 800 μm away from the injection site, was illuminated at a single excitation wavelength (380 nm). Fluorescence was collected by a 50X objective (N.A. 0.9), filtered by a longpass filter (490 nm) and converted into an electrical signal by a single photodiode. A bipolar electrode was positioned in SR of area CA1 to stimulate afferent CA3 axons to CA1 neurones. An extracellular electrode (1–5 MΩ, filled with 2 M NaCl) was used to record field excitatory postsynaptic potentials (fe.p.s.ps). Slices were stimulated every 20 s and stimulation-induced presynaptic calcium transients ($[Ca_{pre}]_t$) and fe.p.s.ps were simultaneously measured. Three traces were averaged to improve signal-to-noise ratio. The amplitude of calcium transients (ΔF) was measured as the difference between peak and resting fluorescence (F). Signals were corrected for dye bleaching and diffusion by forming the ratio $\Delta F/F$, which represents the equilibrium-state of Ca^{2+} ions binding to the calcium indicator. This amplitude of the fluorescence calcium transient is proportional to the volume-averaged Ca^{2+} influx into presynaptic terminals evoked by a single action potential (Wu & Saggau, 1994a; Sinha *et al.*, 1997). Autofluoresence of the brain slice was measured and subtracted from total fluorescence signal. When testing for changes in presynaptic resting Ca^{2+} concentration, ratio measurements were performed (excitation wavelength 360 nm and 380 nm). The selective presynaptic loading of the Ca indicator was verified by applying the glutamatergic antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) and D-aminophosphonovalerate (D-APV, 25 μM), which did not alter $\Delta F/F$ while completely blocking the fe.p.s.p. ($n=3$) (Figure 1). The maximal slope of the fe.p.s.p. was taken as the measure of synaptic transmission based on the assumption, that the slope of the fe.p.s.p. is proportional to the e.p.s.c. In those experiments where ω-

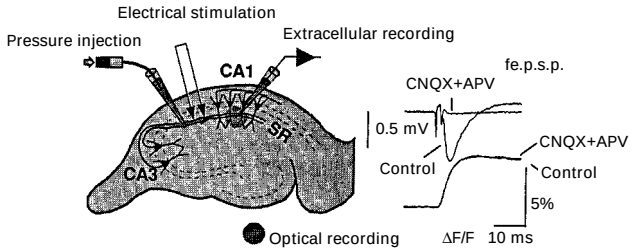

Figure 1 Selective presynaptic loading with Ca indicator. Schematic diagram of pressure injection of membrane-permeable Ca indicator, which is locally taken up into CA3 axons and fills presynaptic terminals onto CA1 dendrites in *stratum radiatum* (SR). Inset shows verification of successful presynaptic loading by comparing normal (control) with blocked synaptic transmission (10 μM CNQX + 25 μM D-APV). Note that $\Delta F/F$ remained unaltered (traces superimposed) while the fe.p.s.p. was abolished.

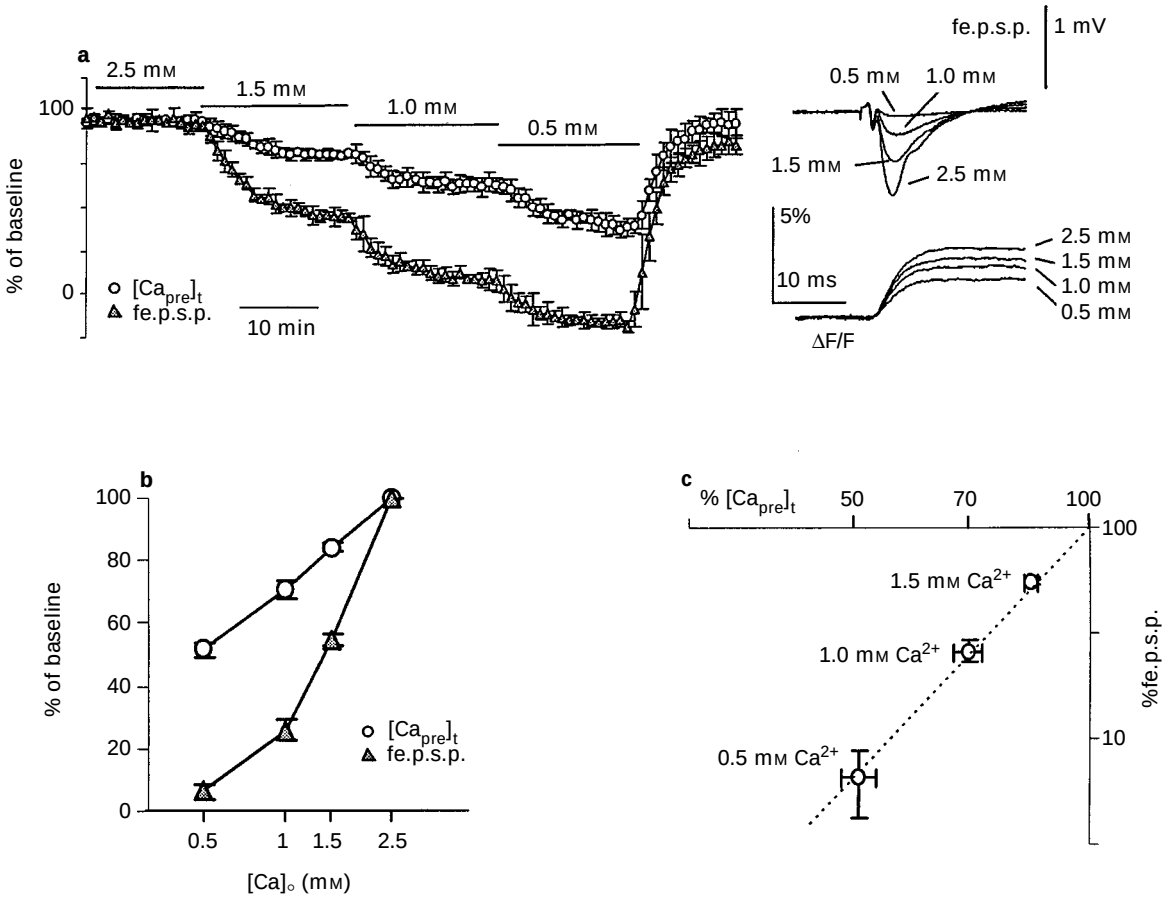

Figure 2 Reduction of $[Ca]_o$ inhibited the $[Ca_{pre}]_t$ and fe.p.s.p. (a) Group data of the time course of normalized slope of fe.p.s.p. and $[Ca_{pre}]_t$ for the reduction of $[Ca]_o$ ($n=6$). Inset shows sample traces taken under control conditions and during the steady-state of each $[Ca]_o$ tested. (b) Semi-log plot of % fe.p.s.p. *vs* $\%[Ca_{pre}]_t$ for different $[Ca]_o$ tested. (c) Log-log plot of %fe.p.s.p. *vs* $\%[Ca_{pre}]_t$ for different $[Ca]_o$ tested. The slope of the regression line is $m=4.0$ ($r^2=0.99$).

