Eur Biophys J (1994) 22:391–398

European Biophysics Journal

Muscarinic inhibition of high-voltage-activated calcium channels in excised membranes of rat hippocampal neurons

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Received: 18 March 1993 / Accepted in revised form: 10 September 1993

Abstract. The effect of muscarine on voltage-gated calcium channels was investigated in outside-out patches from rat hippocampal neurons in culture. By clamping the excised patches at −60 mV holding potential, single and multiple Ca channel currents were recorded, and these displayed features similar to the high-voltage-activated Ca current, with unitary conductance of 6.4 pS in 50 mM external Ca^{2+}. These channels turned out to be insensitive both to Bay K 8644 and to ω-conotoxin. In excised patches muscarine caused a marked reduction in the probability of opening of this class of Ca channels without significant changes in the unitary current amplitude. Interestingly, the degree of current inhibition was reduced by depolarization, thus suggesting a voltage-dependent inhibitory action of the agonist. We conclude that in hippocampal neurons one of the possible ways of HVA Ca channel modulation by muscarine occurs through activation of a substratum localized within the plasma membrane of the cell, independent of the involvement of diffusible intracellular messengers.

Key words: Hippocampal neurons – Ca channel – Muscarine

Introduction

Activation of muscarinic receptors can alter the properties of voltage-dependent Ca conductances in both peripheral and central neurons (Misgeld et al. 1986; Gähwiler and Brown 1987; Wanke et al. 1987; Toselli and Lux 1989; Tse et al. 1990; Bernheim et al. 1991). However, uncertainty exists about the kind(s) of intracellular pathways involved in modulation. Recent observations obtained from macroscopic Ca currents suggest that, in sympathetic neurons, activation of muscarinic receptors can affect HVA Ca channels with at least two distinct modalities: one is voltage-dependent and membrane-delimited, while the other is voltage-independent and coupled to a diffusible second messenger (Bernheim et al. 1991). Indeed, the second modality of muscarinic action could be demonstrated in on-cell patches (Mathie et al. 1992). In hippocampal neurons, it was proposed as a working hypothesis that muscarinic inhibition of Ca channels, beside other possibilities, could occur through activation of a membrane-delimited pathway (Toselli et al. 1989). In order to test such an hypothesis, it was of interest to determine whether muscarinic inhibition of Ca channels could also be observed in excised outside-out patches, as would be expected if a membrane-delimited modulatory pathway is involved. Therefore we measured calcium currents from outside-out membrane patches from hippocampal neurons and examined the action of muscarine on Ca-channel activity. A preliminary account of this work has been reported previously in abstract form (Toselli and Taglietti 1992 b).

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Materials and methods

Primary cultures of rat hippocampal neurons were obtained from 18 to 19-day-old foetuses as already described (Segal 1983; Toselli et al. 1989). The bath solution contained (in mmol/l): $CaCl_2$ 5, NaCl 130, $MgCl_2$ 2, glucose 20, hepes/NaOH 10 (pH 7.4), tetrodotoxin 3×10^{-3}. During outside-out patch recordings the bath solution was changed as follows (mmol/l): $CaCl_2$ 50, NaCl 52, $MgCl_2$ 2, glucose 15, hepes/NaOH 10 (pH 7.4), tetrodotoxin 3×10^{-3}. Glass pipettes were coated at their tips with Sylgard to reduce capacitance and were made to have a resistance of 4–10 MΩ, depending on the size of the membrane patch to be isolated. For experiments with excised patches they were filled with (in mmol/l): CsCl 130, TEA-Cl 20, EGTA 10, $MgCl_2$ 2, hepes/CsOH 10 (pH 7.4), GTP 0.1, Mg-ATP 4, creatine phosphate 5, creatine phosphokinase 20 units/ml. The ATP-regenerating system reduced and slowed down but did not eliminate Ca current run-down. In cell-attached patch recordings the pipette contained (in mmol/l): $CaCl_2$ 50, NaCl 52, $MgCl_2$ 2, TEA 15, hepes/NaOH 10 (pH 7.4).

Unitary current records, obtained at room temperature with a LIST LM/EPC7 patch-clamp amplifier, were digitized at sampling intervals of 40 μs using a 12 bit A/D converter (LabMaster) interfaced with an Olivetti M380/C PC and low pass-filtered at 1 KHz. Capacitive and leakage currents of each record were reduced by analogue circuitry and then almost completely eliminated by subtracting the average of 16 sweeps obtained in the presence of 200 μM Cd.

