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Effects of muscarinic acetylcholine receptor activation on membrane currents and intracellular messengers in medium spiny neurones of the rat striatum

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Abstract

Acetylcholine, acting through muscarinic receptors, modulates the excitability of striatal medium spiny neurones. However, the underlying membrane conductances and intracellular signalling pathways have not been fully determined. Our aim was to characterize excitatory effects mediated by M1 muscarinic acetylcholine receptors in these neurones using whole-cell patch-clamp recordings in brain slices of postnatal rats. Under voltage-clamp, muscarine evoked an inward current associated with an increase in cell membrane resistance. The current, which reversed at −85 mV, was sensitive to the M1 receptor antagonist pirenzepine. Blocking the potassium conductance attenuated the response and the residual current was further reduced by ruthenium red (50 μM) and reversed at +15 mV. Simultaneous recordings from cholinergic interneurones and medium spiny neurones in conjunction with spike-triggered averaging revealed small unitary excitatory postsynaptic currents in four of 39 cell pairs tested. The muscarine-induced inward current was attenuated by a phospholipase C (PLC) inhibitor, U73122, but not by a protein kinase C inhibitor, chelerythrine, or by the intracellular calcium chelator 1,2-bis(2-aminophenoxy) ethane-*N,N,N′,N′*-tetra-acetic acid, suggesting that the current was associated with PLC in a protein kinase C- and Ca^{2+}-independent manner. The phosphatidylinositol 4-kinase inhibitor wortmannin (10 μM) reduced the recovery of the inward current, indicating that the recovery process was dependent on the removal of diacylglycerol and/or inositol 1,4,5 triphosphate or resynthesis of phospholipid phosphatidylinositol 4,5-bisphophate. Ratiometric measurement of intracellular calcium after cell loading with fura-2 demonstrated a muscarine-induced increase in calcium signal that originated mainly from intracellular stores. Thus, the cholinergic excitatory effect in striatal medium spiny neurones, which is important in motor disorders associated with altered cholinergic transmission in the striatum such as Parkinson's disease, is mediated through M1 receptors and the PLC-dependent pathway.

Introduction

Acetylcholine (ACh) is an abundant neurotransmitter in the neostriatum (Calabresi *et al.*, 2000a; Zhou *et al.*, 2002). It is synthesized and released by giant aspiny interneurones that constitute ∼2% of the entire neuronal population of the neostriatum (Kawaguchi, 1993, 1997). Despite their relatively low number, the cholinergic interneurones provide extensive innervation of the neostriatum through arborized axons (Kawaguchi, 1992, 1993, 1997; Kawaguchi *et al.*, 1995; Zhou *et al.*, 2002). The GABAergic medium spiny neurones, which form the main cell population of the neostriatum, constitute striatal projection neurones which receive inputs from cholinergic interneurones (Calabresi *et al.*, 2000a). These synaptic inputs modulate the activity of medium spiny neurones and an imbalance within the striatum between ACh and dopamine released from axons originating from the substantia nigra pars compacta has been strongly implicated in Parkinson's disease (e.g. Graybiel, 1990).

Although both nicotinic and muscarinic acetylcholine receptors (mAChRs) have been localized within the neostriatum, only mAChRs have been detected postsynaptically in medium spiny neurones (Hill *et al.*, 1993; Hersch *et al.*, 1994; Zhou *et al.*, 2001). Of the five mAChR subtypes identified with molecular techniques (m1–m5), m1 and m4 receptors have been located on medium spiny neurones, with the former exhibiting more abundant expression (Hersch *et al.*, 1994; Yan *et al.*, 2001). The m1 receptor corresponds to the pharmacologically identified M1 mAChR (Buckley *et al.*, 1989) which couples to the G_q protein (Peralta *et al.*, 1988; Bonner, 1989). This protein can activate phospholipase C (PLC) which converts membrane phospholipid phosphatidylinositol 4,5-bisphophate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP_3). The main function of DAG is to activate protein kinase C (PKC), while IP_3 releases Ca^{2+} from intracellular stores through IP_3 receptors.

Acetylcholine has a variety of pre- and postsynaptic effects on striatal medium spiny neurones. Presynaptically, it inhibits both glutamate and GABA release from cortical inputs through mAChRs (Sugita *et al.*, 1991; Hsu *et al.*, 1995) and enhances dopamine release through nicotinic receptors (Zhou *et al.*, 2001). Postsynaptically, ACh inhibits P-, N- and L-type Ca^{2+} channels (Howe & Surmeier, 1995) and enhances *N*-methyl-D-aspartate responses (Calabresi *et al.*, 1998) via mAChRs. Postsynaptic mAChRs also modulate A-type K^+ conductance in a membrane potential-dependent manner (Akins

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et al., 1990). Other studies have suggested that activation of postsynaptic M1 receptors can directly depolarize medium spiny neurones by reducing basal K^+ conductance (Dodt & Misgeld, 1986; Hsu *et al.*, 1996; Calabresi *et al.*, 1998).

Most of the postsynaptic effects of ACh have been attributed to the activation of PKC and/or IP_3-induced Ca^{2+} release from intracellular calcium stores. However, the mechanism of the direct excitatory effects still remains unclear. The aim of this study was to characterize this mAChR-mediated excitation of medium spiny neurones and to investigate the possible intracellular pathway(s) mediating this response.

Materials and methods

Striatal slice preparation

Transverse slices (250 μm) containing the neostriatum were prepared from postnatal day 14–24 Wistar rats. The choice of this age group was based on a previous study which demonstrated that the density of M1 mAChRs in the rat striatum had already reached adult levels by the second postnatal week (Aubert *et al.*, 1996). Procedures followed the guidelines of the University of Auckland Animal Ethics Committee. Rats were decapitated under CO_2 anaesthesia and their brains quickly removed and submerged in ice-cold cutting solution containing (in mM): sucrose, 240; $NaHCO_3$, 26; KCl, 3; $CaCl_2$, 0.1; NaH_2PO_4, 2.5; $MgSO_4$, 1.3 and glucose, 10 (oxygenated with 95% O_2 and 5% CO_2). Slices were cut with a Vibratome (VT 1000S; Leica) and incubated at room temperature (22–24 °C) for up to 8 h in an oxygenated solution containing (in mM): NaCl, 115; piperazine-1,4-bis [2-ethanesulphonic acid], 20; KCl, 5; $CaCl_2$, 0.1; $MgCl_2$, 1; ascorbic acid, 0.1; pyruvic acid, 1 and glucose, 25, pH 7.25.

Whole-cell patch-clamp recording

Recordings from medium spiny neurones were conducted using a tight seal whole-cell patch-clamp configuration in combination with infrared differential interference contrast visualization of patched cells (E600FN microscope; Nikon, Japan). In most recordings, the pipette solution contained (in mM): K gluconate, 125; NaCl, 5; $CaCl_2$, 1; HEPES, 10; MgATP, 2; Na_3GTP, 0.3 and 1,2-bis(2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (BAPTA) (tetrapotassium salt), 10. For testing the effects of 2-aminoethoxydiphenylborane (2-APB), the pipette solution contained (in mM): K gluconate, 140; NaCl, 5; $MgCl_2$, 1; HEPES, 10; MgATP, 2; Na_3GTP, 0.3 and BAPTA, 0.25. In electrophysiological experiments with concurrent measurements of intracellular calcium, 0.25 mM BAPTA was replaced by 0.25 mM EGTA. In some experiments, K^+ conductances were blocked using a pipette solution containing (in mM): Cs methanesulphonate, 100; tetraethylammonium chloride, 30; $CaCl_2$, 1; HEPES, 10; MgATP, 2; Na_3GTP, 0.3 and EGTA, 10. All pipette solutions had an osmolarity of 275–285 mOsm (pH 7.25). The recording chamber was continuously perfused at 3.5 mL/min with an artificial cerebrospinal fluid (ACSF), bubbled with 95% O_2 and 5% CO_2, which contained (in mM): NaCl, 126; $NaHCO_3$, 26; KCl, 3; $CaCl_2$, 2.6; NaH_2PO_4, 2.5; $MgSO_4$, 1.3 and glucose, 20. In some experiments, the standard ACSF was modified by adding 2 mM $BaCl_2$, reducing NaH_2PO_4 to 1.25 mM and removing $MgSO_4$ or by replacing Ca^{2+} with Mg^{2+} and adding 1 mM EGTA (Ca^{2+}-free ACSF). All recordings were at 33 °C.

