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M3-like muscarinic receptors mediate Ca^{2+} influx in rat mesencephalic GABAergic neurones through a protein kinase C-dependent mechanism

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Abstract

GABAergic neurones in the mesencephalon are important regulators of dopamine neurones. Cholinergic projections from mesopontine nuclei preferentially synapse onto these GABAergic neurones, thus suggesting that ACh can regulate dopamine neurones indirectly by modulating GABAergic interneurones. Muscarinic receptors mediate excitation of these interneurones through a Ca^{2+}-dependent mechanism. Using a mesencephalic primary culture model, we show here that muscarine (10 μM) increases intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in GABAergic interneurones. Compatible with previous anatomical data, our pharmacological studies further suggest that the M3 receptor is the primary mediator of this increase. The rise in $[Ca^{2+}]_i$ induced by muscarine was not activity-dependent but required influx of Ca^{2+} from the extracellular medium. Consistent with the known coupling of the M3 receptor to PKC, the effect of muscarine was blocked by bisindolylmaleimide, a selective PKC antagonist. The effect of muscarine was inhibited by SKF 96365 and verapamil, drugs known to block non-selective cationic channels such as those formed by transient receptor potential (TRPC) proteins. Finally, GABAergic neurones were found to be immunopositive for TRPC1, 3, 5 and 6. Taken together, these results suggest that the Ca^{2+}-dependent regulation of mesencephalic GABAergic neurones by muscarinic receptors requires activation of some receptor-operated Ca^{2+} channels through a PKC-dependent mechanism.

Keywords: Muscarinic; Midbrain; Acetylcholine; Primary cultures; Calcium imaging; TRPC

1. Introduction

Central dopamine (DA) pathways such as the nigro-striatal and meso-cortico-limbic systems are implicated in motor activity and motivational processes (Kalivas and Stewart, 1991). Although the ventral part of the mesencephalon is composed principally of DA neurones, about 20% are GABAergic neurones (Bayer and Pickel, 1991; Johnson and North, 1992b). Such GABAergic interneurones are key targets of opiate drugs, which activate DA neurones by inhibiting local GABAergic neurones (Johnson and North, 1992a).

The ventral mesencephalon receives abundant cholinergic afferents from mesopontine nuclei (Clarke et al., 1987; Oakman et al., 1995). Interestingly, the majority (65%) of cholinergic fibres in this area impinge on DA

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transporter-negative neurones that are presumed to be mostly GABAergic (Garzon et al., 1999). Although acetylcholine (ACh) is thought to directly excite DAergic neurones (Imperato et al., 1986; Gronier et al., 2000), this preferential innervation of GABAergic neurones suggests that in part, ACh also exerts its effects on DAergic neurones by affecting local GABAergic interneurones. ACh can act through nicotinic ionotropic receptors or through muscarinic G-protein-coupled receptors. Muscarinic receptors control GABA release in several structures of the central nervous system (CNS) (Baba et al., 1998; Guo and Chiappinelli, 2001) including the ventral tegmental area (Grillner et al., 2000). Of the five cloned muscarinic receptors, only M3 and M5 mRNAs, part of the M1-like family, have been reported to be present in the ventral mesencephalon. However, their specific cellular localization has not yet been explored (Vilaro et al., 1990; Weiner et al., 1990; Zubieta and Frey, 1993). It has been demonstrated that DAergic neurones are rapidly excited through muscarinic M1-family receptors located in the ventral mesencephalon (Lacey et al., 1990). In addition, a delayed enhancement in DA release is mediated through M5 receptors located in the ventral mesencephalon (Forster et al., 2002; Gronier et al., 2000). Although the net effect of muscarinic activation in the ventral mesencephalon is excitatory, the specific role of local GABAergic neurones in muscarinic regulation has not been studied.

We have recently discovered that muscarine enhances the firing rate of mesencephalic GABAergic neurones by a Ca^{2+}-dependent mechanism (Michel et al., 2004). Here we study the nature of this Ca^{2+}-dependent mechanism to better understand how muscarinic receptors regulate GABAergic neurones. M3 and M5 muscarinic receptors are coupled to $G_{q/11}$ proteins and stimulate phospholipase C leading to activation of PKC and release of Ca^{2+} from intracellular stores (van Koppen and Kaiser, 2003). Muscarinic receptors can also activate Ca^{2+} influx through some store- and voltage-independent Ca^{2+} channels (Felder et al., 1992; Zhu et al., 1998), often referred to as receptor-operated Ca^{2+} channels. Molecular characterization of receptor-operated Ca^{2+} channels is not complete, but Ca^{2+} influx resulting from muscarinic activation may result from opening of TRPC channels (Zitt et al., 2002). Here we show that muscarinic receptor activation causes influx of Ca^{2+} from the extracellular medium. This influx requires PKC activation and depends on receptor-operated channels blocked by SKF 96365 and verapamil.

2. Methods

2.1. Cell culture

Experiments were performed using primary cultures of mesencephalic neurones as previously described (Cardozo, 1993; Sulzer et al., 1998; Congar et al., 2002). Briefly, coverslips coated with collagen/poly-L-lysine were covered with a monolayer of mesencephalic astrocytes, over which the neurones were subsequently plated. To prepare astrocyte cultures, neonatal (P0–P2) Sprague–Dawley rats were cryoanesthetized. Brains were quickly removed from the skull and placed in ice-cold dissociation solution. A 2-mm^3 block of ventral midbrain tissue was rapidly isolated from a 1-mm-thick slice cut at the level of the midbrain flexure. The tissue was then digested with papaïn (Worthington Biochemical Corp., Lakewood, NJ, USA) for 60-min at 37 °C before being gently triturated using glass pipettes of decreasing diameters until a single-cell suspension was obtained. Cells grown in culture flasks and maintained in Basal Medium Eagle with Earl's Salts (Invitrogen, Burlington, ON, Canada) supplemented with penicillin/streptomycin, GlutaMAX-1 (Invitrogen), 10% foetal calf serum (Invitrogen) and Mito+ serum extender (VWR Canlab, Montréal, QC, Canada). After the first 48 h, culture flasks were vigorously washed with cold medium to remove most neurones, leaving only tightly adherent astrocytes. After reaching confluence, astrocytes were gently trypsinized, washed, collected, counted and plated at a concentration of 100 000 living cells per ml. Twenty-four hours after plating, astrocytes covered most of the available surface and further division was inhibited with 5-fluoro-2-deoxyuridine (FUDR).

To prepare neurones, neonatal (P0–P2) Sprague–Dawley rats were cryoanesthetized and the ventral mesencephalon was rapidly isolated. As for the astrocyte preparation, the tissue was digested with papaïn for 30 min at 37 °C, before being gently triturated. The dissociated cells were then collected by centrifugation, counted and diluted at a density titrated to 240 000 living cells per ml in order to optimise neuronal viability and connectivity. Further astrocyte division was inhibited by a second addition of FUDR 24 h after neurones were plated. Neurone cultures were maintained in Neurobasal A/BME (2:1), penicillin/streptomycin, GlutaMAX-1, 10% foetal calf serum (Hyclone Laboratories, Logan, UT, USA) and B27 serum extender (Invitrogen). Kynurenate (0.5 mM) was added to the culture medium 7 days after neurones were plated in order to prevent excitotoxicity. Cell cultures were incubated at 37 °C in a 5% CO_2 atmosphere. Experiments were performed on neurones between 10 and 30 days after plating.