The run-down of Ca channels was relatively fast in excised patches when compared with that observed in whole-cell recordings where a current decrease of less than 10% was generally observed within 15 min. Using a protocol in which depolarizing test pulses of 140 ms duration at 0.2 Hz were applied to the membrane patches, a sizeable run-down was observed, on average, after less than 1 min from the beginning of recordings when the ATP-regenerating system was absent from the intracellular solution. This caused us to discard about 90% of successful outside-out patches. In the presence of the ATP-regenerating system, although with variability among different patches, sizeable run-down was clearly delayed. Stable Ca channel activity could be recorded for about 4 min and in any case for no longer than 10 min (see also Aosaki and Kasai 1989; Shen and Surprenant 1991). This reduced the percentage of discarded records to about 70%.

When the action of muscarine was tested on single channel records, a variable number of sweeps was obtained before, during and after agonist application. Data analysis, however, was restricted to those patches in which the average number of openings per sweep differed by less than 20% between control and washout. As a rule, a quantitative analysis of the action of the agonist was done by comparing the single channel activity recorded during perfusion with muscarine and during washout following agonist application, in order to compensate for the current run-down.

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), except ω-conotoxin, fraction GVIA, which was obtained from Peninsula Laboratories and Bay K 8644, which was a gift from Dr. Seuter, Bayer AG. External solutions were exchanged by fast perfusion using a multi-barreled ejection pipette of about 100 μm opening, which was positioned at a few tens of microns from the membrane patch.

Opening and closing transitions were detected by establishing a baseline for the closed state and amplitude levels for open state(s); excursions of data points of more than 50% from the baseline to the first open channel amplitude level were interpreted as an opening. Stimulation, acquisition and data analysis were done using pClamp software (Axon Instruments).

Results

Ca currents in outside-out macropatches

Using pipettes with a final tip diameter of about 1.5 μm, excised outside-out membrane patches containing a large number of Ca channels could be isolated (Fig. 1). Several lines of evidence suggest that these patches contained two types of Ca currents (low-voltage activated, LVA, and high-voltage activated, HVA) similar to those previously described in the whole-cell configuration (Toselli and Taglietti 1992a). First, starting from a holding potential of −90 mV, current inactivation was fast and complete at potentials more negative than +10 mV and became slow and incomplete at more positive voltages (Fig. 1A); secondly, the peak-current/voltage relationship was biphasic in the range between −50 and +10 mV reaching a maximum at about +30 mV (Fig. 1C, circles); thirdly, changing the holding potential from −90 mV to −60 mV produced suppression of the fast and completely inactivating component, i.e. the LVA Ca current. The remaining component showed features of the HVA Ca current (Fig. 1B): it turned on at potentials positive to −30 mV, inactivation was slow and incomplete and the peak-current/voltage relationship became monophasic in the range between −50 and +10 mV, reaching a maximum at about +30 mV (Fig. 1C, triangles).

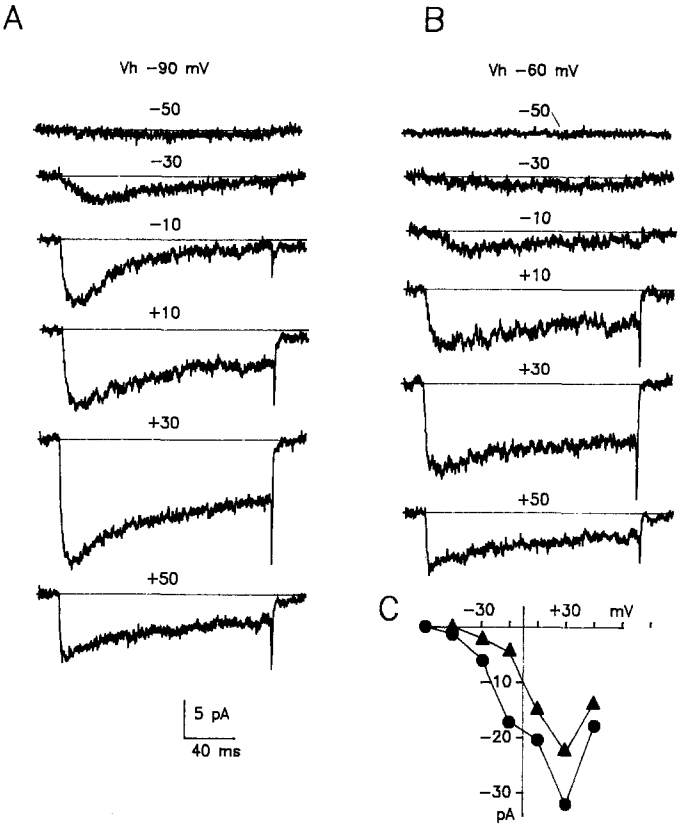

Fig. 1A–C. Ca currents in an outside-out macropatch from a hippocampal neuron recorded at the potentials indicated. The patch of membrane was clamped at −90 mV holding potential in **A** and at −60 mV in **B**. **C**, Peak I–V relationship of records in **A** (*circles*) and **B** (*triangles*)

Since the interest of this study was focused on the effect of muscarine on the HVA Ca current, in the next experiments membrane patches were clamped at −60 mV holding potential, to avoid contamination with the LVA current component.