The acquisition of voltage-clamp data was conducted using a Multiclamp 700A amplifier, Digidata 1322A, Minidigi 1A and pClamp 9.0 software (Axon Instruments, CA, USA) and a PC. Offline data analysis was performed using Clampfit 9.0 or Axograph 4.4 (Axon Instruments). Baseline and peak current amplitudes were measured by averaging the current for 5 and 3 s, respectively. For measurement of recovery of the current induced by muscarine, the current values were measured at the 'pretreatment recovery time' and normalized to the peak current response. In most experiments, a two-tailed paired Student's *t*-test was used for statistical analysis. An unpaired Student's *t*-test was used to compare the amplitudes of muscarine-evoked currents recorded with pipettes containing 0.25 and 10 mM BAPTA. In experiments in which wortmannin was used, a one-sample *t*-test was employed (null hypothesis, 100% recovery). A P-value < 0.05 was considered significant. All values are expressed as mean ± SEM.

Spike-triggered averaging

For spike-triggered averaging experiments, single-unit extracellular recording from cholinergic interneurones was conducted concurrently with whole-cell patch-clamp recording from medium spiny neurones. A glass microelectrode (5 MΩ) containing (in mM): NaCl, 150; KCl, 3; $CaCl_2$, 2.4; HEPES, 10 and $MgCl_2$, 1 (pH 7.3) was positioned onto the putative cholinergic interneurone under infra-red differential interference contrast microscopy. Spontaneous action potentials were recorded with an NL 104 AC amplifier (Digitimer) and acquired to a PC using Digidata 1322A. Whole-cell current responses were averaged online using 90–200 action potentials as the trigger source (pClamp 9.0). These experiments were conducted in the presence of the ionotropic glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione and 2-amino-5-phosphonovaleric acid and the $GABA_A$ receptor antagonist bicuculline to eliminate currents generated by glutamatergic and GABAergic synapses.

Measurement of intracellular Ca^{2+}

For measurement of intracellular free calcium, 0.25 mM fura-2 (Molecular Probes Inc., USA) was included in the pipette solution. Imaging was performed with a 40 × water immersion objective (Nikon), beam splitter (510 nm) and a barrier filter (HQ540/50; Chroma, USA). UV excitation was provided by a xenon lamp (175W Lambda L.S.; Sutter Instruments, USA) connected to a filter wheel (Optospin; Cairns Research, UK) containing 340 and 380 nm excitation filters (D340/10x and D380/10x; Chroma). The fluorescence signal was acquired using a cooled CCD camera (SenSys 1401G2E; Roper Scientific, USA) and Imaging Workbench software (Axon Instruments). Image pairs were collected at 1 Hz. The averaged pixel values of the region containing the soma and proximal dendrites ('region of interest') were measured after background subtraction and $[Ca^{2+}]_i$ was expressed as a 340/380 nm ratio (Grynkiewicz *et al.*, 1985). Correlation analyses of current and calcium responses were conducted using linear regression analysis. Comparisons of the calcium signal before and after various treatments were made with Wilcoxon matched-pairs sign-ranks tests.

Drug applications

Muscarine (100 or 200 μM) was pressured ejected onto the recorded cell. A glass capillary (4 μm tip) was placed ~50 μm from the soma and pulses of pressure (10 psi, 5 s) were used to apply the drug. In some cases, muscarine (5–20 μM) was applied through the bath perfusion system.

All other drugs, which were freshly prepared from stock solutions, were applied through bath perfusion. Stock solutions were stored at

−20 °C and used within 2 weeks of preparation. For drugs dissolved in dimethylsulphoxide [U73122, chelerythrine, 2-APB, bicuculline, cyclopiazonic acid (CPA) and wortmannin], the concentration of the solvent did not exceed 0.2%. U73122 was dissolved in chloroform, dried with N_2 and redissolved in dimethylsulphoxide on the day of the experiment.

Chelerythrine chloride, (+)-muscarine chloride, wortmannin, pirenzepine dihydrochloride, 4-aminopyridine and DL-2-amino-5-phosphonovaleric acid were purchased from Sigma; U73122, 2-APB, 6-cyano-7-nitroquinoxaline-2,3-dione disodium salt, (+)-bicuculline, L-sulpiride and scopolamine hydrobromide were obtained from Tocris and CPA was acquired from Alomone Laboratories (Israel).

Intracellular labelling with biocytin

In some experiments, the tip of the patch pipette was prefilled with standard pipette solution and the pipette was then backfilled with the same solution containing 2% biocytin. After recording, the slice was fixed overnight with 4% formalin in phosphate-buffered saline. The subsequent histochemical protocol was performed as described previously (Lin *et al.*, 2003). In brief, the slice was treated with 1% H_2O_2 and 0.3% Triton-X 100 and incubated with ExtrAvidin-Peroxidase (1 : 1000; Sigma) overnight. Sections were then reacted with 3,3′-diaminobenzidine and cleared with methyl salicylate. Images were acquired with an AxioCam HRc camera (Zeiss).

Results

Identification of medium spiny neurones and their response to muscarine

Striatal medium spiny neurones were identified using previously described electrophysiological and morphological criteria (Jiang & North, 1991; Kawaguchi, 1997; Calabresi *et al.*, 2000b). In current-clamp, they exhibited a hyperpolarized membrane potential (−78.0 ± 0.3 mV, $n = 11$) and, in response to depolarizing current steps, a slow ramp-like depolarization leading to a delayed action potential (Fig. 1B). In voltage-clamp, the membrane input resistance was 112.1 ± 7.9 MΩ ($n = 23$). Their cell bodies, as visualized with infra-red differential interference contrast microscopy, were 10–20 μm in diameter. In cells filled with biocytin ($n = 9$) multiple primary dendrites (four to six) were observed (Fig. 1A) and 'rough' dendritic surfaces were apparent under high magnification (40 ×), indicating the presence of dendritic spines. In accordance with previous reports (Jiang & North, 1991; Kawaguchi, 1997; Calabresi *et al.*, 2000b), these cells were the most abundant cell type in the striatum.

In voltage-clamp recordings ($V_{hold} = -70$ mV), the bath application of muscarine induced a concentration-dependent nondesensitizing inward current (5 μM, −16.3 ± 4.9 pA; 10 μM, −30.8 ± 8.2 pA; 20 μM, −55.0 ± 20.2 pA; $n = 4$ for each concentration). In response to local application of 100 and 200 μM muscarine (using an injection pipette), slow inward currents were evoked with respective amplitudes of −11.7 ± 1.6 pA ($n = 11$; Fig. 1C1 and D1) and −22.1 ± 2.3 pA ($n = 11$; Fig. 2A1). The response showed insignificant rundown with repeated application of muscarine (100 μM, 5-min interval) and remained at 98.4 ± 8.3% of the control value at 5 min and 92.9 ± 6.0% at 10 min ($P > 0.05$ for both time points, $n = 11$). The current evoked by 100 μM muscarine was blocked by the nonselective mAChR antagonist scopolamine (10 μM, $n = 6$, Fig. 1C2) and by the M1 receptor-selective antagonist pirenzepine (0.5–1 μM, $n = 12$, Fig. 1D2), confirming that the response was mediated by muscarinic M1 receptors (Hsu *et al.*, 1996). In current-clamp recordings, the local application of muscarine induced a small depolarization (0.98 ± 0.20 mV; $n = 12$) that was sensitive to both scopolamine and pirenzepine (not illustrated).

Properties of muscarine-induced membrane current

Previous studies have demonstrated that the inward current or cell depolarization evoked by muscarine is due to a reduction of potassium conductance (Dodt & Misgeld, 1986; Hsu *et al.*, 1996; Calabresi *et al.*, 1998). Consistent with this, the current induced in our experiments by bath application of muscarine (10 μM) was associated with an increase in cell membrane resistance from 89.6 ± 13.1 to 103.7 ± 17.6 MΩ ($n = 6$). The current reversal potential was −84.9 ± 3.3 mV ($n = 10$; Fig. 2C). In addition, application of the K^+ channel blocker Ba^{2+} (2 mM) significantly reduced the amplitude of the current evoked by pressure microinjection of 200 μM muscarine (from −22.1 ± 2.3 to −14.2 ± 1.7 pA; $n = 11$; Fig. 2A2 and B). Barium application on its own increased cell membrane resistance from 95.2 ± 24.6 to 231.2 ± 53.2 MΩ ($n = 5$).