Electrophysiological recordings were performed on single isolated GABAergic neurones in "microdot" cultures. In this model, cultured neurones are plated on micro-droplets of substrate that limit synaptic connectivity to cells within the droplet (100–200 μm in diameter). In some droplets, single GABAergic neurones can be found that establish synaptic connections onto their own dendrites and cell body, thus allowing the

measurement of GABA-mediated autaptic currents (Bekkers and Stevens, 1991; Michel and Trudeau, 2000; Bergevin et al., 2002). To prepare microdot cultures, coverslips were coated first with poly-L-ornithine and then with agarose (0.05%). Micro-droplets of collagen were then applied with a micro-sprayer. Astrocytes and neurones were then plated in much the same way as for standard cultures, although final cell concentrations for astrocytes and neurones were 60 000 and 100 000 living cells per ml, respectively. These cell dilutions were established to maximize the number of microdots containing single neurones.

All procedures were approved by the animal ethical committee of the Université de Montréal.

2.2. Intracellular calcium imaging

Cells were loaded for 50–60 min at room temperature with Fura-2 AM (5 μM) (Molecular Probes Inc., Eugene, OR, USA) and pluronic acid (0.2%). Fluorescence images were collected through a Nikon Eclipse TE-200 inverted microscope coupled to a Hamamatsu Orca-II digital cooled CCD camera. Ratio fluorescence excitation at 340/380 nm was driven by a fast optical switch (DG4) (Sutter Instruments, Novato, CA, USA) and images were recorded with an Inovision workstation using Isee software (Inovision Corporation, Raleigh, NC, USA). Images were captured at a frequency of 0.2 Hz. Ratio values were converted to Ca^{2+} concentrations using Grynkiewicz's equation (Grynkiewicz et al., 1985) and an in situ calibration protocol to determine R_{max} and R_{min} values. Cells were perfused with normal extracellular saline containing (in mM): NaCl 140, KCl 5, $MgCl_2$ 2, $CaCl_2$ 2, *N*-(2-hydroxyethyl) piperazine-*N*-(2-ethanesulfonic acid) (HEPES) 10, glucose 10, sucrose 6, (pH of 7.35; 300 mOsm). Drugs were diluted and applied in standard saline solution. In most experiments, tetrodotoxin (TTX; 0.5 μM) (Alomone Labs, Jerusalem, Israel) was added to the extracellular medium to inhibit spontaneous action potentials and restrict analysis to neurones directly activated by the muscarinic receptor agonist. At the end of each experiment, saline containing 40 mM K^+ was applied for 1 min to depolarise neurones and verify their viability. Finally, a digital phase contrast micrograph of the field was acquired and fluorescent micro-spheres (Duke Scientific Corp., Palo Alto, CA, USA) were deposited locally using a patch pipette to facilitate the localization of the imaged neurones following immunolabelling to determine whether neurones were GABAergic (anti-GABA antibody) or DAergic (anti-tyrosine hydroxylase antibody).

As muscarinic effects on $[Ca^{2+}]_i$ virtually did not desensitise (see Section 3 and Fig. 1C, D) most of the imaging experiments followed the same basic procedure involving three successive applications of muscarine. The first application provided a control response for each cell. The second application of muscarine was performed in the presence of the test drug. The last application of muscarine permitted an evaluation of the extent of drug washout and effect reversibility. In all cases, the maximal elevation in $[Ca^{2+}]_i$ in response to muscarine was determined relative to baseline, irrespective of the fact that neurons showed a monotonic or oscillatory-like response.

2.3. Electrophysiology

Calcium currents were recorded at room temperature on microdot cultures. Signals were recorded with a Warner PC-505 or PC-501 patch-clamp amplifier (Warner Instrument Corp, Hamden, CT, USA), filtered at 1 kHz, digitised at 5 kHz and analysed with Pclamp 8 software (Axon Instruments, Foster City, CA, USA). Patch pipettes were prepared with borosilicate glass and had a resistance of 5 ± 0.5 MΩ. Intrapipette solution contained (in mM): CsF 68, CsCl 56, $MgCl_2$ 2.2, EGTA 4.5, creatine phosphate (tris salt) 14, ATP (Mg salt) 4, GTP (tris salt) 0.3, HEPES 9 (pH 7.35 adjusted with CsOH 50%; 300 mOsm). The phenotype of the recorded neurone was first tested by recording autaptic synaptic currents in normal saline. To evoke autaptic synaptic currents, isolated neurones were stimulated every 15 s with a brief (1 ms) voltage command pulse to 20 mV from a holding potential of −40 to −70 mV. Blockade of the synaptic response with SR95531 (5 μM), a selective and potent antagonist of $GABA_A$ receptors, confirmed the GABAergic nature of the recorded neurone. Calcium currents were isolated and recorded at a holding potential of −60 mV with an extracellular solution containing (in mM): tetraethylammonium chloride (TEACl) 160, $BaCl_2$ 5, HEPES 10 and tetrodotoxin (TTX) 0.5 (pH of 7.35 adjusted with TEAOH 20%; 300 mOsm). At the end of each experiment, cadmium (1 mM), a broad-spectrum blocker of voltage-dependent Ca^{2+} channels, was applied to confirm that Ca^{2+} currents were correctly isolated. In experiments where action potentials were recorded, the extracellular saline contained (in mM): NaCl 140, KCl 5, $MgCl_2$ 2, $CaCl_2$ 2, HEPES 10, glucose 10, sucrose 6 (pH 7.35 adjusted with NaOH 2 N; 300 mOsm). Action potentials were recorded in microdot cultures with a K^+ methylsulfate intrapipette solution containing (in mM): KMeSO4 120, KCl 20, NaCl 5, phosphocreatine (tris salt) 10, ATP (Mg salt) 4, GTP (tris salt) 0.5, HEPES 10, EGTA 0.1, (pH 7.35; 295 mOsm).

2.4. Immunocytochemistry

The phenotype of neurones was determined, when necessary, by post hoc immunocytochemical double labelling. Cells were fixed in paraformaldehyde (4%),