Muscarine had a depressive and reversible action on HVA Ca currents in outside-out macropatches. In 6 out

of 7 cells a maximum of inhibition was observed at test potentials between −10 and +10 mV (Fig. 2A), this inhibition being less evident at more positive potentials (Fig. 2B). For a quantitative description of this effect, the current-voltage relationship was estimated (Fig. 2C). Muscarine was found to reduce the peak amplitude of the HVA current by an average of 38% ± 8% at +10 mV ($n=6$), and the partially inactivated current at the end of a 140 ms pulse was similarly depressed (42% ± 9%). At +30 mV the percentage of inhibition was reduced to 29% ± 14% at the peak of the current and was negligible at the end of the step. At +50 mV both the peak current and that measured at the end of the step were not significantly inhibited by the agonist. These results are consistent with those obtained in whole-cells recordings (Toselli and Taglietti 1993), suggesting a voltage- and time-dependent modulatory action of muscarine on HVA channels. In one cell however, the inhibitory action of muscarine did not follow the previously described features: for instance the percentage of inhibition did not decrease significantly by increasing the amplitude of voltage step (Fig. 2D–E).

Interestingly, muscarine clearly decreased Ca channel activity only when GTP was available in the patch pipette. In fact, in 11 excised patches not internally perfused with GTP there was no significant decrease of Ca current during muscarine application, the percentage of inhibition being 4% ± 7% at +10 mV.

Whole-cell Ca currents from hippocampal neurons can be pharmacologically dissected into three components on the basis of their sensitivity to ω-conotoxin (ω-CgTx) and to the dihydropyridine agonist Bay K 8644: a ω-CgTx-sensitive and Bay K insensitive component, a ω-CgTx-insensitive and Bay K sensitive component and a third component, insensitive to both ω-CgTx and Bay K (Toselli and Taglietti 1992a). For a pharmacological characterization of the muscarine-inhibited current observed in outside-out macropatches, sensitivity to Bay K and to ω-CgTx were tested. The example tracings in Fig. 3 indicate a lack of effect of either Bay K (5 μM) or ω-CgTx (5 μM) on Ca currents elicited at +20 mV after focal perfusion of the drugs for about 1 min. On the average we measured a decrease of the peak current of 3% ± 10% and 5% ± 8% with Bay K and ω-CgTx respectively ($n=4$).

Single HVA Ca channels in outside-out patches

To resolve unitary Ca currents in outside-out patches, we used pipettes of tip diameters less than 1 μm. Also in this case, to avoid contamination with the LVA component, Ca currents were elicited from a holding potential of −60 mV. Examples of single-channel recordings at different test potentials are shown in Fig. 4A. No channel activity was observed for depolarizing pulses to −50 mV. Unitary currents began to appear at test potentials of −30 mV; however, a considerable number of sweeps failed to show channel activity, indicating a low probability of channel opening at this test potential. At higher voltages, the frequency of openings increased and current amplitude became progressively smaller. Single channel activity was completely abolished by patch perfusion with 200 μM cadmium (Fig. 4A, bottom). The averaged single channel current at 0 mV showed slow and in-

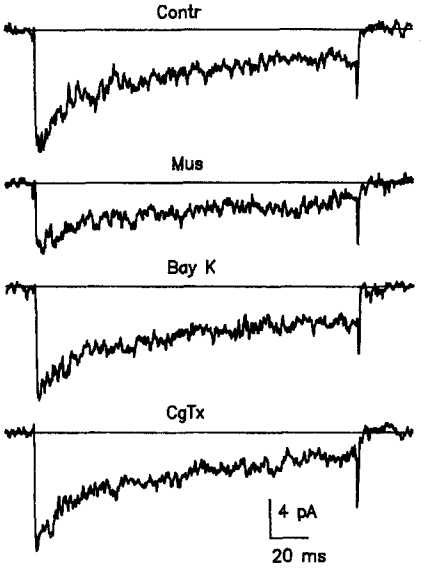

Fig. 3. Effects of Bay K 8644 and of ω-CgTx on Ca currents recorded from an outside-out macropatch. Current traces were elicited by a step pulse to +20 mV from a holding potential of −60 mV during focal perfusion of the outer membrane patch with control saline (Contr), 30 μM muscarine (Mus), 5 μM Bay K (Bay K) and with 5 μM ω-CgTx (CgTx). Traces obtained during washout with control saline after drug applications are not shown for simplicity