The failure of Ba^{2+} to completely block the muscarine-evoked inward current suggested the involvement of another conductance unrelated to K^+. To investigate this possibility, recordings were made with pipettes containing Cs^+ (100 mM) and tetraethylammonium (30 mM). In the presence of intracellular Cs^+ and tetraethylammonium, an inward current could still be evoked in response to local application of 200 μM muscarine (−17.0 ± 2.8 pA, $n = 14$; Fig. 3A1). Application of the drug at different holding potentials demonstrated that the current reversed at +15 mV ($n = 8$; Fig. 3B). This current was not diminished in the presence of 2 mM Ba^{2+} (94.9 ± 5.7% of control, $P > 0.05$, $n = 7$; Fig. 3A2) or 2 mM Ba^{2+} with 0.2 mM Cd^{2+} (94.3 ± 12.1%, $n = 7$, $P > 0.05$; Fig. 3C). Ruthenium red (50 μM), a blocker of cationic conductance, significantly reduced the response after 3 min of bath application (to 88.6 ± 4.7% of control, $n = 9$; $P < 0.05$; Fig. 3C). Another cationic channel blocker, lanthanum (La^{3+} 100 μM), was associated with only a small and nonsignificant reduction in the response (to 87.8 ± 8.9% of control; $n = 9$, $P > 0.05$; Fig. 3C).

Spike-triggered averaging

Putative cholinergic interneurones in the striatum were identified under infra-red differential interference contrast optics by their large somata (> 30 μm). In accordance with previous reports (Bennett & Wilson, 1999; Bennett *et al.*, 2000), these cells were tonically active in the slice preparation (mean firing rate 3.9 ± 0.4 Hz, $n = 13$). Their spontaneous action potentials were used as triggers for averaging membrane currents recorded in nearby (50–150 μm) medium spiny neurones (Fig. 4A). In the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (20 μM), 2-amino-5-phosphonovaleric acid (10 μM) and bicuculline (20 μM), spike-triggered averaging revealed scopolamine (10 μM)-sensitive excitatory postsynaptic currents in four of 39 cholinergic interneurone/medium spiny neurone pairs tested. The excitatory postsynaptic current amplitude was 180 ± 35 fA and the peak synaptic current occurred 35 ± 5 ms after the onset of spikes recorded from interneurones (Fig. 4B). The application of 4-aminopyridine (100 μM; $n = 11$) or L-sulpiride (10 μM; $n = 17$), agents that might be expected to increase ACh release from presynaptic terminals, did not increase the detection of functional synaptic connections nor enhance the amplitude of unitary excitatory postsynaptic currents when tested in two of four synaptically connected pairs.

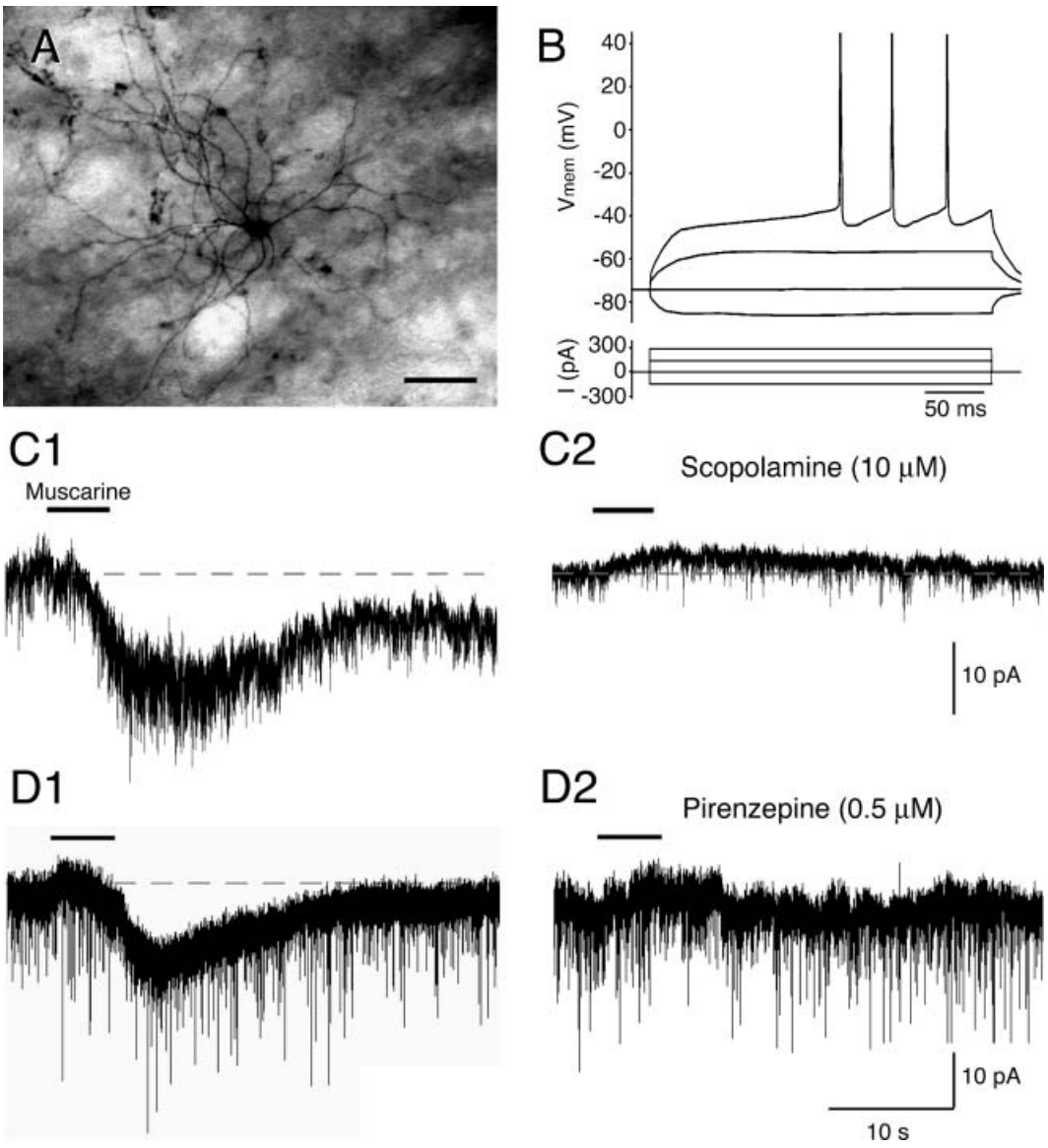

FIG. 1. Morphology and electrophysiological properties of striatal medium spiny neurones. (A) A neurone filled with biocytin and visualized with an ExtrAvidin-3,3′-diaminobenzidine reaction. The cell had multiple dendrites with extensive dendritic arborizations. Scale bar, 50 μm. (B) Delayed action potentials, preceded by a ramp-like depolarization, in response to a depolarizing current step. (C1 and D1) Inward current evoked in two different cells by local microinjection of muscarine (100 μM, 5 s). The response was blocked by the nonselective muscarinic acetylcholine receptor antagonist scopolamine (10 μM; C2) or the specific M1 receptor antagonist pirenzepine (0.5 μM; D2).

Effects of modulators of the phospholipase C pathway

As M1 mAChRs are known to be coupled to PLC through the G_q protein (Peralta *et al.*, 1988; Bonner, 1989), several intracellular inhibitors of the PLC pathway were tested. Application of the PLC inhibitor U73122 significantly reduced the amplitude of the muscarine-evoked inward current in a concentration-dependent manner; 5 μM U73122 reduced the response to 60.9 ± 8.0% ($n = 7$) and 10 μM to 36.9 ± 9.6% ($n = 7$) of control after 10–15 min of preperfusion (Fig. 5A and E). This result supports the notion that the response to muscarine is mainly mediated through the activation of PLC. The PKC inhibitor chelerythrine (5–10 μM) did not alter the amplitude of the muscarine-induced current after 20 min of preperfusion (100.3 ± 6.8%; $n = 10$), arguing against the involvement of PKC in the response (Fig. 5B and E). Another second messenger, intracellular Ca^{2+}, was also unlikely to be involved as the responses could be evoked in the presence of both low (0.25 mM) and high (10 mM) intracellular BAPTA (Fig. 5C1 and D1). With 0.25 mM intracellular BAPTA, the peak amplitude evoked by 100 μM muscarine (−19.9 ± 2.9 pA, $n = 14$) was not significantly different from the response recorded with 10 mM intracellular BAPTA (−16.4 ± 1.7 pA, $n = 20$).