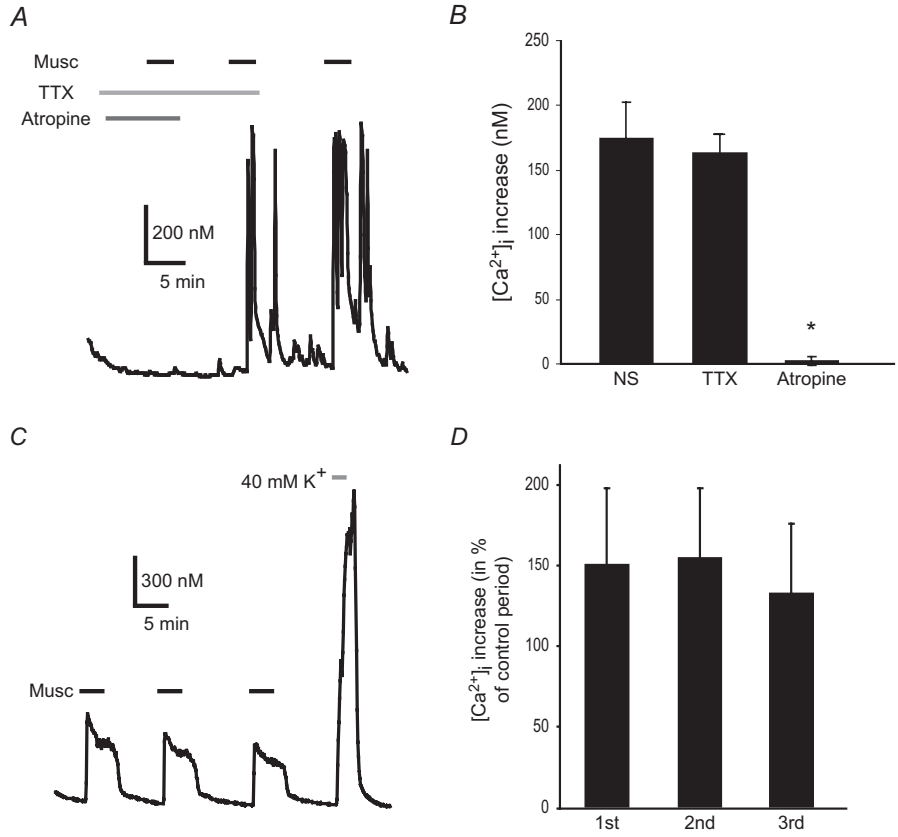


Fig. 1. Muscarine (10 μM) induces a reproducible increase in $[Ca^{2+}]_i$. (A) Example of relative levels of $[Ca^{2+}]_i$ in a representative neurone. Muscarine (10 μM) increased $[Ca^{2+}]_i$ in this GABAergic neurone, an effect that was reversibly blocked by atropine (1 μM). Note that TTX (0.5 μM) did not prevent the effect of muscarine. (B) Bar graph summarizing the effects of muscarine on all responsive GABAergic neurones in the presence of TTX, in normal saline (NS) or in the presence of atropine. (C) Representative experiment showing the effect of three successive applications of muscarine separated by 10 min intervals. K^+ saline (40 mM) was applied at the end of all experiments to demonstrate that the neurone was still metabolically active and able to buffer large loads of intracellular Ca^{2+}. (D) Bar graph summarizing the average $[Ca^{2+}]_i$ increase ($n = 7$) for three successive applications of muscarine. *$p < 0.05$ in comparison to control.

permeated with Triton X-100 (0.1%) and non-specific sites were blocked with a solution containing BSA (0.5%), Triton X-100 (0.1%) and goat serum (5%). Primary mouse monoclonal (1:100) or rabbit polyclonal (1:500) (Sigma) antibodies raised against GABA were used to identify GABAergic neurones. Anti-tyrosine hydroxylase (TH) mouse monoclonal (1:1000) (Pelfrez Biological, AK, USA) or rabbit polyclonal (1:5000) (Sigma) antibodies allowed identification of DAergic neurones. Fluorescent secondary antibodies used included Alexa-488, Alexa-546 or Alexa-647 (1:200) (Molecular Probes) or the DAB-HRP system (Jackson Immuno-Research Laboratories Inc. PA, USA). TRPC antibodies (1:500) were all from Alomone Labs, except anti-TRPC5 that was kindly provided by Dr. William Schilling from Case Western Reserve University School of Medicine, (Cleveland, OH, USA) (Goel et al., 2002). Images of immunofluorescent labelling were acquired using a point-scanning confocal microscope from Prairie Technologies LLC (Middleton, WI, USA). Excitation was achieved using the 488 nm line of an argon ion laser and with the 633 nm line of a helium–neon laser.

2.5. Data processing

As not all neurones were responsive to muscarine under our conditions, we included in our final analyses only neurones identified as "responsive". To determine objectively if a cell was responsive or not, we determined whether the recorded signal changed by at least two standard deviations away from control level in response to muscarine. A third of all recorded neurones were thus discarded.

Data presented in the text are expressed as mean ± SEM. Statistical comparisons were performed using Student's paired *t*-test (when comparing the response to the first application of muscarine (control) to the second response in presence of test drugs) or analyses of variance (ANOVA) with Tukey's post hoc test. In figures, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

2.6. Products

All chemicals were obtained from Sigma–Aldrich (Oakville, ON, Canada) except when indicated.

3. Results

3.1. Muscarinic receptors directly regulate $[Ca^{2+}]_i$ in mesencephalic GABAergic neurones

We have shown recently that muscarine, a selective agonist for muscarinic receptors, induces a sustained increase in firing rate in isolated mesencephalic GABAergic neurones in culture. This excitatory effect was also shown to be Ca^{2+}-dependent (Michel et al., 2004).

To elucidate the nature of the Ca^{2+} signal that underlies the muscarine-induced increase in firing rate, we monitored intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) using Fura-2 and Ca^{2+} imaging. The level of $[Ca^{2+}]_i$ was measured in individual cells, and the GABAergic or DAergic phenotype of imaged neurones was determined post hoc by immunocytochemistry. The average basal level of $[Ca^{2+}]_i$ across all neurones was 152 ± 11 nM ($n = 274$) in normal saline containing TTX (0.5 μM). In GABAergic neurones, muscarine increased $[Ca^{2+}]_i$ above control by 187 ± 15 nM (223% of control; $n = 199$; $p < 0.01$) (Fig. 1A, B). In some neurons, the effect of muscarine appeared as an oscillatory-like response with multiple peaks (Fig. 1A), while in others, the response was more monotonic (Fig. 1C). In a subset of neurons, muscarine was applied twice; first in the presence of TTX and then after washout of TTX. The first agonist application caused an average increase of 165 ± 16 nM above control ($n = 55$). After washout of TTX, the second application of muscarine caused a similar effect on average (151 ± 25 nM above control) ($n = 55$). In a second series of experiments, muscarine was tested first in normal saline and second in the presence of TTX. In these experiments, the first application of muscarine increased $[Ca^{2+}]_i$ above control by 282 ± 105 nM ($n = 12$). The second application of muscarine in the presence of TTX increased $[Ca^{2+}]_i$ above control by 157 ± 41 nM ($n = 12$). Because the difference between the effect of muscarine in normal saline or in the presence of TTX was not significantly different in either series of experiments ($p > 0.05$), all data were pooled (Fig. 1B). The effect of muscarine therefore did not require cell firing.

The specificity of muscarine's effect was confirmed by the complete inhibition of the muscarine-evoked increase in $[Ca^{2+}]_i$ by atropine (1 μM), a muscarinic antagonist without selectivity amongst the five muscarinic receptor subtypes ($101 \pm 3\%$ of control) ($n = 9$; $p < 0.05$) (Fig. 1A, B). The response to muscarine did not show any obvious desensitisation as three successive applications of muscarine evoked comparable responses ($150 \pm 47\%$, $154 \pm 44\%$ and $132 \pm 43\%$ of control; $n = 7$) (Fig. 1C, D). A brief 1 min pulse of 40 mM K^+ saline was applied at the end of each recording session to depolarise neurones and evoke a brief $[Ca^{2+}]_i$ elevation, thus confirming their viability.

3.2. Role of muscarinic receptor subtypes

To date, among the five cloned muscarinic receptors, only M3 and M5 mRNAs have been detected in the ventral mesencephalon (Vilaro et al., 1990; Weiner et al., 1990; Wang et al., 2004). No previous study has directly determined the presence and functional role of either receptor directly in mesencephalic GABAergic neurones (Grillner et al., 2000; Yeomans et al., 2001). Because muscarinic agonists are not sufficiently selective to discriminate among various muscarinic receptors, we tested the ability of muscarinic receptor antagonists to block muscarine-induced increases in $[Ca^{2+}]_i$.