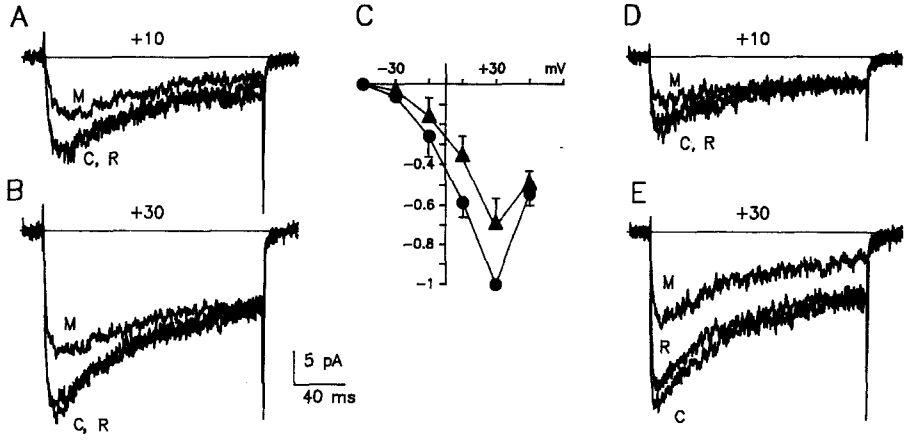

Fig. 2A–B. Effect of muscarine (10 μM) on HVA Ca currents from an outside-out macropatch recorded at +10 mV and +30 mV in control conditions (C), during external perfusion of muscarine (M) and during wash-out (R). Holding potential: −60 mV. **C** Normalized Ca current average amplitude ($n=6$) versus voltage measured at the peak of control currents (*circles*) and at the corresponding time in the presence of muscarine (*triangles*). **D–E** muscarine-sensitive Ca currents from another outside-out macropatch recorded as in **A** and **B** respectively, where the action of muscarine displays different features

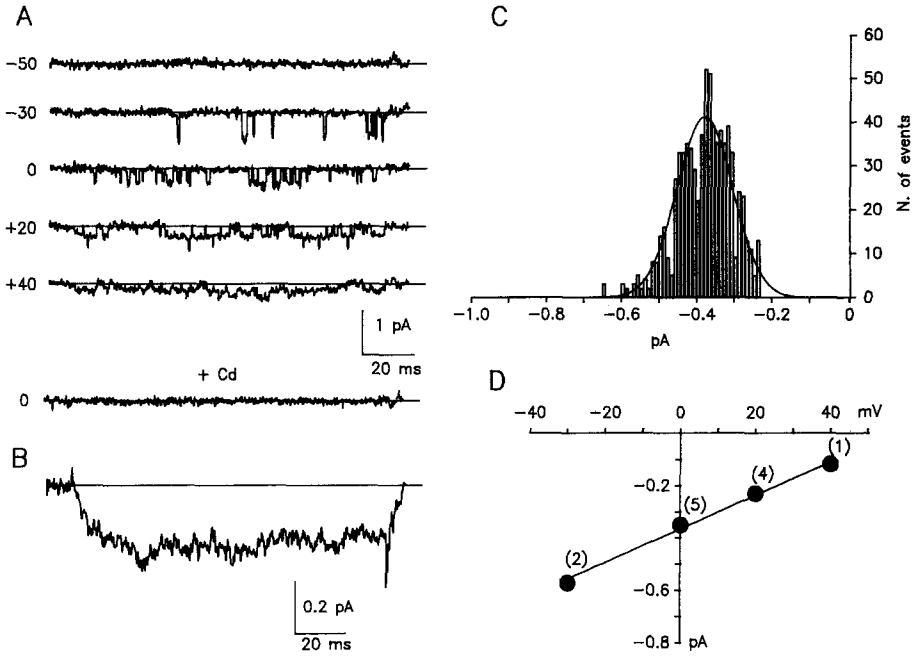

Fig. 4A–D. Single Ca channel currents in an outside-out patch. **A** Example tracings of single-channel activity recorded at the potentials indicated from a holding potential of −60 mV. The bottom trace shows that channel activity (here at 0 mV) is abolished in the presence of 200 μM cadmium. **B** Average over 64 samples of single channel currents recorded at 0 mV. **C** Amplitude histogram of single channel openings recorded at 0 mV and fitted with a Gaussian function (mean = −0.38 ± 0.07 pA). **D** I-V relationship for HVA channels. Data points represent means of the values collected from a number of patches indicated within brackets. The solid line was obtained by best fitting and gives a slope conductance of 6.4 pS in 50 mM external Ca^{2+}

complete inactivation during a 130 ms depolarizing step (Fig. 4B).