In the presence of 0.25 mM intracellular BAPTA, the application of 2-APB, a drug reported to inhibit IP_3-induced calcium release (Hamada *et al.*, 1999), significantly reduced the muscarine-evoked current (to 68.5 ± 6.9% of control; $n = 8$; Fig. 5C1 and C2). The response was also significantly reduced with 10 mM intracellular BAPTA (to 82.9 ± 6.0% of control; $n = 9$; Fig. 5D1 and D2).

These results indicate that the cellular cascade leading to the muscarine-induced inward current involves PLC but is independent of PKC activation or release of intracellular Ca^{2+}. The main function of

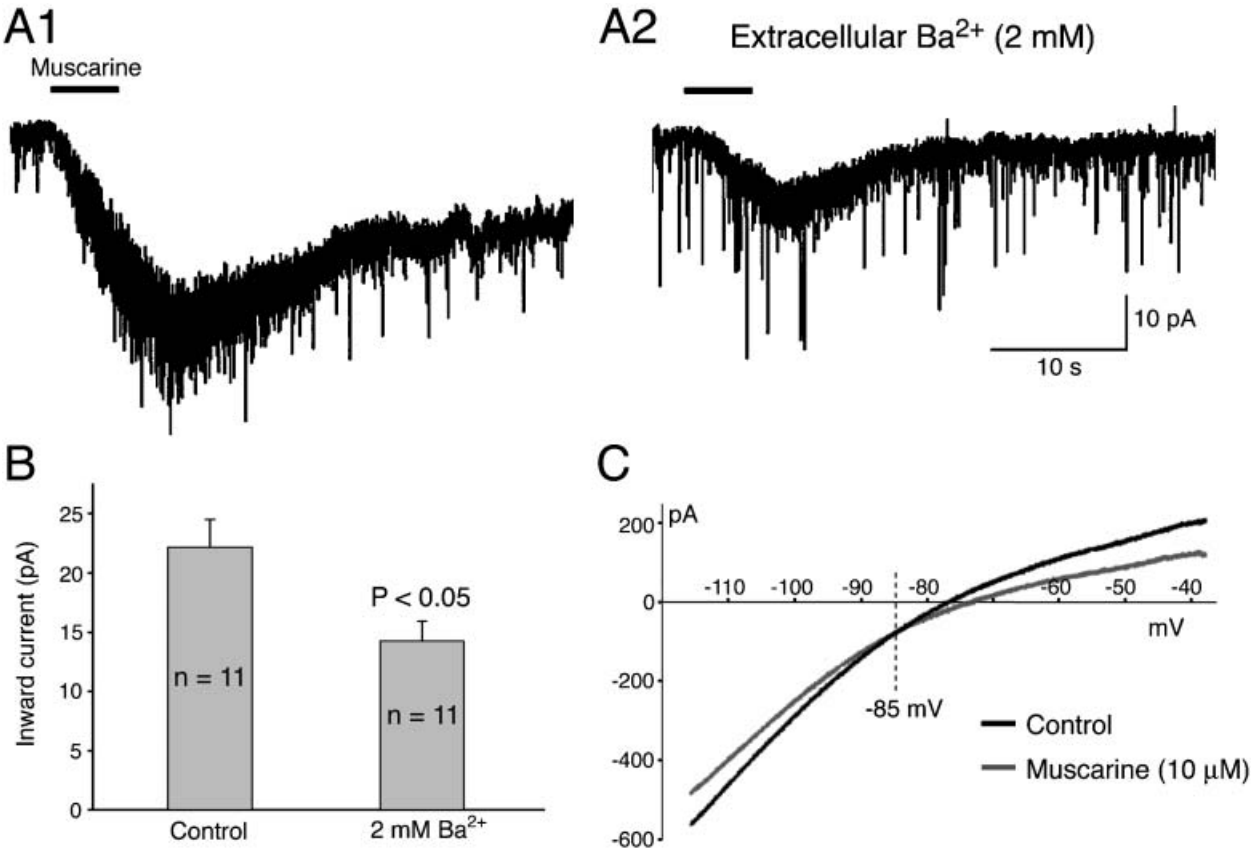

FIG. 2. Inhibition of a potassium conductance is partly responsible for the muscarine-induced inward current. (A) The current recorded in response to local microinjection of muscarine (200 μM; $V_{hold} = -70$ mV; A1) was reduced by the nonselective potassium channel blocker Ba^{2+} (2 mM; A2). (B) Group data showing that the current was reduced by extracellular Ba^{2+} from −22.1 ± 2.3 to −14.2 ± 1.7 pA. (C) A typical I–V relationship recorded before (black line) and during (grey line) bath application of 10 μM muscarine. The current reversal potential was −85 mV. An increase in membrane resistance is reflected in the reduction in slope.

PLC is the hydrolysis of the membrane phospholipid PIP_2 into DAG and IP_3. To assess whether the inward current evoked by muscarine was associated with the hydrolysis of PIP_2, we tested the effect of wortmannin, an inhibitor of phosphatidylinositol 4-kinase (a PIP_2 resynthesis enzyme), on the muscarine-evoked current. At 10 μM, wortmannin (10–15 min preperfusion) significantly reduced the recovery of the current after the conclusion of muscarine application (Fig. 6A1 and A2). The response to muscarine remained at 73.1 ± 10.7% of the peak amplitude at the mean pretreatment recovery time of 75 s ($n = 6$). At 1 μM, the inhibition was smaller and nonsignificant, with recovery to 22.5 ± 12.7% ($n = 6$) at the same time. These results indicate that the continuous presence of DAG and/or IP_3 or the absence of PIP_2 are required to sustain the muscarine-induced current. The removal of hydrolysis products of PIP_2, or regeneration of PIP_2, is required for recovery of the response.

Effects of muscarine on intracellular calcium

To further explore the relationship between calcium levels and the electrophysiological responses to muscarine, changes of intracellular calcium were measured using the ratiometric technique after loading cells with fura-2 through a patch pipette. Only cells which showed a reproducible increase in calcium following local application of 100 μM muscarine at 5 min intervals were used. In these cells, the 340 : 380 fluorescence ratio increased from 0.15 ± 0.01 to 0.26 ± 0.03 ($n = 41$). In general, the peak amplitude ($R^2 = 0.091$) and time to peak ($R^2 = 0.129$) of the calcium responses were poorly correlated with the associated inward currents (Fig. 7A1 and B1).

Application of the pirenzepine (0.25 μM, 10 min; $n = 6$) significantly reduced the muscarine-induced calcium and current responses (to 15.7 ± 7.4 and 30.5 ± 12.6% of pretreatment levels, respectively; $P < 0.05$; Fig. 7A). The inhibitory effect of pirenzepine on the intracellular calcium increase implies that the rise in calcium is associated with the activation of M1 receptors.

To identify the source of the intracellular calcium increase, eight cells were tested in the presence of 10 μM CPA, an inhibitor of endoplasmic reticulum calcium ATPase. Pretreatment with CPA for 10 min markedly reduced the muscarine-induced calcium increase (to 2.4 ± 4.6% of the pretreatment level, $P < 0.01$), while the current was not significantly affected (88.5 ± 8.7% of control, $P > 0.05$; Fig. 7B). In a separate set of experiments, the contribution of extracellular Ca^{2+} to the muscarine-evoked increase in intracellular calcium was assessed by the application of Ca^{2+}-free ACSF. In the absence of extracellular Ca^{2+}, the muscarine-induced calcium signal was not significantly reduced ($P > 0.05$, $n = 7$; not illustrated).

To test whether the inhibitory effects of 2-APB on muscarine-induced inward currents were associated with an inhibition of IP_3-induced calcium release, 12 cells were tested with 2-APB (100 μM; 10 min). In these cells, the calcium response remained at 93.8 ± 32.1% of the pretreatment level ($P > 0.05$), indicating that the muscarine-induced calcium increase in these cells was not sensitive to 2-APB.