First, AFDX-116 (500 nM) and tropicamide (100 nM) did not inhibit muscarine's effect on $[Ca^{2+}]_i$ ($132 \pm 21\%$ and $90 \pm 26\%$ of control, respectively; $p > 0.05$) (Fig. 2A). This suggests that M2 and M4 muscarinic receptors are unlikely to be implicated in the rise in $[Ca^{2+}]_i$ induced by muscarine. We next tested pirenzepine (10 and 100 nM, $n = 11$), known to block preferentially the M1 receptor, 4-DAMP (10 and 100 nM, $n = 5$ and 10, respectively), known to block preferentially the M3 receptor, and oxybutynin (10 and 100 nM, $n = 10$), another potent M1 and M3 antagonist that displays particularly low affinity for the M5 receptor (Watson et al., 1999; Caulfield and Birdsall, 1998; Eglen et al., 1999). Pirenzepine reduced the effect of muscarine to $54 \pm 10\%$ of control at 100 nM ($p < 0.05$) and to $42 \pm 18\%$ of control at 10 nM ($p < 0.05$). 4-DAMP reduced the effect of muscarine to $6 \pm 5\%$ of control at 100 nM ($p < 0.01$) and to $62 \pm 25\%$ of control at 10 nM ($p > 0.05$). Finally, oxybutynin reduced the effect of muscarine to $23 \pm 9\%$ at 100 nM ($p < 0.01$) and to $48 \pm 17\%$ of control at 10 nM (Fig. 2B). Their respective potency indicates that M3 receptors are likely to be playing the major role. The pharmacological profile of responses evoked in DAergic neurones was not examined in detail due to the lower number of neurones detected.

3.3. Role of extracellular Ca^{2+} influx

To characterize the origin of the increase in $[Ca^{2+}]_i$ induced by muscarinic receptor activation, we first tested the involvement of intracellular Ca^{2+} stores using thapsigargin (1 μM for 30 min), which depletes intracellular Ca^{2+} stores by inhibiting the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase pump. In mesencephalic cultures, thapsigargin, used under the same conditions, blocks intracellular Ca^{2+} mobilization caused by the peptide neurotensin (Trudeau, 2000). Although this treatment caused a small increase in $[Ca^{2+}]_i$ by itself (Fig. 3B), it did not reduce the ability of muscarine to increase $[Ca^{2+}]_i$ ($134 \pm 28\%$ of the first muscarine-induced response; $n = 9$; $p > 0.05$) (Fig. 3A, B). Compatible with the alternate hypothesis of a major

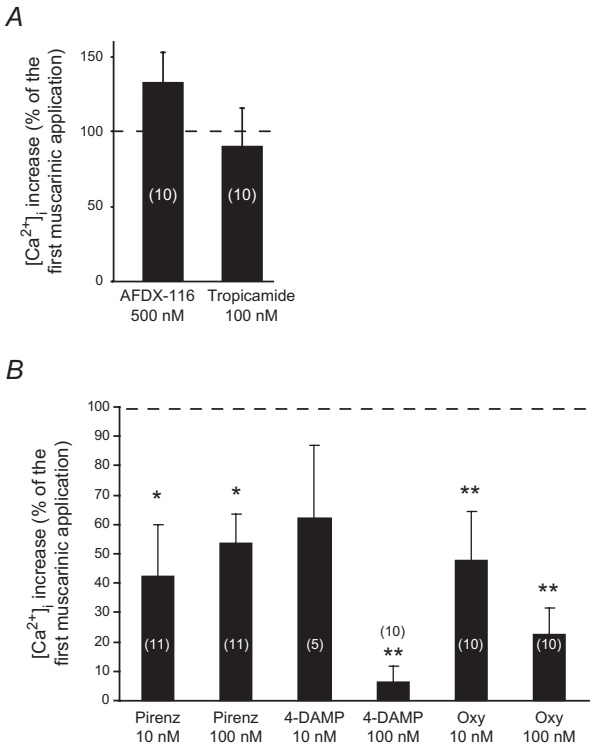


Fig. 2. Inhibition of the effect of muscarine with selective muscarinic receptor blockers. (A) Bar graph showing the lack of effect of AFDX-116 and tropicamide, two antagonists that block M2 and M4 muscarinic receptors, respectively. The neurones were tested twice with muscarine, with or without the antagonist. The data are presented in percent of the amplitude of a first application of muscarine. (B) Bar graph summarizing the effects of antagonists that inhibit M1 (pirenzepine), M3 (4-DAMP) and M1-4 (oxybutynin) muscarinic receptors. The data are presented in percent of the amplitude of the response to a first application of muscarine. The data are expressed in nM to illustrate the estimated changes in absolute levels in relation to the control period. *$p < 0.05$, **$p < 0.01$ in comparison to control.

role for Ca^{2+} influx, short applications of Ca^{2+}-free saline blocked the effect of muscarine on $[Ca^{2+}]_i$ ($8 \pm 6\%$ of control; $n = 24$; $p < 0.001$) (Fig. 3A, C). Finally, broad-spectrum inorganic Ca^{2+} channel blockers including gadolinium (Gd^{3+}) (50 μM) and cobalt (1 mM) blocked muscarine-evoked $[Ca^{2+}]_i$ increase. The block was complete with Gd^{3+} ($-3 \pm 1.3\%$ of control; $n = 9$; $p < 0.01$), while only $22 \pm 6\%$ of the control response remained in the presence of cobalt ($n = 12$; $p < 0.001$) (Fig. 3A). These results demonstrate that $[Ca^{2+}]_i$ elevation induced by muscarinic receptor activation in mesencephalic GABAergic neurones arises mostly from extracellular Ca^{2+} influx.

3.4. Involvement of a PKC-dependent mechanism

Activation of M1, M3 or M5 muscarinic receptors leads to the activation of phospholipase C and the