Amplitude distributions associated with the single channel recordings of Fig. 4A were obtained using 16 to 32 samples for a variable number of experiments at each test potential. One example of an amplitude histogram for the data obtained at 0 mV is illustrated in Fig. 4C. The relationship between single-channel current and test potential was linear between −30 and +40 mV with a slope conductance (γ) of 6.4 pS in 50 mM Ca (Fig. 4D). This estimate of γ is close to that of the HVA Ca channel in other neurons where Ca was used as charge carrier in cell-attached experiments (Lux and Nagy 1981; Hagiwara and Ohmori 1983; Lux and Brown 1984). A second Ca conductance (23 pS in 110 mM external Ba^{2+}) was observed by others in several neuronal cell types (Fox et al. 1987; Plummer et al. 1989; Aosaki and Kasai 1989; Fisher et al. 1990; O'Dell and Alger 1991; Mogul and Fox 1991). No trace of this high conductance was found in our records. Interestingly however a lack or a very fast disappearance of this conductance in excised patches has been described (Fenwick et al. 1982; Codina et al. 1987; Carbone and Lux 1987; Shen and Surprenant 1991). To check this point we ran some control experiments in cell-attached configuration in the presence of 50 mM Ca. The patches were depolarized by 10 mV from the resting potentials that in cultured hippocampal neurons (5–8 days) were observed to range between −50 and −60 mV (see also Segal 1983). This was equivalent to holding the patch at −60 to −50 mV, causing steady-state LVA channel inactivation. Unitary HVA Ca currents were evoked by depolarizing pulses of +50 mV, and from the analysis of the single channel activity obtained from 3 separate patches we measured two different mean unitary current amplitudes of 0.31 ± 0.06 pA and 0.58 ± 0.04 pA, the former comparing well with that measured at 0 mV in excised patches (Fig. 5). Interestingly, single Ca channel

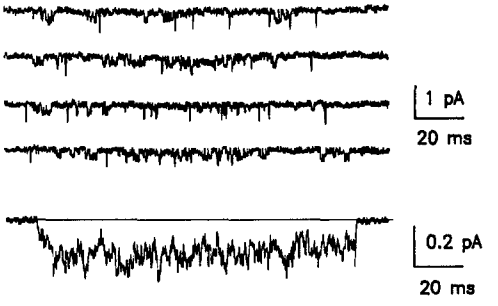

Fig. 5. HVA single Ca channel currents recorded in a cell attached patch in 50 mM Ca. The cell was held at a potential level 10 mV more positive than the resting potential. Step depolarization (+50 mV) was delivered from this level. The bottom trace is the average current from 36 records

activity could be measured under these conditions for more than 15 min without observing sizeable run-down.

Modulation of single HVA Ca channels by muscarine

Muscarine reduced the number of openings of the HVA channels evoked by depolarization of outside-out patches, as is evident from a visual inspection of single channel traces in Fig. 6. A reduction in the averaged current is also evident (Fig. 6, bottom). By step depolarizations from −60 mV to 0 mV, we measured a reduction of the averaged current by 68% ±17% ($n = 5$) when outside-out patches were perfused with 10 μM muscarine.

The amplitude distribution of unitary currents measured at 0 mV during washout with control saline had a maximum at −0.37 ± 0.04 pA ($n = 5$), which did not differ significantly from that measured during application of muscarine (−0.35 ± 0.03 pA). Thus muscarinic modu-