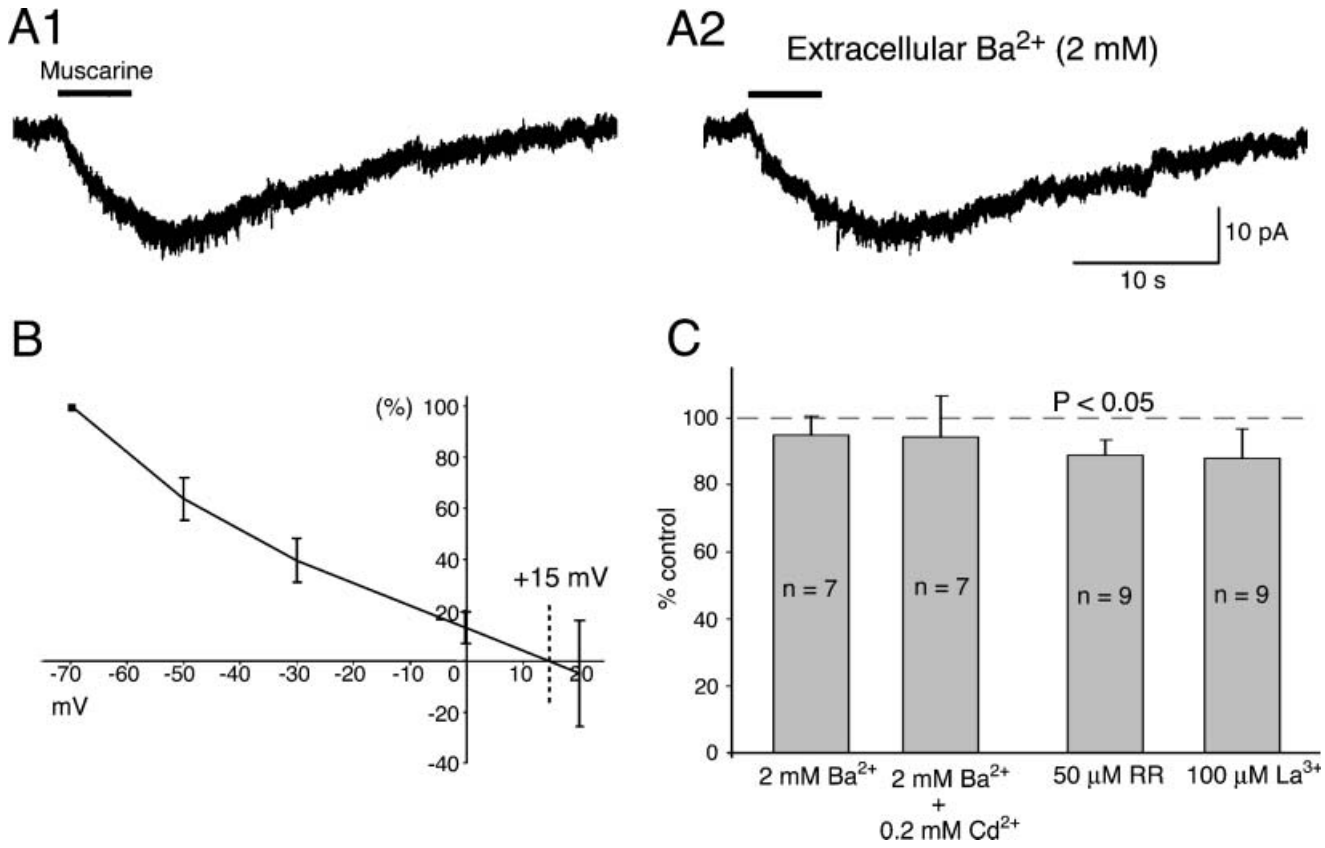

FIG. 3. Properties of muscarinic acetylcholine receptor-mediated inward current recorded in medium spiny neurones after blocking K^+ channels. (A) When recorded with a pipette solution containing 100 mM Cs^+ and 30 mM tetraethylammonium ($V_{hold} = -70$ mV), muscarine (200 μM) induced an inward current (A1) that was insensitive to 2 mM extracellular Ba^{2+} (A2). (B) I–V relationship of the muscarine-evoked response recorded with the same pipette solution. The drug-induced current was recorded at V_{hold} of −70, −50, −30, 0 and +20 mV and normalized to the response evoked at −70 mV. The mean value of the reversal potential was +15 mV ($n = 8$). (C) The muscarine-induced current was not significantly affected after K^+ channel blockade by 2 mM extracellular Ba^{2+} or 2 mM Ba^{2+} and 0.2 mM Cd^{2+}. A significant reduction was observed in the presence of ruthenium red (RR, 50 μM) but not lanthanum (La^{3+}, 100 μM).

Discussion

Muscarinic acetylcholine receptor activation reduces potassium conductance and activates nonselective cationic channels in medium spiny neurones

It has been reported that the depolarization of medium spiny neurones by the activation of M1 mAChRs is due to inhibition of a potassium conductance (Dodt & Misgeld, 1986; Hsu *et al.*, 1996; Calabresi *et al.*, 1998). Our study confirms this finding by showing that the muscarine-evoked inward current is sensitive to the potassium channel blocker Ba^{2+} and reverses at a value (−85 mV) near the equilibrium potential for K^+, as calculated from the Nernst equation (−105 mV). In many other types of neurone, activation of M1 mAChRs inhibits a K^+ conductance described as the M-type potassium current (I_M) (Dutar & Nicoll, 1988; Sims *et al.*, 1988). However, the conductance mediating the muscarine-induced current in striatal neurones appears to be distinct from I_M as the response persisted at membrane potentials below −75 mV (see Fig. 2C), at which I_M normally inactivates (Bosma & Hille, 1989; Hsu *et al.*, 1996; Calabresi *et al.*, 1998; Stemkowski *et al.*, 2002). The responses observed in our study are thus more likely to be mediated by inhibition of 'leak' potassium channels (Hsu *et al.*, 1996). Nevertheless, as the M-current channel subunit KCNQ2 has been identified in medium spiny neurones (Cooper *et al.*, 2001), the muscarine-induced effects may reflect inhibition of both the I_M and 'leak' K^+ current.

In addition to inhibition of potassium channels, our results indicate that activation of M1 mAChRs increases a nonselective cationic conductance, as seen in a variety of other neurones and nonexcitable cells (Hasuo *et al.*, 1996; Yajeya *et al.*, 1999; Ito & Dulon, 2002; Lee *et al.*, 2003; Sydorenko *et al.*, 2003). However, activation of these cationic channels is not limited to muscarinic agonists as neurotransmitters acting on different metabotropic receptors (e.g. purinergic receptors and adrenoreceptors) induce a similar response (Ito & Dulon, 2002; Sydorenko *et al.*, 2003). In medium spiny neurones, this Ba^{2+}-insensitive, muscarine-induced, nonselective cationic conductance reversed at +15 mV. Its attenuation by ruthenium red in the presence of strong intracellular calcium buffer suggested that the inhibitory effect was not due to inhibition of intracellular calcium release. However, the identity of these cationic channels remains to be established. One group of channels which might be involved in this response is the transient receptor potential (TRP) channels which have some properties compatible with the results obtained in our study. First, some members of the TRP channel family can be activated by mAChRs and are coupled to the PLC pathway (Schaefer *et al.*, 2000; Clapham *et al.*, 2001; Vennekens *et al.*, 2002; Plant & Schaefer, 2003). Second, TRP channels are relatively nonselective cationic channels that have current reversal potentials between 0 and +10 mV (Clapham *et al.*, 2001; Strubing *et al.*, 2001; Lee *et al.*, 2003; Plant & Schaefer, 2003). Third, some TRP channels can be blocked by ruthenium red through a mechanism which is independent of the modulation of