agatoxin (ω-Aga) IVA was used, slices were cut at 300 μm and 0.1% cytochrome C was added to the bath solution to reduce nonspecific binding of the toxin. Data in each experiment were normalized to baseline before any drug application, then pooled together and expressed as the mean $\pm$ s.d.

Drugs

Carbachol was purchased from Sigma; CNQX and D-APV were obtained from Tocris Cookson; ω-CTx GVIA was purchased from Bachem; ω-Aga IVA was a gift from Pfizer, Inc. (Groton, CT).

Results

Muscarinic receptor activation inhibits $[Ca_{pre}]_t$ and fe.p.s.p.

Presynaptic VDCCs play a key role in control of neurotransmitter release at presynaptic terminals in the central nervous system (reviewed by Dunlap *et al.*, 1995). Activation of presynaptic VDCCs by action potentials results in Ca^{2+} influx into presynaptic terminals. Calcium then cooperatively triggers the release of neurotransmitter. This cooperative property can be best described by a nonlinear power function between Ca^{2+} influx and neurotransmitter release, which has been shown at many synapses including squid giant axon synapse (Katz & Miledi, 1970; Augustine & Charlton, 1986), CA3–CA1 synapse of guinea-pig hippocampus (Wu & Saggau, 1994a) and parallel fibre synapse of rat cerebellar Purkinje neurones (Mintz *et al.*, 1995). The power number (m) between these synapses was found to be 2–4 when either the extracellular Ca^{2+} concentration ($[Ca]_o$) was varied or calcium (Cd^{2+}), a non-specific blocker of VDCCs, was used to manipulate the presynaptic Ca^{2+} influx (for review see Wu & Saggau, 1997). A similar power relationship between $[Ca_{pre}]_t$ and synaptic transmission was found at the CA3-CA1 synapse of the preparation investigated (Figure 2). Figure 2a shows the group data ($n=6$) for normalized $[Ca_{pre}]_t$ (%$[Ca_{pre}]_t$) and normalized fe.p.s.p. (%e.p.s.p.) when $[Ca]_o$ was reduced from 2.5 mM to 0.5 mM. At 0.5 mM $[Ca]_o$, synaptic transmission was almost completely blocked. However, a certain amount of $[Ca_{pre}]_t$ (about 50% of control) could still be detected. The $[Mg^{2+}]_0$ was held constant during manipulation of $[Ca]_o$. No significant changes in the presynaptic fibre volley were observed. The power number $m = \log(\%\text{fe.p.s.p.})/\log(\%[Ca_{pre}]_t)$ for each tested $[Ca]_o$ was 3.6 ± 0.5 ($n=8$), 3.9 ± 0.6 ($n=8$) and 4.2 ± 0.6 ($n=8$) for 1.5 mM and 0.5 mM $[Ca]_o$, respectively. The average power number m was 4.0 ($r^2=0.99$), which was estimated by the slope of a regression line obtained from a double logarithmic plot of %fe.p.s.p. *vs* %$[Ca_{pre}]_t$ (Figure 2c).

Activation of muscarinic receptors reversibly inhibited $[Ca_{pre}]_t$ and the fe.p.sp. This inhibition was not accompanied by