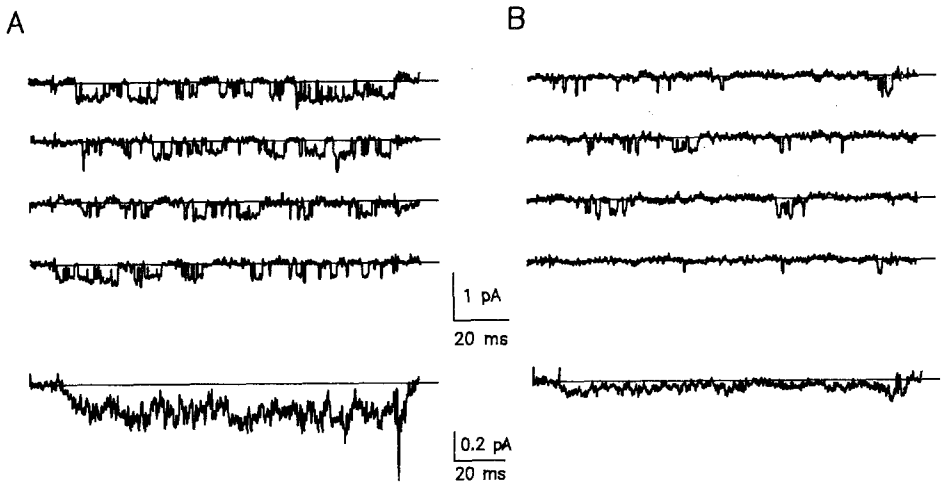

Fig. 6 A, B. Effect of muscarine on unitary Ca currents in an outside-out patch. Tracings were evoked by steps to 0 mV during wash-out **A** and during external perfusion with muscarine **B**. A reduction of the frequency of openings is evident in the presence of the agonist. Comparison was done with responses after application of the agonist in order to compensate for gradual run-down of channel activity with time. The lower traces are ensemble currents from 32 records. Holding potential −60 mV

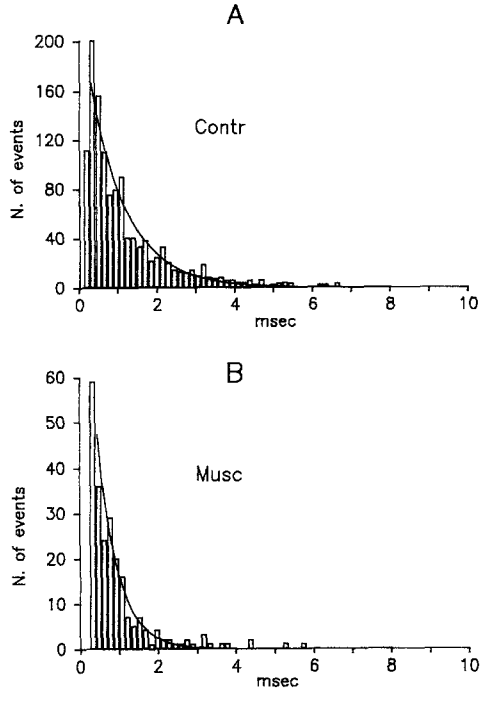

Fig. 7 A, B. Example of open time distributions fitted with single exponentials revealing mean open times of 0.84 ms during washing **A** and of 0.52 ms during muscarine application **B** Data were obtained for step depolarizations to 0 mV from an holding potential of −60 mV

lation does not affect the elementary conductance of HVA Ca channels.

Open time distributions for HVA Ca channels at 0 mV in the presence of muscarine (10 μM) were compared with those during washout with control saline. The distributions were best fitted by single exponentials (Fig. 7) with time constants 0.63 ± 0.17 ms and 0.44 ± 0.12 ms ($n=5$) during washout and in the presence of muscarine respectively, corresponding to a 30% decrease of mean open time with muscarine. In the same patches the frequency of opening (total number of events in the record/duration of the record) was reduced from 0.44 ± 0.14 ms^{-1} during washout to 0.27 ± 0.04 ms^{-1} during muscarine application, which means a 39% decrease of opening frequency with muscarine. Thus, both reduction in the probability of opening and decrease in channel open time contributed to the overall current reduction observed in the presence of muscarine.

Gradual run-down of channel activity a few minutes after the formation of outside-out patches usually prevented adequate comparisons of the action of muscarine at different test potentials in the same patch. In two patches, however, the effect of muscarine on single Ca channels could be adequately recorded both at 0 mV and at +20 mV, obtaining a reduction of the averaged current by 61% and by 32% respectively ($n=2$). Single channel activity recorded in these two patches at 0 mV displayed features similar to those previously described (see Fig. 4) and therefore it is not further shown. Example tracings and averaged currents obtained by step depolarization to +20 mV are shown in Fig. 8. Simple visual inspection of tracings shows that a reduction in opening frequency in the presence of the agonist is more evident at the beginning than at the end of the voltage step. The results obtained at 0 and +20 mV agree qualitatively with those obtained in outside-out macropatches (see above), where a global attenuation in the percentage of current inhibition was evident for test potentials positive to −10 mV, suggesting a voltage-dependent effect of muscarine. This effect was observed in both excised patches where the action of muscarine was tested at +20 mV.