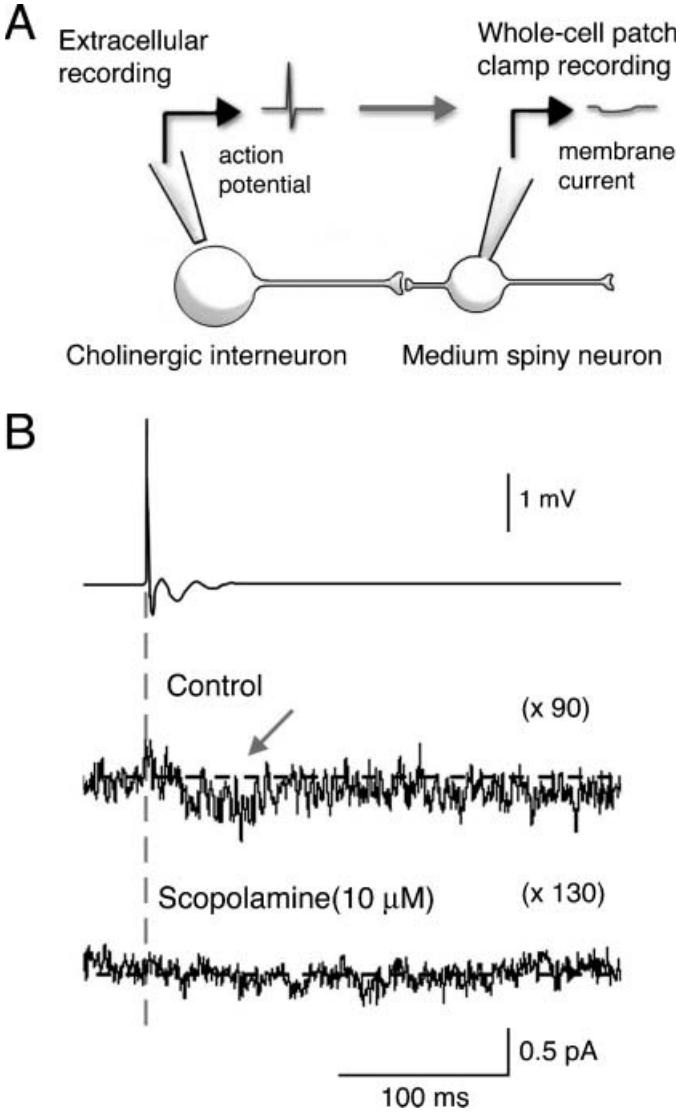

FIG. 4. Spike-triggered averaging reveals a muscarinic acetylcholine receptor-mediated excitatory postsynaptic current (EPSC). (A) A schematic diagram of the experimental arrangement used to reveal acetylcholine-induced EPSCs. Action potentials recorded extracellularly from a cholinergic interneurone (also shown in B, top trace) were used as a trigger source to average the membrane current measured in a nearby medium spiny neurone as a way to increase the signal-to-noise ratio of the synaptic responses. (B) Small unitary EPSC (arrow) detected with spike-triggered averaging. The action potentials recorded from a cholinergic interneurone were used as a trigger (top trace) and averaged currents recorded from a medium spiny neurone in the absence and presence of scopolamine are shown below. The number in brackets indicates the number of averaged responses.

calcium release from intracellular stores (Clapham *et al.*, 2001; Guler *et al.*, 2002; Watanabe *et al.*, 2002; Gee *et al.*, 2003). Lanthanum, another known inhibitor of TRP channels, did not have significant effects on the muscarine-activated cationic current in medium spiny neurones. This does not exclude TRP channels as the inhibitory effect of lanthanum is subunit dependent (Schaefer *et al.*, 2000; Strubing *et al.*, 2001). We have not tested the effects of other known TRP/cationic channel blockers (such as flufenamic acid, SKF96365 and gadolinium). However, as specific antagonists of the TRP channels are not available (apart from the blockers of TRPV1 channels iodo-resiniferatoxin and capsazepine), it is difficult to definitely prove the involvement of these channels. Thus, at this stage, the cationic conductance activated by muscarine in striatal medium spiny neurones can be described only as 'TRP-like'.

The relative proportion of the muscarine-induced cell membrane current evoked through reduction of the K^+ conductance and activation of TRP-like channels could not be determined precisely because of the changes associated with the block of potassium channels by Ba^{2+} (i.e. increase in cell input resistance and reduction of space-clamp error). However, the inhibition of K^+ conductance appears to account for most of the response, as judged by the current reversal potential (measured with a pipette containing K gluconate) which was close to the reversal potential calculated from the Nernst equation for K^+ (see above).

Not only have we demonstrated that activation of M1 mAChRs via exogenous muscarine excites striatal medium spiny neurones through at least two separate ionic mechanisms but we have also provided the first demonstration that ACh released from presynaptic terminals of local cholinergic interneurones excites these neurones. The unitary muscarinic excitatory postsynaptic currents, detected by spike-triggered averaging in a small proportion of tested cell pairs, would evoke only a small depolarization of approximately 20 μV, as calculated from the mean unitary current amplitude of 180 fA and the membrane resistance of 112 MΩ. Thus, a significant synaptic effect would probably require the release of ACh from several cholinergic interneurones firing simultaneously (Graybiel *et al.*, 1994). Alternatively, ACh may exert striatal effects through volume transmission and a nonsynaptic summation process (Descarries *et al.*, 1997).

Muscarine-induced inward current is mediated through the phospholipase C pathway and transient receptor potential (TRP) -like channels but is not associated with protein kinase C or intracellular calcium

In our experiments, the PLC inhibitor U73122 was an effective blocker of the muscarine-induced inward current. Another inhibitor that attenuated the response was 2-APB. It is likely that 2-APB acted through an intracellular calcium-independent mechanism as the inhibitory effect was observed with both low and high concentrations of the intracellular calcium chelator BAPTA. If 2-APB acted by inhibiting the IP_3-induced calcium release, then the effect would be abolished or attenuated when intracellular calcium was strongly buffered by 10 mM BAPTA. 2-APB was initially shown to be an inhibitor of IP_3-induced calcium release (Hamada *et al.*, 1999). However, recent studies have demonstrated several unrelated effects, including the blockade or modulation of TRP channels (Clapham *et al.*, 2001; Iwasaki *et al.*, 2001; Bootman *et al.*, 2002). Given that our data suggest the involvement of TRP-like channels in the muscarine-evoked response (see above), it is possible that 2-APB acted by blocking these channels. In addition, the application of 2-APB failed to significantly alter the muscarine-induced calcium increase, further indicating that 2-APB acted through an intracellular calcium-independent mechanism in these cells.

Three other observations argue against the role of intracellular calcium as a second messenger involved in activation of the muscarine-induced inward current. First, the muscarine-evoked current was poorly correlated with intracellular calcium elevation, as measured by concurrent patch-clamp recording and microfluorometry. Second, buffering intracellular calcium with a high concentration of BAPTA failed to abolish the current. Third, inhibition of the muscarine-induced calcium increase with CPA failed to significantly alter the amplitude of the inward current in the same cells. PKC, the