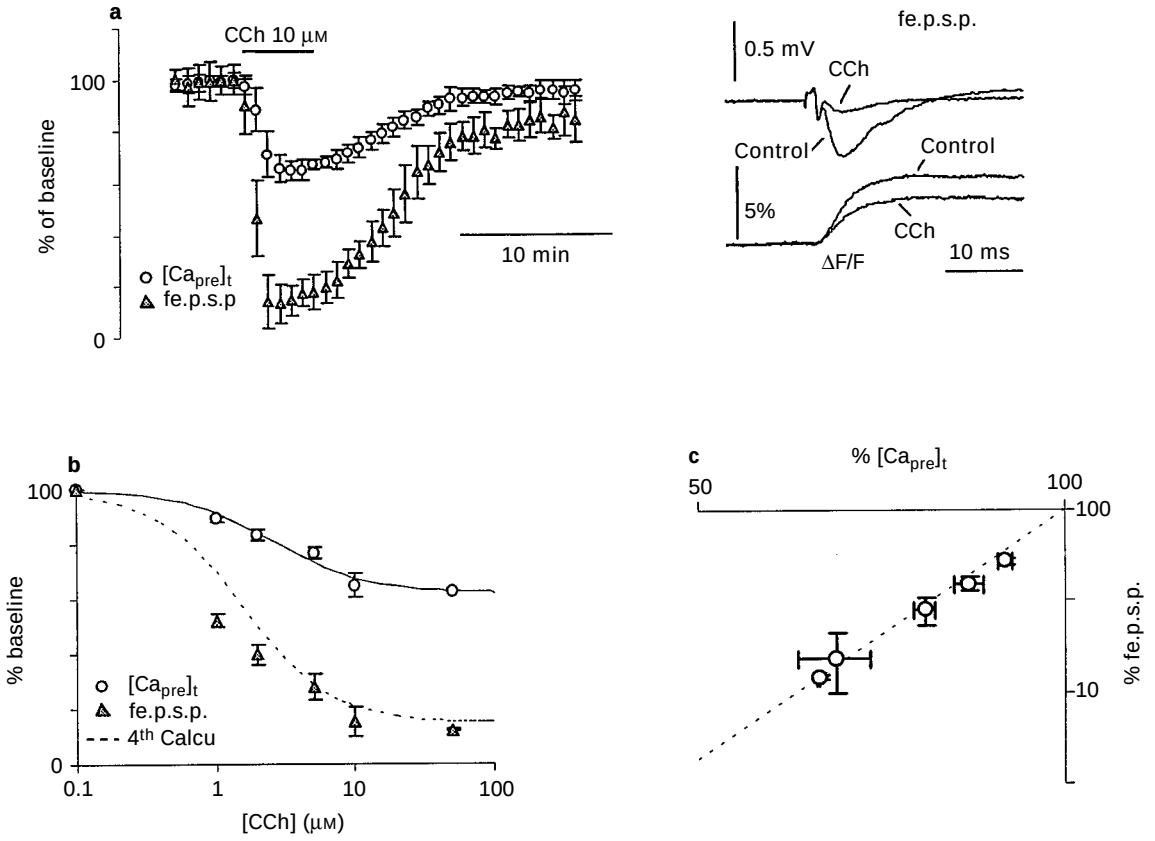

Figure 3 Muscarinic receptor activation inhibited the $[Ca_{pre}]_t$ and fe.p.s.p. (a) Time course of normalized slope of fe.p.s.p. and $[Ca_{pre}]_t$ of grouped experiments. CCh (10 μM), reversibly inhibited the presynaptic $[Ca_{pre}]_t$ and fe.p.s.p. Inset shows sample recordings taken at control and during the steady-state response of CCh. (b) Dose-response curve for effect of CCh on $[Ca_{pre}]_t$ and fe.p.s.p. $[Ca_{pre}]_t$ was reduced to $89.1 \pm 1.2\%$, $83.4 \pm 2.1\%$, $76.7 \pm 1.7\%$, $68.8 \pm 4.4\%$ and $62.8 \pm 0.2\%$ of control by 1 μM ($n=5$), 2 μM ($n=5$), 5 μM ($n=15$), 10 μM ($n=31$), and 50 μM ($n=2$) of CCh, respectively. The corresponding reduction of the fe.p.s.p. was $52.2 \pm 2.5\%$, $39.7 \pm 3.5\%$, $28.1 \pm 4.9\%$, $15.4 \pm 5.5\%$ and $12.1 \pm 0.4\%$ of control, respectively. The dose-response curve for inhibition of $[Ca_{pre}]_t$ was fitted by a Hill equation with $IC_{50} = 2.6$ μM. (R = remaining fraction of $[Ca_{pre}]_t$)

$$([Ca]_t = R_{min} + \frac{IC_{50}^{n_H}}{[CCh]^{n_H} + IC_{50}^{n_H}} \times (R_{max} - R_{min})$$

$n_H = 1.3$, $IC_{50} = 2.6$ μM, $R_{max} = 100\%$, $R_{min} = 62\%$). The dashed line is the 4th power calculation of $[Ca_{pre}]_t$. (c) A log-log plot of mean %$[Ca_{pre}]_t$ *vs* mean %fe.p.s.p. for different concentrations of CCh. The regression line has a slope of 4.5 ($r^2=0.97$).

a change in presynaptic resting Ca^{2+} concentration (data not shown). Figure 3a shows the group data for 9 experiments with a similar time course. Sample traces taken in control and during the peak effect of carbachol (CCh) are shown as an inset. This inhibition was antagonized by 1 μM atropine (muscarinic receptor antagonist, $n=3$, data not shown), confirming the involvement of muscarinic receptors. Recovery after 20 min of wash was about 98% for $[Ca_{pre}]_t$ and 90% for the fe.p.s.p. On average, 10 μM CCh decreased $[Ca_{pre}]_t$ and fe.p.s.p. to about 65% and 15% of baseline, respectively, in the steady-state. The dose-response relationship for the inhibition of $[Ca_{pre}]_t$ (Figure 3b) was fitted with a Hill equation having an IC_{50} of 2.6 μM (solid line in Figure 3b). The mean remaining fraction of $[Ca_{pre}]_t$ *vs* the mean remaining fraction of fe.p.s.p. for different concentrations of CCh is plotted in Figure 3c. The data were fitted by a regression line with a slope of 4.5 ($r^2=0.97$). During the application of 10 μM CCh, spontaneous burst discharges with a low frequency (less than 0.2 Hz) and with magnitudes much smaller than the evoked fe.p.s.p. were observed in area CA1. Such sparsely spontaneous events did not interfere with our stimulation-evoked calcium and fe.p.s.p. signals.

ω-CTx GVIA partially occludes the inhibition of $[Ca_{pre}]_t$ by carbachol

Somatic ω-CTx GVIA-sensitive calcium channels have been shown to be inhibited by muscarinic receptors in cultured hippocampal neurones (Toselli & Taglietti, 1994; 1995). Here, we tested if presynaptic ω-CTx GVIA-sensitive calcium channels are involved in the modulation of synaptic transmission by muscarinic receptors. If ω-CTx GVIA-sensitive Ca channels are involved, then during blockade of these channels, application of CCh should result in a reduced inhibition of $[Ca_{pre}]_t$ relative to control. Figure 4a shows the time course of normalized $[Ca_{pre}]_t$ and fe.p.s.p. of a typical experiment. ω-CTx GVIA partially and irreversibly blocked the $[Ca_{pre}]_t$ and synaptic transmission. This block reached saturation after 15 min of perfusion with 1 μM ω-CTx GVIA. Addition of 10 μM CCh further inhibited $[Ca_{pre}]_t$ but to a lesser extent than without pre-application of ω-CTx GVIA. The group data of 4 such experiments are shown in Figure 4b. ω-CTx GVIA inhibited $[Ca_{pre}]_t$ by $20.5\pm0.4\%$ while the slope of the fe.p.s.p. was reduced by $48.6\pm2.8\%$. The mean steady-state responses of %fe.p.s.p. and $\%[Ca_{pre}]_t$ after application of the toxin gave the power number (m) of 2.9 ± 0.3. The summary of the effects of ω-CTx GVIA on the inhibition of $[Ca_{pre}]_t$ by CCh is shown in Figure 4c. After ω-CTx GVIA, CCh further inhibited the $[Ca_{pre}]_t$ by $22.3\pm1.3\%$. Compared to the amount of inhibition of $[Ca_{pre}]_t$ by CCh in control experiments ($35.2\pm4.4\%$), about 37% of inhibition was abolished by ω-CTx GVIA. Normalized by the amount of ω-CTx GVIA-sensitive $[Ca_{pre}]_t$, the percentage inhibition of ω-CTx GVIA-sensitive $[Ca_{pre}]_t$ by CCh was about 63% ($=(35.2\%-22.3\%)/20.5\%$). This indicates that presynaptic ω-CTx GVIA-sensitive Ca channels are involved in the modulation of synaptic transmission by muscarinic receptors at the CA3–CA1 synapse of rat hippocampus.