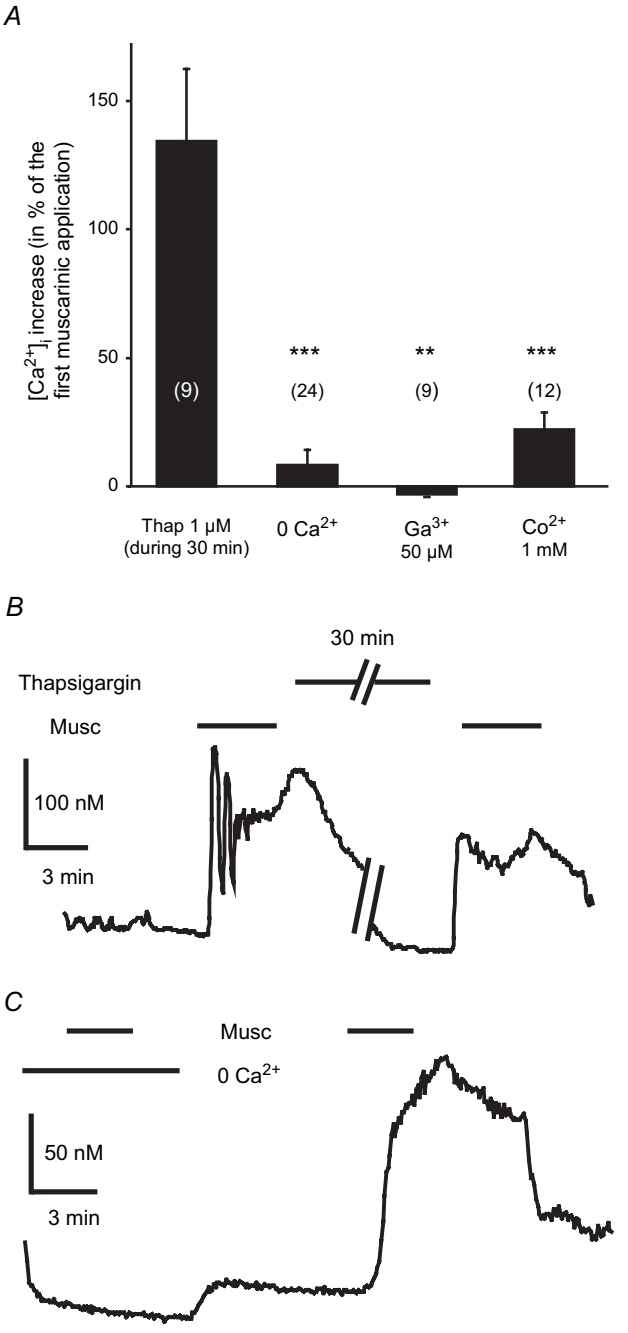
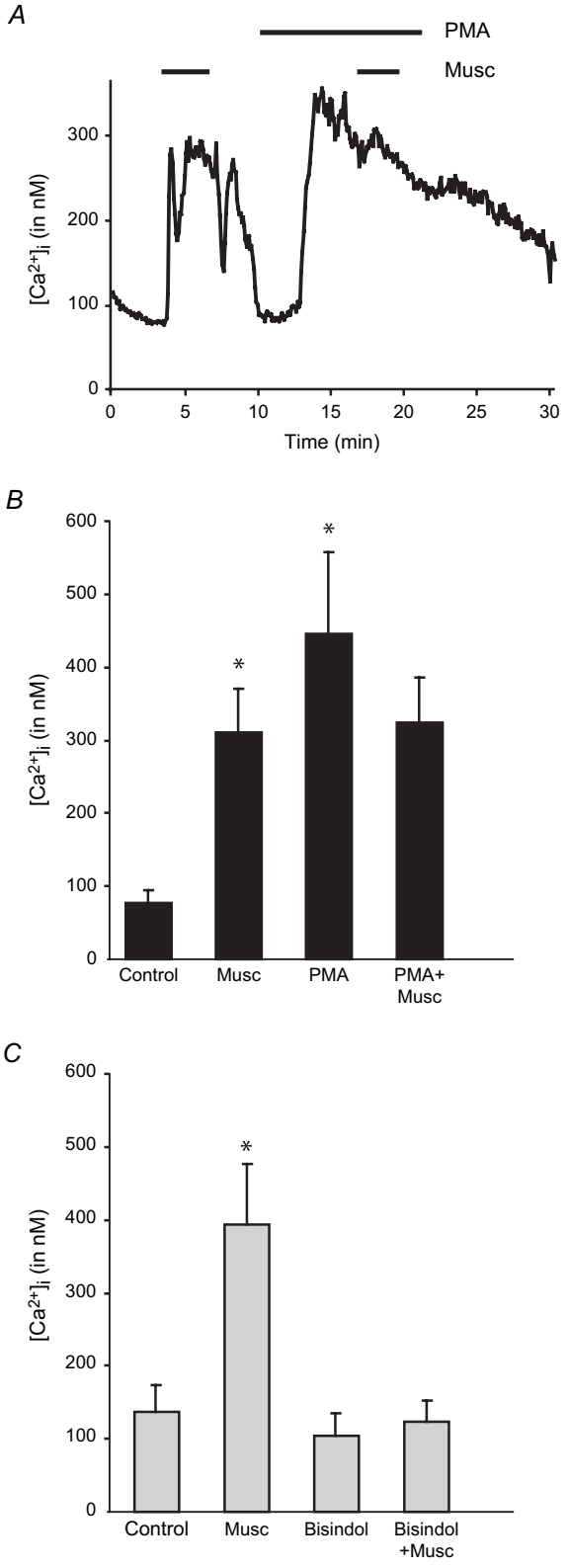


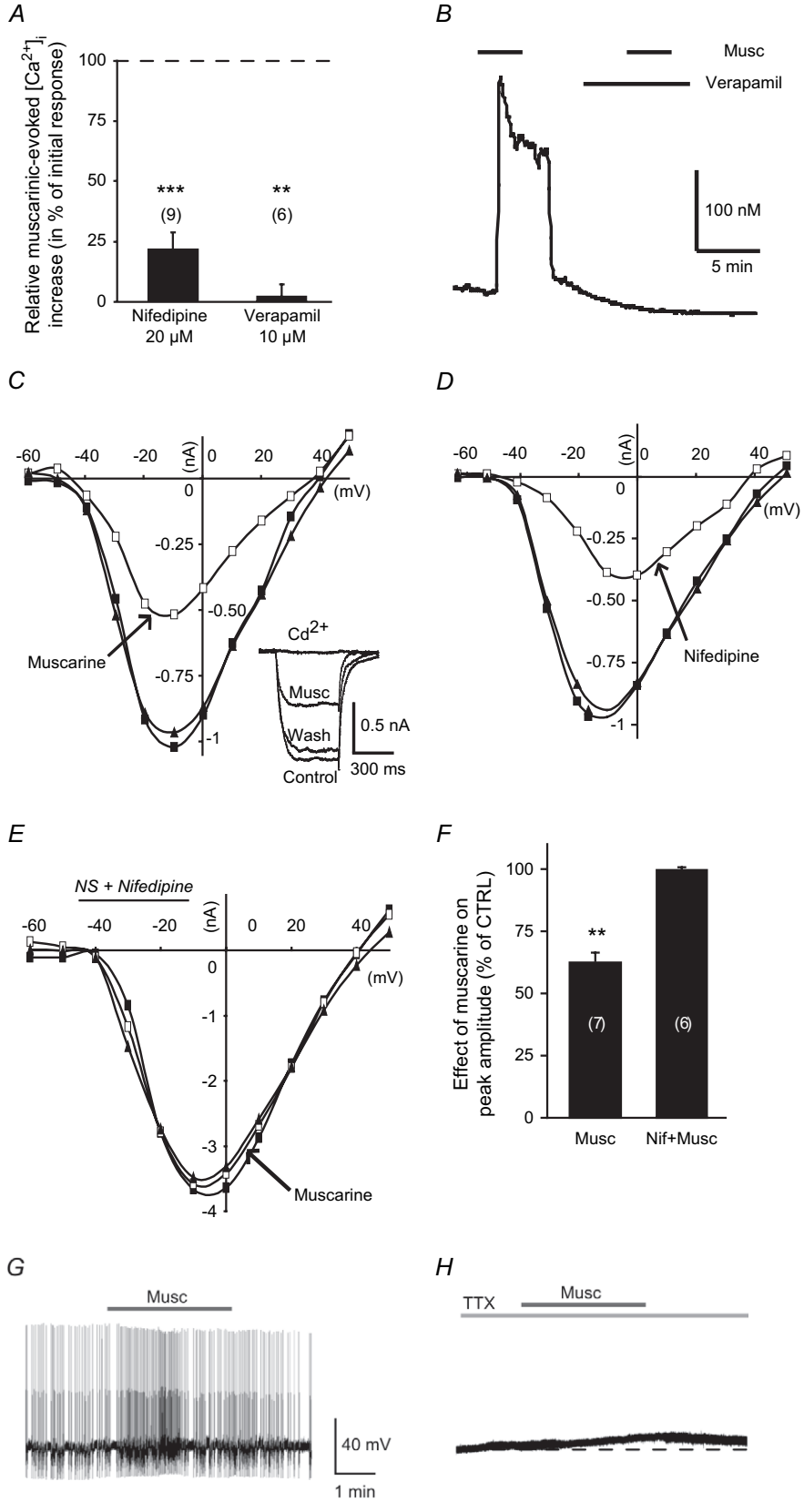
Fig. 3. Muscarinic receptor activation induces Ca^{2+} influx from the extracellular medium. (A) Bar graph summarizing the effect of thapsigargin (Thap), extracellular Ca^{2+} removal (0 Ca^{2+}) and inorganic Ca^{2+} channel blockers on muscarine-induced elevation in $[Ca^{2+}]_i$. Neurons were exposed twice to muscarine. The data are expressed in percent of the amplitude of the first response to muscarine, obtained in normal extracellular medium containing 0.5 μM TTX. (B) Example of variations in $[Ca^{2+}]_i$ in a representative neurone in the presence of thapsigargin (applied for 30 min after the first application of muscarine). (C) Example of variations in $[Ca^{2+}]_i$ in a representative neurone, illustrating the effect of Ca^{2+}-free saline on muscarine-induced elevation in $[Ca^{2+}]_i$. **$p < 0.01$, ***$p < 0.001$ in comparison to control.