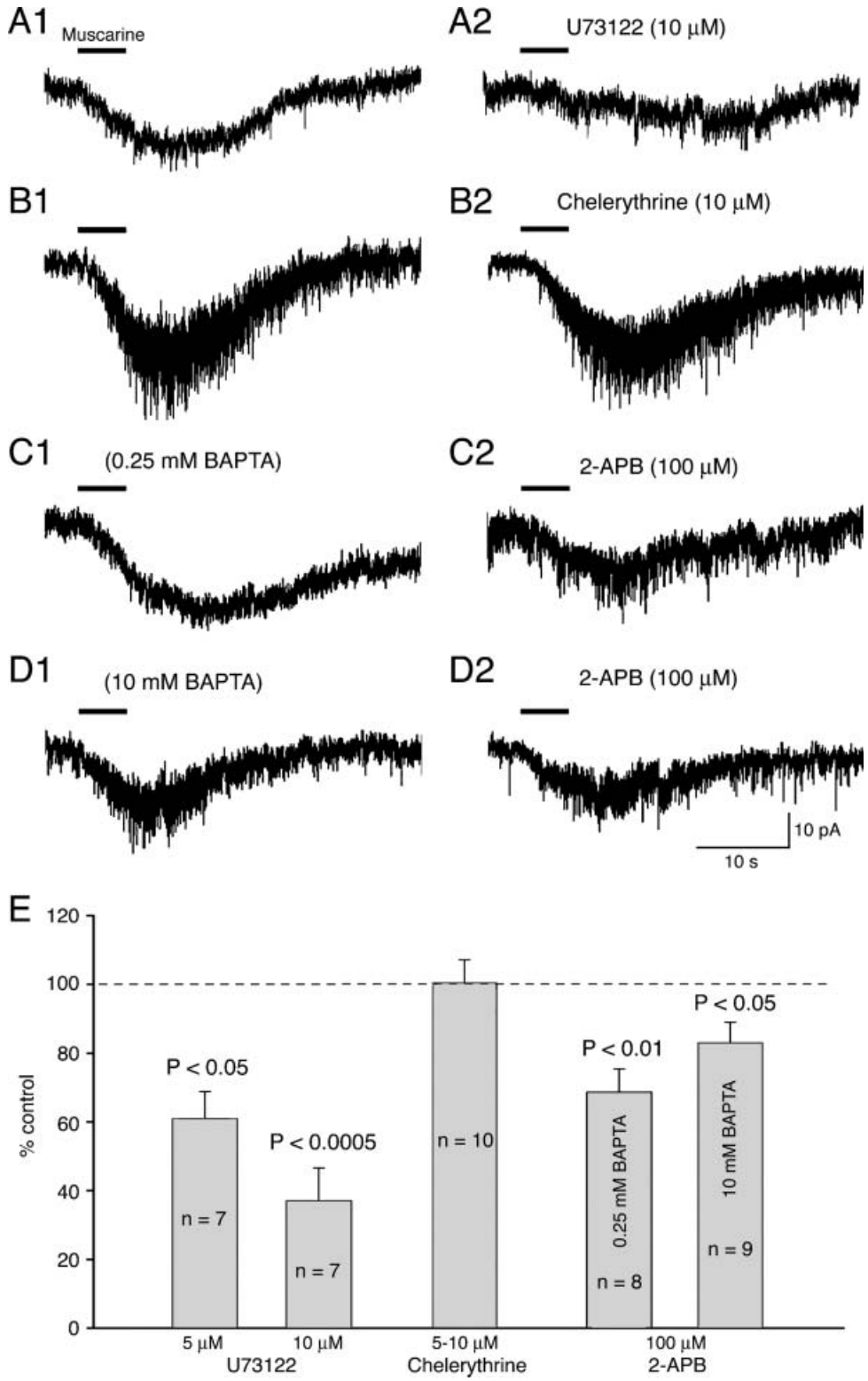

FIG. 5. The effects of the phospholipase C inhibitor U73122, protein kinase C inhibitor chelerythrine and putative inositol 1,4,5 triphosphate receptor/transient receptor potential channel blocker 2-aminoethoxydiphenylborane (2-APB) on the muscarine-evoked inward current. Records in the left column (A1, B1, C1 and D1) are controls. (A2) Inhibition of the response by U73122 (10 μM; 10 min of preapplication). (B2) Chelerythrine (10 μM) did not affect the response, even after 20 min application. (C2) In the presence of 0.25 mM intracellular 1,2-bis(2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (BAPTA), 2-APB (100 μM, 10 min) reduced the amplitude of the inward current evoked by 100 μM muscarine. (D2) 2-APB (100 μM, 10 min) reduced the muscarine-induced current recorded with pipettes containing 10 mM BAPTA. (E) Summary of data showing the effects of U73122 (5 and 10 μM), chelerythrine (5–10 μM) and 2-APB (100 μM) with 0.25 and 10 mM intracellular BAPTA.

other common second messenger in the PLC pathway, is also unlikely to mediate the muscarine-induced current as the PKC inhibitor chelerythrine failed to alter the muscarine-induced current.

Overall, our results show that the muscarine-induced membrane current is associated with the activation of PLC but not of PKC nor with the release of calcium from intracellular stores. Other intracellular messengers which may be involved include the PLC substrate PIP_2 and the PIP_2 hydrolysis products IP_3 and DAG. PIP_2 and DAG have been implicated in the direct modulation of membrane channels (Bosma & Hille, 1989; Marrion, 1994; Stemkowski *et al.*, 2002; Suh & Hille, 2002; Ford *et al.*, 2003). On the other hand, IP_3 has rarely been associated with functions other than the control of intracellular calcium release (Dutar & Nicoll, 1988; Marrion, 1997) and is therefore unlikely to be involved in the muscarine-induced currents.

The regulation of membrane channel function by PIP_2 has been described in a variety of cells (Kobrinsky *et al.*, 2000; Lopes *et al.*, 2002; Ma *et al.*, 2002), including the mAChR-induced inhibition of the M-current in sympathetic ganglia (Stemkowski *et al.*, 2002; Suh & Hille, 2002; Ford *et al.*, 2003) and the activation of TRP channels in photoreceptors (Hardie, 2003). The activity of these channels is normally associated with the availability of PIP_2 in the cell membrane, possibly through a binding site that increases channel stability in either an open or a closed state. As membrane PIP_2 becomes depleted following PLC activation, PIP_2 unbinds from the channel protein causing the channels to move towards their alternative state. When the PIP_2 levels are replenished by PIP_2-resynthesizing protein following the removal of agonists from the receptors, the channels return to their original configuration (Stemkowski *et al.*, 2002; Suh & Hille, 2002; Ford *et al.*, 2003). Consistent with this mechanism are our data showing that wortmannin, a drug that interferes with PIP_2 resynthesis through the inhibition of phosphatidylinositol 4-kinase (Suh & Hille, 2002; Ford *et al.*, 2003), slows the rate of recovery of the muscarine-induced current.

Our results do not exclude the possibility that DAG acts as a second messenger independent of PKC activation (Brose & Rosenmund, 2002). The inhibition of PIP_2 resynthesis by wortmannin can lead to a build up of intracellular DAG and continuous activation/inactivation of membrane channels through a DAG-dependent mechanism. Such DAG-mediated regulation of receptor-activated TRP channels has been suggested previously (Hardie, 2003; Trebak *et al.*, 2003). In addition, DAG can suppress the M-current without activating PKC (Bosma & Hille, 1989; Marrion, 1994). Therefore, both DAG and PIP_2 remain possible candidates as mediators of the muscarine-evoked current in striatal medium spiny neurones.

Muscarinic acetylcholine receptors and intracellular calcium elevation

The elevation of intracellular calcium following M1 receptor activation has been implicated in several of the postsynaptic effects of ACh on medium spiny neurones, including modulation of L-type calcium channels and *N*-methyl-D-aspartate receptors (Howe & Surmeier, 1995; Calabresi *et al.*, 1998). An mAChR-mediated increase in intracellular calcium has also been demonstrated in cultured striatal neurones (Lezcano & Bergson, 2002). However, it is uncertain whether this response was direct or mediated indirectly through membrane depolarization and activation of voltage-gated calcium channels. Our study in brain slices is therefore the first to demonstrate a direct increase of intracellular calcium following muscarinic M1 receptor activation. The calcium signal was acquired from the soma and proximal dendrites under voltage-clamp and hence involvement of voltage-gated channels was unlikely.

Our results with CPA and Ca^{2+}-free ACSF suggest that the muscarine-evoked elevation of calcium originates mainly from intracellular calcium stores. M1 receptor activation is associated with