ω-Aga IVA does not occlude the effect of carbachol

Somatic ω-Aga IVA-sensitive calcium channels have been demonstrated to be inhibited by baclofen, a $GABA_B$ receptor agonist, in cerebellar Purkinje neurones (Mintz & Bean, 1993). However, such an inhibition was not found at the hippocampal CA3-CA1 synapse (Wu & Saggau, 1995). An ω-Aga IVA-sensitive Ca current was also inhibited by muscarinic receptors

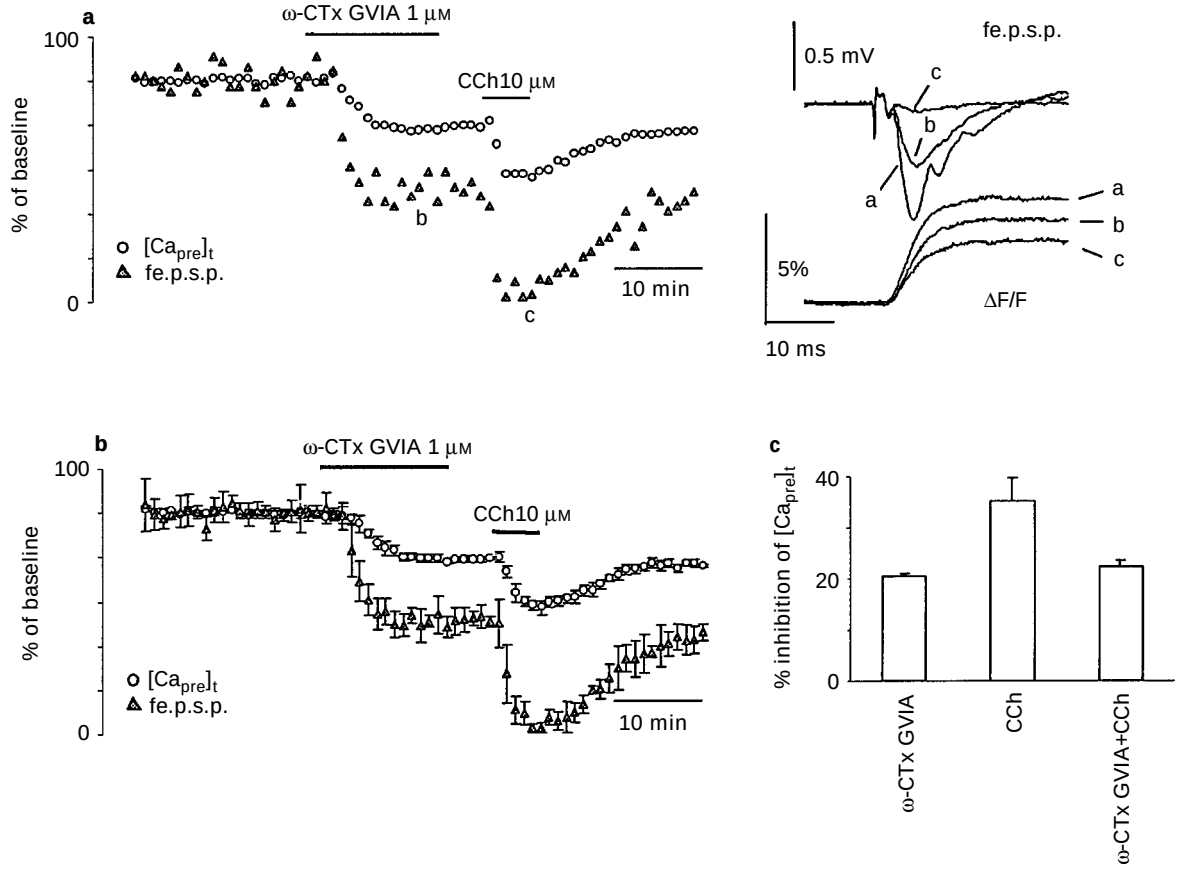

Figure 4 ω-CTx GVIA partially occluded the inhibition by carbachol. (a) A typical experiment showing the effects of ω-CTx GVIA, which partially and irreversibly blocked $[Ca_{pre}]_t$ and synaptic transmission. CCh further inhibited $[Ca_{pre}]_t$ but less than without pre-application of ω-CTx GVIA. The inset shows sample recordings taken at the times indicated. (b) Summary data for four experiments. (c) Inhibition of $[Ca_{pre}]_t$ by CCh with and without pretreatment with ω-CTx GVIA. ω-CTx GVIA partially occluded the inhibition by CCh.

in acutely isolated striatal neurones (Howe & Surmeier, 1995). In order to determine whether presynaptic ω-Aga IVA-sensitive Ca channels were modulated by activating presynaptic muscarinic receptors, the same experimental strategy as above was used. ω-Aga IVA (1 μM) was applied to 300 μm thick brain slices. The results are shown in Figure 5a. The inset shows sample traces taken under control conditions, after application of ω-Aga IVA and during application of CCh. On average, the toxin inhibited $[Ca_{pre}]_t$ to $65.3 \pm 3.1\%$ of baseline ($n=5$) (Figure 5c). The fraction of the remaining fe.p.s.p. was $15.1 \pm 4.5\%$. The calculated power number for ω-Aga IVA was m = 4.4. Figure 5c compares the mean inhibition of $[Ca_{pre}]_t$ with and without pre-application of ω-Aga IVA. In contrast to ω-CTx GVIA, ω-Aga IVA had little effect on the inhibition of $[Ca_{pre}]_t$ by CCh. After pretreatment with ω-Aga IVA, exposure to CCh still inhibited $[Ca_{pre}]_t$ by $34.3 \pm 3.8\%$ of control calcium influx ($n=4$), while in the absence of the toxin, this inhibition was 35%. Thus, in spite of the large inhibition of $[Ca_{pre}]_t$ by ω-Aga IVA ($34.7 \pm 3.1\%$), this did not result in a significant alteration in the inhibition of $[Ca_{pre}]_t$ by CCh. This suggests that presynaptic ω-Aga IVA-sensitive Ca channels are not inhibited by muscarinic receptors at the CA3–CA1 synapse of rat hippocampal slices.