production of second messengers such as IP3 and diacylglycerol (DAG). These messengers then lead to mobilization of intracellular Ca^{2+} stores and/or activation of protein kinase C (PKC). Although our previous results suggest that most of the muscarine-induced elevation in $[Ca^{2+}]_i$ arises from Ca^{2+} influx, this does not exclude a role for PKC, especially considering that Ca^{2+} influx through some receptor-operated Ca^{2+} channels has previously been reported to be PKC-dependent (Premkumar and Ahern, 2000). To test this hypothesis, we determined whether direct activation of PKC using phorbol 12-myristate 13-acetate (PMA) (500 nM) mimics the effect of muscarine. Indeed, PMA significantly increased $[Ca^{2+}]_i$ (446 ± 113% of control; $n = 8$; two-way ANOVA; main effect of PMA, $F(1,31) = 7.1$, $p < 0.05$) (Fig. 4A, B see also Fig. 6B). After reaching a peak, the response gradually declined. Moreover, PMA-induced elevation in $[Ca^{2+}]_i$ completely occluded the effect of muscarine (13 ± 5% of control; $n = 8$; two-way ANOVA, interaction factor $F(1,31) = 6.1$, $p < 0.05$) (Fig. 4A, B). For these experiments, the baseline level was considered to be the average of the pre-muscarine and post-muscarine periods, thus accounting for the gradual decline of $[Ca^{2+}]_i$ following the initial rise caused by PMA. In a separate experiment, PMA was used at a lower concentration of 100 nM. At this concentration PMA failed to cause a significant rise in $[Ca^{2+}]_i$ (143 ± 42% of control; $p > 0.05$) ($n = 4$), suggesting that this concentration was insufficient to robustly activate the Ca^{2+} entry mechanism. At this concentration, PMA had variable effects on muscarine-evoked intracellular Ca^{2+} elevation and although it reduced Ca^{2+} entry, this was not statistically significant (47 ± 25% of control; $n = 4$, two-way ANOVA, interaction factor $F(1,15) = 0.1$, $p > 0.05$) (not shown).

The implication of PKC in muscarine-evoked elevations of $[Ca^{2+}]_i$ in GABAergic neurones was confirmed by showing that the PKC inhibitor bisindolylmaleimide (10 μM) blocked the effect of muscarine (19 ± 8% of control; $n = 7$; two-way ANOVA; interaction factor $F(1,27) = 5.6$, $p < 0.001$) (Fig. 4C). Unexpectedly, although PMA also occluded the ability of muscarine to cause $[Ca^{2+}]_i$ elevation in DAergic neurones (5 ± 13% of baseline; $n = 5$, two-way ANOVA, interaction factor $F(1,19) = 6.1$, $p < 0.05$), muscarine's effect was PKC-independent in these neurones since bisindolylmaleimide was without any effect (98 ± 13% of control, $n = 11$) (results not shown).

Fig. 4. Role of PKC in muscarine-induced Ca^{2+} influx in GABAergic neurones. (A) Example of variations in $[Ca^{2+}]_i$ in a representative GABAergic neurone illustrating the effect of 500 nM PMA. This PKC activator increased $[Ca^{2+}]_i$ and occluded any further effect of muscarine. (B) Bar graph illustrating the effect of the PKC activator PMA (500 nM) on muscarine-induced elevation of $[Ca^{2+}]_i$ in mesencephalic GABAergic neurones. (C) Bar graph illustrating the effect of the PKC inhibitor bisindolylmaleimide (Bisindol) on muscarine-induced elevation of $[Ca^{2+}]_i$ in mesencephalic GABAergic neurones. *$p < 0.05$ in comparison to control.


A
Relative muscarinic-evoked [Ca2+]i increase (in % of initial response)
100
75
50
25
0
***
(9)
**
(6)
Nifedipine 20 µM
Verapamil 10 µM
B
Musc
Verapamil
100 nM
5 min
C
-60 -40 -20 (nA) 0 20 40
0
-0.25
-0.50
-0.75
-1
(mV)
Muscarine
Cd2+
Musc
Wash
Control
0.5 nA
300 ms
D
-60 -40 -20 (nA) 0 20 40
0
-0.25
-0.50
-0.75
-1
(mV)
Nifedipine
E
NS + Nifedipine
-60 -40 -20 (nA) 0 20 40
0
-1
-2
-3
-4
(mV)
Muscarine
F
Effect of muscarine on peak amplitude (% of CTRL)
100
75
50
25
0
**
(7)
(6)
Musc
Nif+Musc
G
Musc
40 mV
1 min
H
TTX
Musc

3.5. Lack of involvement of voltage-dependent calcium channels

Considering that broad-spectrum Ca^{2+} channel blockers such as Gd^{3+} and cobalt can prevent muscarine-induced $[Ca^{2+}]_i$ elevation in GABAergic neurones, a possibility is that muscarinic receptor activation enhances the function of voltage-dependent Ca^{2+} channels (VDCCs), perhaps shifting their voltage-dependency to more negative potentials. Considering that L-type voltage-dependent Ca^{2+} channels are abundant in the somatodendritic compartment of most neurones, we evaluated whether nifedipine and verapamil, two potent antagonists of L-type Ca^{2+} channels, could interfere with the effect of muscarine. Both drugs, used at concentrations commonly used (20 μM and 10 μM, respectively) (Bonci et al., 1998) were effective and mostly blocked the ability of muscarine to elevate $[Ca^{2+}]_i$ ($16 \pm 5\%$ of control for nifedipine; $n = 9$; $p < 0.001$, and $1 \pm 4\%$ of control for verapamil; $n = 6$; $p < 0.01$) (Fig. 5A, B).

To clarify the possible implication of VDCCs, we determined the effect of muscarine on pharmacologically isolated Ca^{2+} currents in single GABAergic neurones, recorded in the whole-cell configuration. Current–voltage relationships were constructed. Incompatible with the hypothesis of a facilitation of VDCCs, muscarine instead inhibited the amplitude of Ca^{2+} currents by $37 \pm 3\%$ (evaluated at −10 mV; $n = 7$; $p < 0.01$) (Fig. 5C, F). L-type channels carried a major component of the measured currents because nifedipine (10 μM) inhibited the current by $60 \pm 7\%$ ($n = 6$, $p < 0.05$) (Fig. 5D). Furthermore, the current inhibited by muscarine was mostly L-type because pre-application of nifedipine completely occluded any additional inhibition of the Ca^{2+} current by muscarine ($100 \pm 1\%$ of control; $n = 6$) (Fig. 5E, F). These results allow us to conclude that although muscarinic receptors can regulate L-type Ca^{2+} channels in mesencephalic GABAergic neurones, the modulation is in a direction opposite to that expected for a contribution to muscarine-induced Ca^{2+} elevation. Nifedipine and verapamil are therefore likely to mediate their effect by blocking other, non voltage-gated Ca^{2+} channels. As a final test of the involvement of voltage-dependent channels in muscarine-induced Ca^{2+} elevation, we performed membrane potential recordings from isolated GABAergic neurons. Although as previously reported, muscarine caused an increase in firing rate (Fig. 5G), this was accompanied by only minor membrane depolarisation when assessed in the presence of TTX (Fig. 5H). On average, muscarine caused a small depolarisation of 1.6 ± 1.1 mV ($n = 7$), a value not likely to be sufficient to open voltage-gated Ca^{2+} channels in neurons with a resting potential of -51.8 ± 3.0 mV.