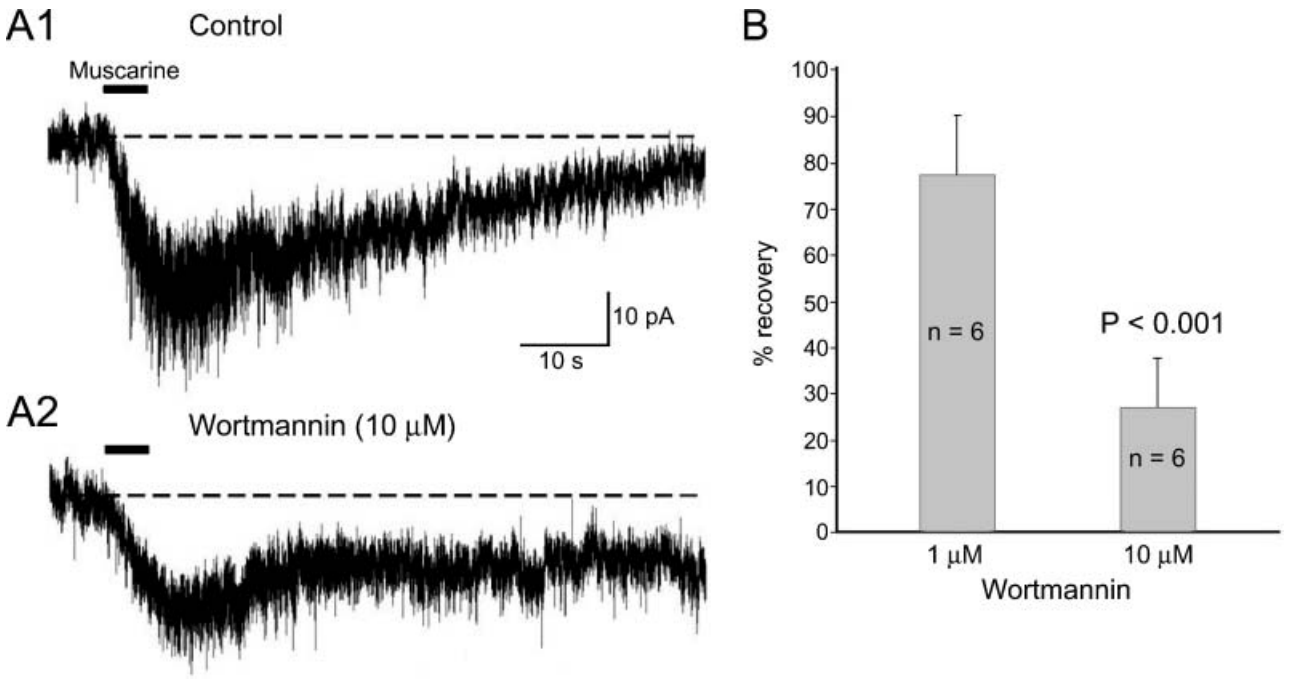

FIG. 6. Effect of wortmannin on the muscarine-induced inward current. (A) Wortmannin (10 μM, 10 min application) inhibited the recovery of the muscarine-induced inward current (A2) compared with control (A1). (B) The inhibition was concentration dependent. The 'recovery' value of the current was measured at the timepoint at which the control (pretreatment) response recovered to 100% (75 s).

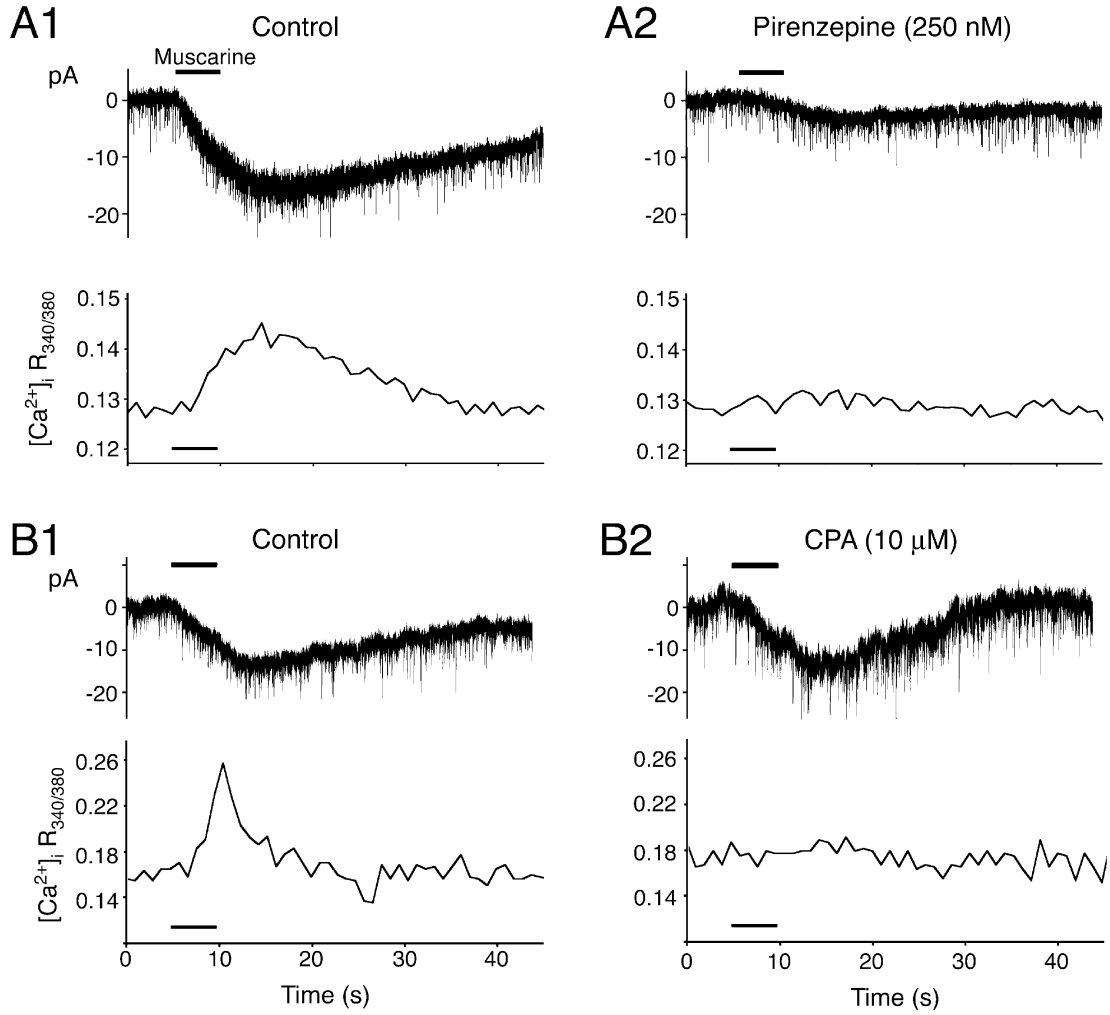

FIG. 7. Effects of pirenzepine and cyclopiazonic acid (CPA) on the intracellular calcium elevation and inward current. (A1 and B1) Examples of the muscarine-evoked elevation of intracellular calcium as detected with the ratiometric fura-2 technique (bottom traces). Upper traces show the simultaneously recorded membrane current. (A) In the cell illustrated in A1 and A2, both the inward current and Ca^{2+} signal were greatly reduced by the application of the M1 receptor-selective antagonist pirenzepine (250 nM). (B) Example of cell in which CPA (10 μM, 10 min application) completely abolished the calcium signal evoked by muscarine application but had only a small effect on the amplitude of the muscarine-induced membrane current.

activation of the PLC pathway, which can lead to IP_3-mediated calcium release from intracellular stores. IP_3 receptor expression has been reported in striatal medium spiny neurones (Martone *et al.*, 1997). It is unclear whether, at least in some neurones, calcium also originated from the extracellular space as the muscarine-induced calcium increase in the absence of extracellular calcium was highly variable among neurones although the group mean difference was insignificant.

The level of intracellular calcium in medium spiny neurones is important in the control of a variety of striatal functions. Calcium elevation can activate the calcium-dependent protein phosphatase 2B which controls DARPP-32 (Greengard, 2001). DARPP-32 is an intracellular phosphoprotein with multiple regulatory functions which is stimulated by the D1 dopamine receptor-mediated activation of protein kinase A and is inhibited by protein phosphatase 2B (Olianas & Onali, 1996; Greengard, 2001). Thus, the mAChR-induced increase in intracellular calcium may inhibit the D1 receptor-induced phosphorylation of DARPP-32 through calcium-dependent activation of protein phosphatase 2B. This particular intracellular pathway may be the basis for the beneficial effects of anticholinergic agents used in the treatment of Parkinson's disease, in which the balance between the pathways leading to dopaminergic and cholinergic receptors is disrupted.

The dissection of these intracellular pathways, especially the involvement of intracellular calcium, may also have important implications for the induction of corticostriatal long-term potentiation, as studies have indicated M1 mAChR activation to be a requirement for this phenomenon (Calabresi *et al.*, 1999).

Acknowledgements

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Abbreviations

ACh, acetylcholine; ACSF, artificial cerebrospinal fluid; 2-APB, 2-aminoethoxydiphenylborane; BAPTA, 1,2-bis(2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid; CPA, cyclopiazonic acid; DAG, diacylglycerol; IP_3, inositol 1,4,5 triphosphate; mAChR, muscarinic acetylcholine receptor; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; TRP, transient receptor potential.

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