Similar mechanisms underlie presynaptic inhibition of $[Ca_{pre}]_t$ by muscarinic and adenosine receptors

So far, we have shown that activation of presynaptic muscarinic receptors inhibits ω-CTx GVIA but not ω-Aga IVA-sensitive Ca channels. This is similar to the activation of presynaptic adenosine receptors at the CA3–CA1 synapse of guinea-pig hippocampus (Wu & Saggau, 1994b). If these two G-protein-coupled receptors would share a common point in their pathway at the presynaptic terminal, this would result in a similar inhibition of $[Ca_{pre}]_t$. In order to compare these two receptor systems, we first tested the action of adenosine in the rat hippocampus. In the rat, 100 μM adenosine inhibited the $[Ca_{pre}]_t$ and corresponding fe.p.s.p. by $38.0 \pm 1.8\%$ and $96.6 \pm 4.5\%$ ($n=8$), respectively, similar to the guinea-pig, (Figures 6a and b). Like the activation of muscarinic receptors, the inhibition of $[Ca_{pre}]_t$ by adenosine receptors was partially occluded by ω-CTx GVIA, while ω-Aga IVA had little effect, as shown in Figures 6c and e. The effects of these two Ca channel blockers on the inhibition of $[Ca_{pre}]_t$ by muscarinic and adenosine receptors are summarized in Figure 6d and f. In the presence of ω-CTx, application of adenosine inhibited $[Ca_{pre}]_t$ by $25.8 \pm 1.0\%$ ($n=4$). Thus, about 32% (=(38% – 25.8%)/38%) of the inhibition was abolished by the toxin. Normalized by the amount of ω-CTx GVIA-sensitive $[Ca_{pre}]_t$, the percentage inhibition of ω-CTx GVIA-sensitive $[Ca_{pre}]_t$ by adenosine was about 59% (=(38% – 25.8%)/20.5%). Subsequent application of adenosine after ω-Aga IVA elicited an inhibition of the $[Ca_{pre}]_t$ of $34.0 \pm 1.2\%$ ($n=4$). Normalized for the amount of ω-Aga IVA-sensitive $[Ca_{pre}]_t$, adenosine resulted in only 12% inhibition of ω-Aga IVA-sensitive $[Ca_{pre}]_t$ (12% = (38% – 34%)/34.7%). Thus, inhibition of ω-Aga IVA-sensitive Ca channels by adenosine was much smaller than the inhibition of ω-CTx GVIA-sensitive Ca channels (59%). Furthermore, in agreement with the similar profiles of Ca channel involvement, the inhibition of $[Ca_{pre}]_t$ by muscarinic and adenosine receptors occluded each other. As shown in Figure 7, 100 μM adenosine and 10 μM CCh alone both inhibited $[Ca_{pre}]_t$ by about 40%. In the presence of a saturating concentration of adenosine, the effect of CCh was completely occluded. This occlusion was independent of the sequence of

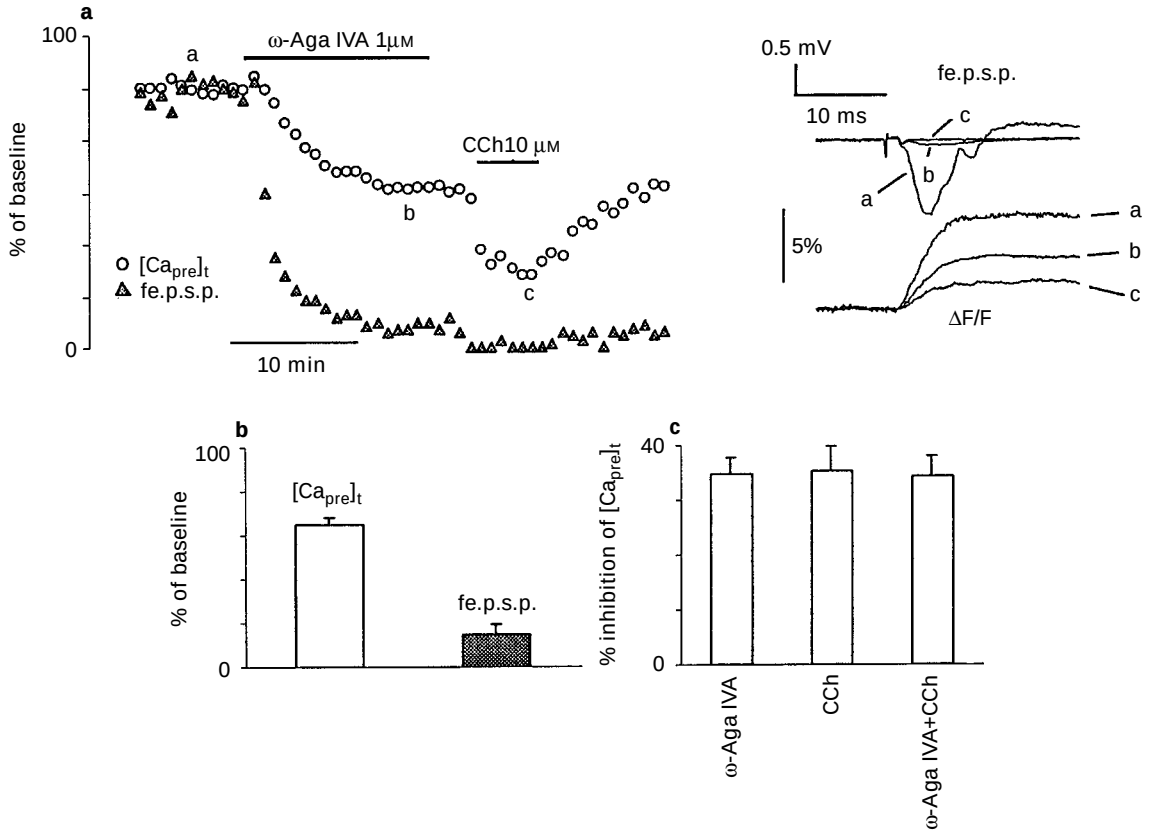

Figure 5 ω-Aga IVA did not occlude the inhibition of $[Ca_{pre}]_t$ by carbachol. (a) Time course of normalized fe.p.s.p. and $[Ca_{pre}]_t$ in a typical experiment showing the effects of ω-Aga IVA. The toxin irreversibly reduced the $[Ca_{pre}]_t$ and synaptic transmission, but had little effect on the inhibition of $[Ca_{pre}]_t$ by CCh. CCh still elicited a similar amount of inhibition on $[Ca_{pre}]_t$ as in the absence of the toxin. The inset shows sample recordings taken at times indicated. (b) Summary of data from five experiments. ω-Aga IVA largely inhibited $[Ca_{pre}]_t$ and synaptic transmission. (c) Effect of ω-Aga IVA on inhibition by CCh of $[Ca_{pre}]_t$. Despite the large inhibition of the $[Ca_{pre}]_t$ by ω-Aga IVA, this did not result in a significant change in the amount of inhibition by CCh.

drug application. In the presence of CCh, adenosine did not elicit any further inhibition of $[Ca_{pre}]_t$.