3.6. Potential involvement of receptor-operated channels

In addition to blocking L-type Ca^{2+} channels, agents such as Gd^{3+} and verapamil can also block receptor- and store-operated channels, such as those assembled by the recently identified members of the TRP channel family. Indeed, several recent studies have demonstrated that these channels are Ca^{2+}-permeable, that they can be activated by G-protein-coupled receptors like muscarinic receptors, and that they can be blocked by inorganic cations such as Gd^{3+}, by verapamil and by other more specific antagonists such as SKF 96365 (Harteneck et al., 2000; Merritt et al., 1990; Zhu et al., 1998). We found that SKF 96365 (25 μM) also blocked most of the muscarine-induced elevation of $[Ca^{2+}]_i$ ($20 \pm 2\%$ of control; $n = 18$, $p < 0.001$) (Fig. 6A). Finally, Zn^{2+} (10 μM), suggested to act as a blocker of some receptor- and store-operated channels (Prothero et al., 2000; Kaneko et al., 2002) was found to be ineffective ($135 \pm 51\%$ of control; $n = 14$, $p > 0.05$) (Fig. 6A). Together, these results suggest that receptor-operated Ca^{2+} channels are involved in the effect of muscarine on Ca^{2+} influx in mesencephalic GABAergic neurones, and that verapamil, the most effective antagonist identified in our experiments, probably acts by blocking TRP-like receptor-operated channels.

Considering our finding that PKC is required for the ability of muscarine to elevate $[Ca^{2+}]_i$, we finally tested whether verapamil could prevent the ability of the PKC

Fig. 5. Muscarine-induced Ca^{2+} influx does not involve facilitation of L-type voltage-dependent Ca^{2+} channels. (A) Bar graph summarizing the effects of the L-type VDCC blockers nifedipine and verapamil on muscarine-evoked elevations in $[Ca^{2+}]_i$. The data are presented as percent of the amplitude of the response evoked by a first application of muscarine. (B) Variations in $[Ca^{2+}]_i$ in a representative neurone illustrating the effect of verapamil (10 μM) on muscarine-evoked elevation in $[Ca^{2+}]_i$. (C) Current–voltage (I/V) relationship illustrating the effect of muscarine (10 μM) on pharmacologically isolated Ca^{2+} currents in a representative GABAergic neurone. The black squares (■) represent the control condition, open squares (□) the current in presence of muscarine and black triangles (▲) the current after washout of the drug. Inset: Example of a set of traces from the same cell in response to a voltage step to −10 mV. The broad-spectrum Ca^{2+} channel blocker Cd^{2+} was added at the end of each experiment to confirm the selective isolation of Ca^{2+} currents. (D) I/V curve illustrating the effect of nifedipine (10 μM) on Ca^{2+} currents. (E) I/V curve illustrating the lack of effect of muscarine after pre-application of nifedipine. (F) Bar graph summarizing the inhibitory effect of muscarine on peak Ca^{2+} current amplitude in response to a voltage step to −10 mV, with or without pre-application of nifedipine (Nif). The data are presented in percent of control amplitude. (G) Patch-clamp recording from an isolated GABAergic neurone. Muscarine increased firing rate but only caused minor membrane depolarisation. (H) Recording from the same neurone as in G, but in the presence of TTX. Muscarine caused only minor membrane depolarisation. ** $p < 0.01$, ***$p < 0.001$ in comparison to control.

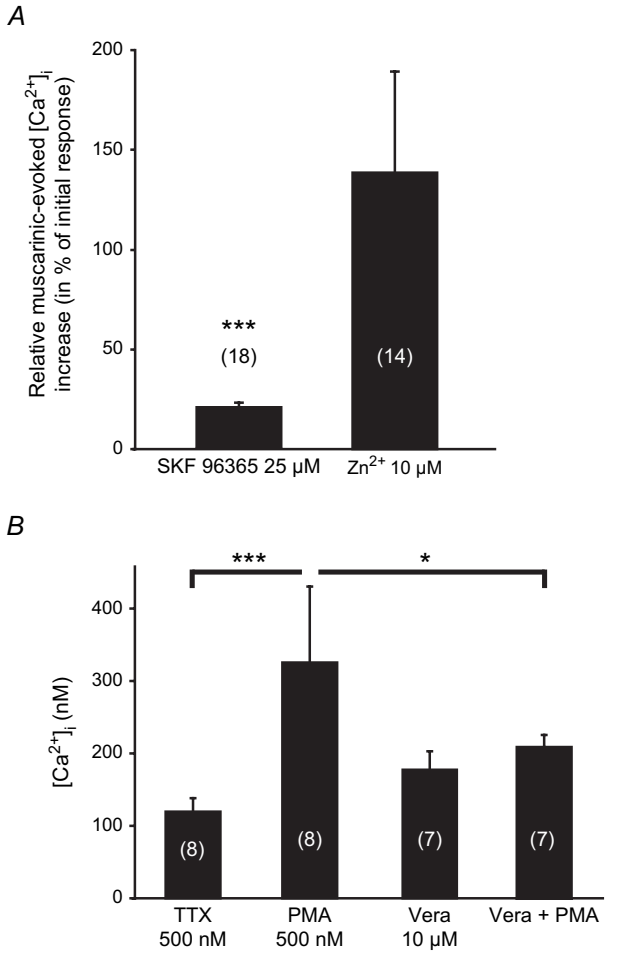


Fig. 6. Evidence for the implication of PKC-sensitive receptor-operated Ca^{2+} channels. (A) Bar graph illustrating the inhibitory effect of SKF 96365 (25 µM) and the lack of effect of Zn^{2+} (10 µM) on muscarine-induced Ca^{2+} influx. The data are expressed in percent of the response to a first application of muscarine. (B) Bar graph summarizing the effect of PMA (500 nM) on $[Ca^{2+}]_i$. Verapamil (Vera) prevented the effect of PMA. Resting level of intracellular Ca^{2+} in the presence of 500 nM TTX alone is also shown for reference. The data are expressed in estimated concentrations of intracellular Ca^{2+}. ***$p < 0.001$, *$p < 0.05$, two-way ANOVA.

activator PMA to mimic the effect of muscarine. In agreement with this hypothesis, PMA produced no significant effect on $[Ca^{2+}]_i$ in the presence of verapamil (10 µM) (two-way ANOVA, interaction factor $F(1,29) = 5.5$, $p < 0.05$) (Fig. 6B). We conclude that the receptor-operated Ca^{2+} influx pathway activated by muscarinic receptors and by PKC are likely to be the same.