As with macropatches (see Fig. 3), Bay K and ω-CgTx were tested in three outside-out patches where single Ca channel currents, elicited at −20 mV, showed sensitivity to muscarine. Mean open times were 0.51 ms, 0.59 ms and 0.54 ms for the control, during Bay K and ω-CgTx application respectively, while the frequencies of opening were 0.39, 0.30 and 0.34 ms^{-1} respectively. These data, together with a simple visual inspection of the example tracings in Fig. 9, indicate that while muscarine significantly reduced the number of openings, application of either Bay K (5 μM) or ω-CgTx (5 μM) did not significantly affect single Ca channel activity, thus corroborating the previously described results obtained with macropatches that this muscarine-sensitive channel is Bay K and ω-CgTx insensitive.

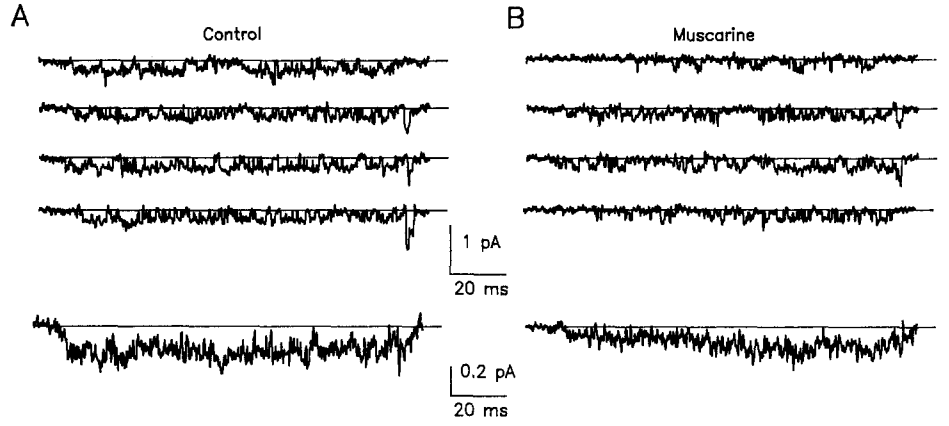

Fig. 8 A, B. Effect of muscarine on unitary Ca currents in an outside-out patch for a voltage step to +20 mV. Example tracings of single channel currents were recorded during washout **A** and during external perfusion with muscarine **B**. Notice that a reduction of the opening frequency in the presence of the agonist is more evident at the beginning than at the end of the voltage step to +20 mV. Holding potential − 60 mV

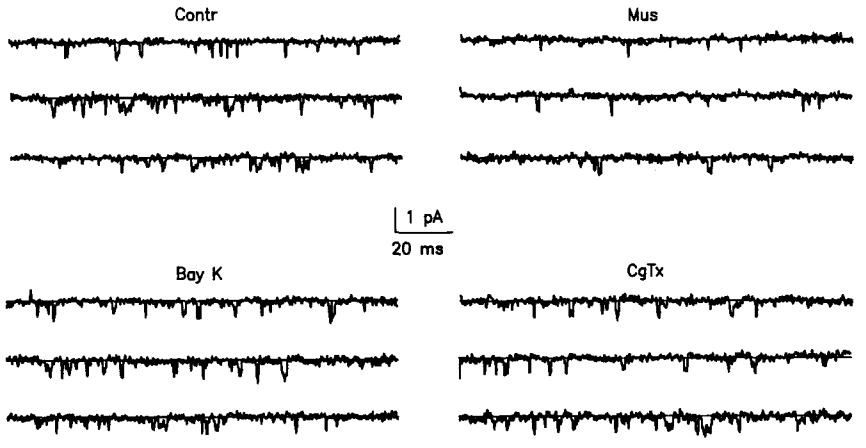

Fig. 9. Effects of Bay K 8644 and of ω-CgTx on single Ca-channel currents recorded from an outside-out patch. Current traces were elicited by a step pulse to −20 mV from an holding potential of −60 mV during focal perfusion of the outer membrane patch with control saline (Contr), 30 μM muscarine (Mus), 5 μM Bay K (Bay K) and with 5 μM ω-CgTx (CgTx). The time of delivery of the drugs was about 1 min

Discussion

The present data, obtained from the analysis of results from outside-out patches, corroborate previous observations from whole-cell recordings that at least two types of voltage-activated Ca channels can be isolated in hippocampal neurons (Yaari et al. 1987; Meyers and Barker 1989; Mogul and Fox 1991; Thompson and Wong 1991; Toselli and Taglietti 1992a). They correspond to the LVA and HVA Ca conductances found in many neuronal cell-types. HVA Ca channels, isolated from LVA channels by clamping the outside-out membrane macropatches at −60 mV holding potential, displayed an activation threshold of −30 mV and slow and incomplete inactivation.