Discussion

Muscarinic receptor activation inhibits fe.p.s.p. by reducing $[Ca_{pre}]_t$

The inhibition of high-threshold VDCCs by muscarinic receptors has been shown in rat sympathetic neurones (Beech *et al*., 1992; Mathie *et al*., 1992), cultured hippocampal neurones (Toselli & Taglietti, 1994; 1995a) and other central neurones (Howe & Surmeier, 1995). The same mechanism may also occur at the presynaptic terminal. Indeed, we detected a reduction of action potential-induced calcium indicator fluorescence transients ($\Delta F/F$) when the muscarinic receptor agonist carbachol was applied to hippocampal slices (Figure 3a). The decrease of $\Delta F/F$ cannot be accounted for by reduced excitation of presynaptic axon fibres. A slight reduction of presynaptic volley (about 5%), observed during the application of CCh, is much less than the amount of decrease in $\Delta F/F$ produced by CCh (35%). In control experiments, when the stimulation intensity was varied, the $\Delta F/F$ showed a linear relationship with the size of presynaptic volley (data not shown). Therefore, the reduced $\Delta F/F$ reflects the decrease of presynaptic calcium influx rather than a reduction in the recruitement of axon fibres.

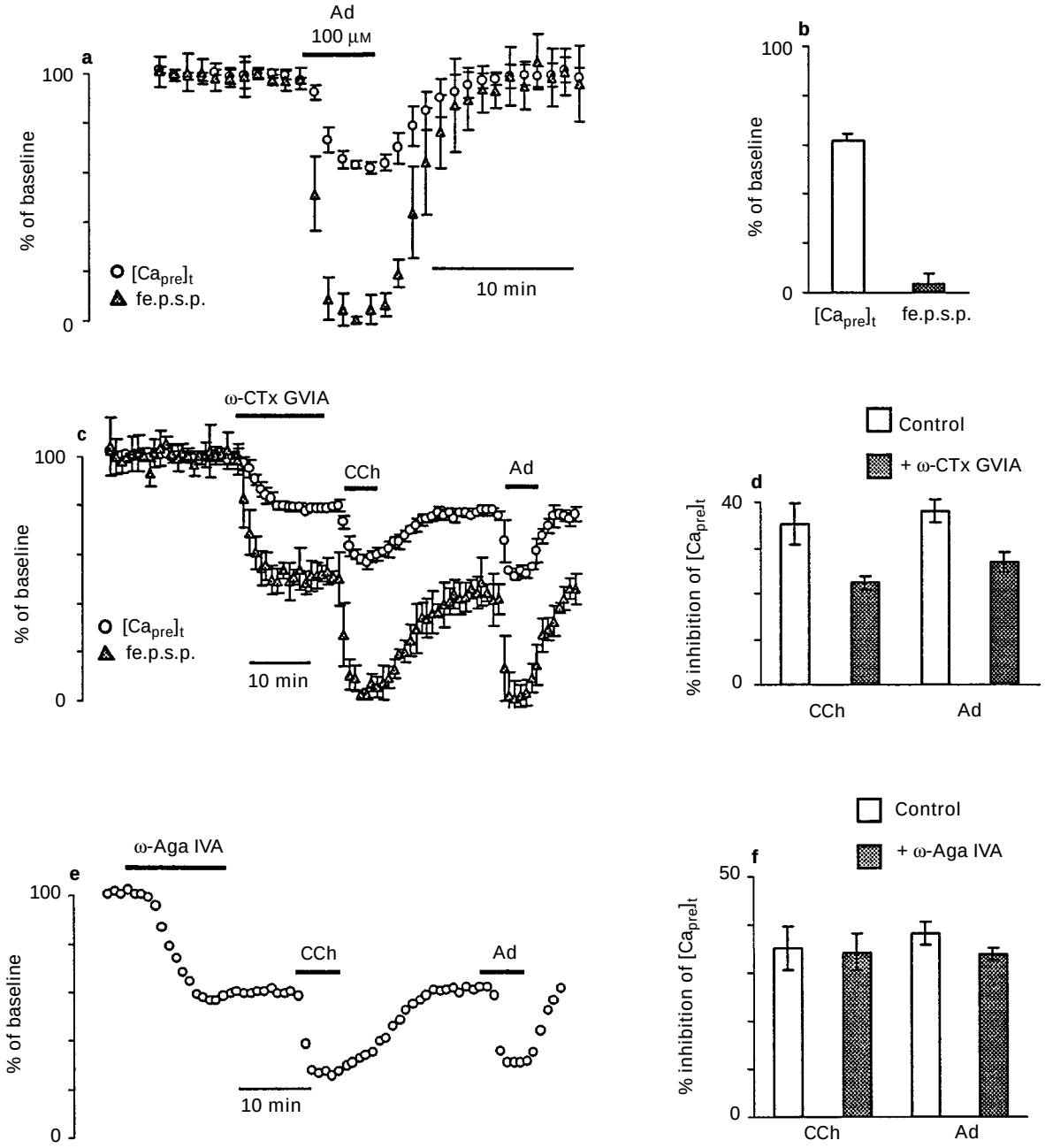

Figure 6 Similar mechanism for inhibition of $[Ca_{pre}]_t$ by activation of muscarinic and adenosine receptors. (a) Group data to show the inhibition by adenosine (Ad, 100 μM, $n=8$), which reduced both the $[Ca_{pre}]_t$ and the slope of the fe.p.s.p. fully recovered after 20 min wash. (b) Summary of data from 8 experiments. (c) Average time course of $[Ca_{pre}]_t$ modulation by CCh and adenosine after pretreatment with ω-CTx GVIA ($n=4$). (d) Summary of data for experiments in (c). Similar to CCh, adenosine further inhibited $[Ca_{pre}]_t$ by $25.8 \pm 1.0\%$. Compared to the amount of inhibition of $[Ca_{pre}]_t$ by adenosine under control conditions, ω-CTx GVIA partially blocked the effect of adenosine. (e) Time course of normalized $[Ca_{pre}]_t$ in a typical experiment showing the effects of CCh and adenosine after pretreatment with ω-Aga IVA. ω-Aga IVA did not block the inhibition of $[Ca_{pre}]_t$ by CCh or adenosine. (f) Summary of data from four experiments. ω-Aga IVA slightly reduced the inhibition of $[Ca_{pre}]_t$ by adenosine by about 12%.

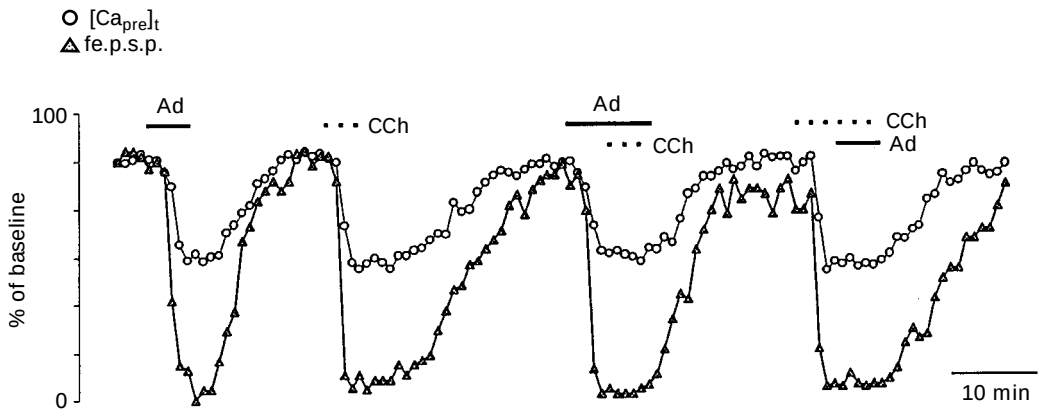

Figure 7 Activation of muscarinic and adenosine receptors occluded each other in the inhibition of $[Ca_{pre}]_t$. A typical experiment testing for occlusion of inhibition of $[Ca_{pre}]_t$ by CCh (10 μM) and adenosine (Ad, 100 μM).

Presynaptic VDCCs control neurosecretion in a non-linear way (Dodge & Rahamimoff, 1967; Katz & Miledi, 1970; Augustine & Charlton, 1986; Wu & Saggau, 1994a; Mintz *et al*., 1995; Borst & Sakmann, 1996). The inhibition of $[Ca_{pre}]_t$ has been shown to contribute to the reduction of action potential evoked transmitter release by various neuromodulators such as adenosine, baclofen and neuropeptide Y in the mammalian central nervous system (for review see Wu & Saggau, 1997). During application of these neuromodulators, a close to 4th power relationship between $[Ca_{pre}]_t$ and fe.p.s.p. was found. Here, a similar power relationship ($m = 4.5$) was also observed during application of CCh (Figure 3c), indicating that the inhibition of $[Ca_{pre}]_t$ contributes to the reduction of transmitter release by activation of muscarinic receptors. The power number for the effect of CCh was slightly greater than that obtained in control experiments where $[Ca_{pre}]_t$ was decreased by reducing $[Ca]_o$ ($m = 4.0$, Figure 2c). In general, an increased power number during the application of a neuromodulator suggests that an additional mechanism downstream to Ca influx is involved (for review see Wu & Saggau, 1997). If this additional mechanism is assumed to be a Ca-independent inhibition of the release process, about 20% inhibition of the release machinery by mucarinic receptors is estimated. Such a calcium-independent reduction in the frequency of me.p.s.cs by muscarinic receptors was observed at synapses onto CA3 pyramidal neurones of cultured hippocampal slices (Scanziani *et al*., 1995). Interestingly, these authors found no evidence for inhibition of Ca channels by muscarinic receptors. However, in addition to the use of cultured hippocampal slices, there are substantial differences in the experimental approaches used. Scanziani *et al*. tonically depolarized neurones by raising $[K^+]_o$ to activate VDCCs and induce Ca-dependent release, while we employed single action potentials to evoke release. In normal and high $[K^+]_o$, the former found no significant difference in the reduction of me.p.s.c. frequency during activation of muscarinic receptors and concluded that presynaptic VDCCs are not involved in muscarinic presynaptic inhibition. However, it is not clear if the inhibition of presynaptic VDCCs by muscarinic receptors was altered by the employed tonic depolarization, since the G-protein-mediated inhibition of calcium channels has been demonstrated to be voltage-dependent in several preparations (Hille, 1994). At a cerebellar synapse, a combination of Ca-dependent and Ca-independent mechanisms was shown to be involved in the inhibition of synaptic transmission by baclofen, while adenosine receptor reduced synaptic strength by only modulating presynaptic calcium channels (Dittman & Regehr, 1996). On the other hand, presynaptic inhibition by activation of $GABA_B$ receptors has been shown to affect only Ca channels at hippocampal CA3-CA1 synapses (Wu & Saggau, 1995) and inhibitory synapses at CA1 pyramidal neurones (Doze *et al*., 1995). Therefore, it seems likely that different mechanisms of presynaptic inhibition may be expressed to different extents across synapses. A postsynaptic contribution to this additional inhibition could not be completely ruled out. Activation of muscarinic receptors has been shown to cause postsynaptic membrane depolarization by modulating various K^+ conductance at CA1 neurones (Dutar & Nicoll, 1988). The decreased driving force due to such a membrane depolarization could also result in a reduction of the postsynaptic currents. In summary, inhibition of the $[Ca_{pre}]_t$ and an inhibitory mechanism downstream to $[Ca_{pre}]_t$ contribute to the presynaptic inhibition of synaptic transmission. However, the reduction in $[Ca_{pre}]_t$ plays the dominant role in the inhibition of action potential evoked synaptic transmission by muscarinic receptors at the CA3-CA1 synapse.