We have shown that muscarine decreased Ca channel activity in outside-out patches, thus substantiating observations from whole-cell recordings about an inhibitory action of the agonist on HVA Ca currents described in peripheral as well as central neurons (Gähwiler and Brown 1987; Wanke et al. 1987; Toselli and Lux 1989; Tse et al. 1990; Bernheim et al. 1991).

The muscarine-sensitive channel investigated here in outside-out patches displayed a slope conductance of 6.4 pS in 50 mM Ca and insensitivity to Bay K 8644, therefore it cannot be classified as an 'L-type' channel which are Bay K sensitive and display a conductance of about 25 pS (in 110 mM Ba) (Fox et al. 1987; Aosaki and Kasai 1989; Fisher et al. 1990; O'Dell and Alger 1991). Its conductance is closer to that of the 'N-type' channel (13 pS in 110 mM Ba) and the discrepancy between our estimate of γ and that of others might be attributed to differences in the experimental conditions such as, for instance, cell preparation and charge carrier. However, we have shown that this Ca conductance is not significantly affected by application of ω-CgTx. Indeed, Bay K and ω-CgTx insensitive Ca channel subtype(s) have been recently described in central neurons: in some cases they have been shown to be blocked by ω-agatoxin and have been classified as 'P-type' channels (Llinas et al. 1989; Mintz et al. 1992). Possible sensitivity of the Ca channels described here to ω-agatoxin was not examined in this study.

In sympathetic neurons the modulation of HVA Ca channels by muscarinic receptor activation has been shown to occur through at least two different pathways, one membrane delimited and voltage-dependent, and the other voltage-independent and operated through a diffusible messenger (Bernheim et al. 1991). Indeed, the second kind of modulation was confirmed in cell-attached patch experiments which showed a muscarinic inhibition of the two HVA Ca conductances (13 pS and 23 pS in 110 mM Ba^{2+}) found in those neurons (Mathie et al. 1992). Also in cultured hippocampal neurons a slow and voltage-independent, and a faster and voltage-dependent muscarinic modulatory mechanism can be dissected in whole-cell experiments (Toselli and Taglietti 1993). The present data, obtained from excised outside-out patches, corroborate the belief that one of the possible mecha-

nisms of HVA Ca channel modulation by muscarine occurs through a membrane-delimited pathway, without the need for cytoplasmic diffusible messengers.

Our results also suggest that this membrane-delimited modulatory action of muscarine consists of changes in gating kinetics rather than in a reduction of unitary current amplitude. A decrease both in opening frequency and in mean open time could account for the inhibitory action of the agonist. Also in the case of the α-adrenergic inhibition of voltage-gated Ca channels a decrease in opening probability was observed in outside-out patches from submucosal neurons (Lipscombe et al. 1989), as well as in on-cell patches from sympathetic neurons (Shen and Surprenant 1991) when the agonist was directly applied to the isolated patch of membrane.

Interestingly, the muscarinic inhibition that we observed in outside-out patches occurred, as a rule, most effectively at low membrane potentials and it was attenuated at more positive voltages. In this respect it is similar to the action of other agonists all operating through voltage-sensing mechanisms (Kasai and Aosaki 1988; Bean 1989; Grassi and Lux 1989; Elmslie et al. 1990; Pollo et al. 1992). This mechanism of modulation of HVA Ca channels by neurotransmitters would be useful at nerve terminals for controlling Ca entry in a time-limited way and would be appropriate for rapid feedback regulation of transmitter release.

As shown in Fig. 2D–E, in one macropatch muscarine inhibited Ca current in an apparently voltage-independent way. Since it was observed only once, this "anomalous" action of muscarine could not be studied in detail.

Muscarine clearly decreased Ca channel activity when applied to the outside of the excised membrane patch but only when GTP was available to the inside surface of the membrane. This is in agreement with the results obtained in whole-cell recording and indicates that a G-protein is involved in modulation (Toselli et al. 1989). The membrane-delimited, voltage-dependent inhibitory action described here might involve, at the molecular level, direct interaction of the α subunit of a pertussis toxin sensitive G-protein with the channel, as was demonstrated for the modulation of a class of cardiac K channels by muscarine (Codina et al. 1987). At the moment, however, the interaction with some other unknown membrane-bound enzyme cannot be excluded.

Beside such a membrane-delimited pathway, preliminary whole-cell experiments suggest a complex picture for modulation of HVA Ca channels by muscarinic agonists. We believe that further studies utilizing on-cell patches will be useful to clarify a possible involvement of other pathways in the muscarinic modulation of HVA Ca channels in hippocampal neurons.

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