Two pharmacologically distinct presynaptic VDCCs

At the CA3-CA1 synapse of rat hippocampal slices, application of ω-CTx GVIA has been shown to block partially synaptic transmission (Luebke & Dunlap, 1993; Takahashi & Momiyama, 1994; Wheeler *et al*., 1994). We found that the maximal reduction of $[Ca_{pre}]_t$ by ω-CTx GVIA to be about 20.5%. The corresponding remaining fe.p.s.p, was about 51.4% of baseline, which results in the power number, $m = 2.9 \pm 0.3$. Application of ω-Aga IVA (1 μM) produced about 35% inhibition of $[Ca_{pre}]_t$. The average remaining fraction of fe.p.s.p. was about 15% of baseline. This is consistent with earlier findings at the CA3-CA1 synapse (Takahashi *et al*., 1994; Wheeler *et al*., 1994). A 4th power calculation of average inhibition of the $[Ca_{pre}]_t$ predicted an inhibition of fe.p.s.p. to 18% of baseline, which is close to the measured value. Our data also indicate that at the rat CA3-CA1 synapse about 21% of presynaptic $[Ca_{pre}]_t$ is ω-CTx GVIA-sensitive and about 35% is ω-Aga IVA-sensitive. The remaining 44% of $[Ca_{pre}]_t$ is resistant to both toxins and could not be further reduced by applying antagonists of L-type Ca channels (data not shown). These results are consistent with the previous findings at the same synapse in the guinea-pig. Presynaptic VDCCs, sensitive to ω-CTx GVIA and ω-Aga IVA, as well as those insensitive to both toxins, are co-localized at presynaptic terminals, as the total percentage inhibition of the fe.p.s.ps by both toxins is much more than 100% (Wu & Saggau, 1994c). This is in contrast to a recent study in cultured hippocampal neurones, where a heterogeneous terminal population was found (Reuter, 1995). However, it is unclear to what extent such cultures represent the situation in the brain.

Differential effects of Ca channel blockers on inhibition of $[Ca_{pre}]_t$ by carbachol

Muscarinic receptors have been shown to inhibit ω-CTx GVIA-sensitive Ca channels in a membrane delimited way. The activation of muscarinic receptors shifts the Ca channel activation curve to a more depolarized potential (Mathie *et al*., 1992; Toselli *et al*., 1994; 1995). Reductions in both the

probability of channel opening and the mean open time contributed to the overall current reduction. In our experiments, the difference between muscarinic inhibition of $[Ca_{pre}]_t$ under control conditions and in the presence of ω-CTx GVIA reveals the amount of inhibition of $[Ca_{pre}]_t$ contributed by ω-CTx GVIA-sensitive Ca channels. This difference normalized by total ω-CTx GVIA-sensitive $[Ca_{pre}]_t$, indicates that the percentage inhibition of ω-CTx GVIA-sensitive $[Ca_{pre}]_t$ by muscarinic receptors was about 63%.

ω-Aga IVA-sensitive Ca channels were shown to be inhibited by a G-protein in rat cerebellar Purkinje neurones (Mintz & Bean, 1993) and a pertussis toxin-sensitive G-protein in rat striatal neurones (Howe & Surmeier, 1995). However, in our preparation we did not detect a significant difference in the inhibition of $[Ca_{pre}]_t$ by CCh in the presence or absence of ω-Aga IVA. It was shown that the inhibition of ω-Aga IVA-sensitive Ca channels by muscarinic receptors was BAPTA-insensitive (Howe & Surmeier, 1995). Therefore, it is unlikely that the presence of a high affinity Ca indicator (i.e., Fura-2) within presynaptic terminals, which might somehow prevent the generation of the Ca-dependent second messenger, occluded the inhibition of ω-Aga IVA-sensitive Ca channels by muscarinic receptors.

The observed different percentage inhibitions for ω-CTx GVIA and ω-Aga IVA sensitive-$[Ca_{pre}]_t$ make it highly unlikely that muscarinic receptors activate more K^+ channels during presynaptic membrane repolarization. Otherwise, a similar percentage inhibition for both types of Ca channels should be detected, because a shortening of the action potential duration would equally affect each type of presynaptic VDCCs. To test further if K^+ channels are involved, adenosine was co-applied with CCh. Activation of adenosine receptors has been shown to inhibit only Ca channels (Wu & Saggau, 1994b; Dittman & Regehr, 1996). If CCh activates K^+ channels, an additional inhibition of $[Ca_{pre}]_t$ would be detected in the presence of adenosine due to the reduced duration of the action potential. However, the experimental data did not support this scenario. In the presence of a saturating concentration of adenosine, CCh could not elicit any further inhibition of $[Ca_{pre}]_t$ (Figure 7).

Our results also indicate that of the total inhibition of $[Ca_{pre}]_t$ by muscarinic receptors (35%), only a small part (13%) was due to the inhibition of ω-CTx GVIA-sensitive $[Ca_{pre}]_t$. The remaining 22% was mainly attributed to the inhibition of unidentified Ca channels resistant to both ω-CTx GVIA and ω-Aga IVA. Even though there was a large amount of inhibition of these unidentified Ca channels by muscarinic receptors, we still observed a close to 4th power relationship between $[Ca_{pre}]_t$ and synaptic transmission when muscarinic receptors were activated. This further suggests that these Ca channels play a similar role in triggering neurotransmitter release as the other two well characterized presynaptic VDCCs at the CA3–CA1 synapse of rat hippocampus.

The activation of muscarinic receptors and adenosine receptors converges at some point controlling VDCCs

Several lines of evidence suggest that activation of muscarinic receptors and adenosine receptors converge at some point in their functional pathway to Ca channels. Firstly, activation of muscarinic and adenosine receptors both resulted in about the same maximal inhibition of $[Ca_{pre}]_t$. CCh (50 μM) inhibited $[Ca_{pre}]_t$ by 37% while adenosine (100 μM) inhibited it by about 38%. Secondly, a similar profile of Ca channel types was involved in this modulation: ω-CTx GVIA partially blocked the effect of both drugs while ω-Aga IVA had little effect. Furthermore, similar percentages of inhibition of ω-CTx-sensitive $[Ca_{pre}]_t$ were obtained for activation of both types of receptor (63% for muscarinic receptor, 59% for adenosine receptor). Finally, the effect of CCh and adenosine completely occluded each other. Taken together, these findings suggest that a convergent point exists on the pathway activating muscarinic and adenosine receptors. This common point may be the same G-protein coupled to both receptors, or, different G-protein subunits activated by both receptors may act on the same VDCCs.

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