3.7. Expression of TRPC channels in mesencephalic neurones

TRPC channels are broadly and abundantly expressed in the CNS (Riccio et al., 2002). The substantia nigra reportedly contains mRNA for TRPC1, 5 and 6, with lower levels of TRPC3 and 4, and no TRPC7 (Riccio et al., 2002). However, the precise cellular distribution of these transcripts is not yet well characterized. We used immunocytochemistry to evaluate the presence and distribution of TRPC1, 3, 4, 5 and 6 proteins in isolated mesencephalic neurones. No attempt was made to localize TRPC7 or TRPC2 because they are reportedly not present in rodent midbrain (Riccio et al., 2002). Co-labelling was performed to localize glutamic acid decarboxylase (GAD), a phenotypic marker of GABAergic neurones. In isolated neurone cultures, GABAergic interneurones expressed TRPC1, 3, 5 and 6 (Fig. 7) but not TRPC4 (not shown). DAergic

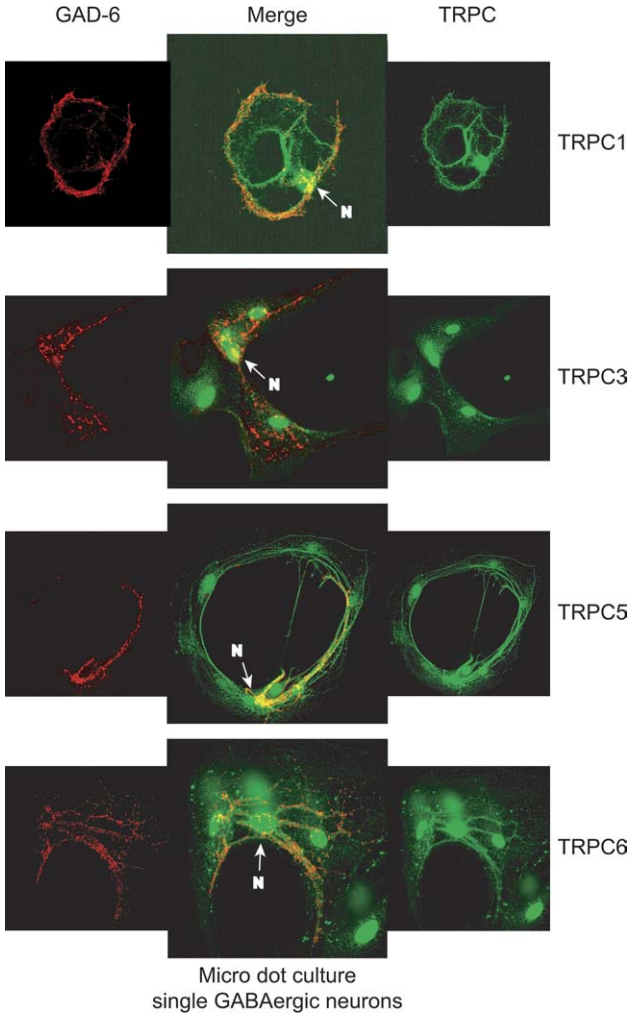


Fig. 7. Expression of TRPC channels in mesencephalic GABAergic neurones. Fluorescence micrographs illustrating the expression of TRPC1, 3, 5 and 6 (in green) in GABAergic neurones co-labelled for glutamic acid decarboxylase (GAD) (in red). The central panel of each series provides a merged image showing signal co-localization (in yellow). Note that GAD labelling was restricted to nerve terminals and did not delineate cell bodies. Experiments were performed in single-neurone microdot cultures, assuring that all signal of neuronal origin originated from GABAergic neurones only (labelled with an N). Note that TRPC1 labelling was restricted to neuronal processes, TRPC3 was mostly nuclear, and TRPC5–6 labelled both neuronal processes and nuclear compartments.

neurones, identified in standard cultures by their expression of tyrosine hydroxylase (TH) expressed the same pattern (not shown). Note that TRPC3 labelling was almost exclusively nuclear, TRPC1 was mostly localized on the cell body and major neuronal processes, while TRPC5 and 6 were found both in nuclei and cell processes (Fig. 7). Glial cells also expressed TRPC3, 5 and 6 but not TRPC1. In astrocytes, the labelling was mostly nuclear, although golgi-like structures also appeared to be immunopositive. Although these data do not prove that muscarinic receptors regulate TRPC-containing channels, they provide data in support of this possibility.

4. Discussion

Muscarinic receptors mediate excitation of mesencephalic GABAergic neurones through a Ca^{2+}-dependent mechanism. Here we have characterized the mechanism mediating the $[Ca^{2+}]_i$ elevation. We found that M3-like muscarinic receptors were the main receptors involved and that their activation leads to Ca^{2+} influx from the extracellular medium. Pharmacological and electrophysiological evidence further suggest that receptor-operated channels mediate this Ca^{2+} influx rather than VDCCs and that PKC activation is required.

4.1. Implication of M3 receptors

Although physiological evidence for a M1-family receptor in the substantia nigra has been previously reported (Lacey et al., 1990), only M3 and M5 receptor mRNA have yet been detected in the ventral tegmentum (Weiner et al., 1990; Zubieta and Frey, 1993). These receptors are all coupled to $G_{q/11}$ proteins that activate phospholipase C-dependent transduction pathways, leading to stimulation of PKC. Here we have provided pharmacological evidence that M3-like receptors are the primary receptor subtype mediating rapid muscarine-induced $[Ca^{2+}]_i$ elevation in GABAergic neurones. First, muscarine-induced Ca^{2+} influx was completely blocked by 100 nM 4-DAMP and partially affected by pirenzepine (10 and 100 nM). Considering that pirenzepine has a K_i of ~10 nM for M1 and ~150 nM for M3 (depending on the binding conditions) (Caulfield and Birdsall, 1998), and that 4-DAMP is recognized as one of the most selective M3 receptor antagonist available (Michel et al., 1989) this argues for a major role of the M3 receptor. The finding that oxybutynin (100 nM), a molecule that is a potent antagonist of the M3 receptor with a relatively low affinity for the M5 receptor, did leave $23 \pm 9\%$ of the response, leaves open the possibility of a minor participation of M5 receptors. In conclusion, our pharmacological data argue for a major role of the M3 receptor. This is highly compatible with the anatomical data arguing for the presence of M3 but not M1, M2 or M4 receptors in this area. Although we cannot exclude that the M5 receptor could have a minor role, we find that in M5-knockout mice, muscarine still elevates intracellular Ca^{2+} in mesencephalic GABAergic neurons (Michel, Yeomans and Trudeau, unpublished results).

Our results need to be considered within the context of several studies by Forster and Blaha (2000, 2003) and Yeomans et al. (2001) in which it was demonstrated that M5 receptors in the ventral mesencephalon are required for a prolonged activation of DA release in vivo induced by electrical stimulation of mesopontine cholinergic nuclei. It should be noted that this release begins 3–5 min after stimulation and peaks after 20–30 min. This time course is much slower that the intracellular Ca^{2+} elevations induced by muscarine in our experiments. It is therefore likely that separate mechanisms are involved. Nonetheless, $[Ca^{2+}]_i$ elevation and excitation mediated by muscarinic receptors in mesencephalic GABAergic neurones could regulate the delayed excitation of DAergic neurones independently mediated through M5 receptors.

4.2. Involvement of a PKC-dependent calcium influx pathway

The elevation in $[Ca^{2+}]_i$ induced by muscarine was strictly dependent on Ca^{2+} influx from the extracellular medium. This influx was, however, not secondary to cell firing since muscarine-induced intracellular Ca^{2+} elevation was not blocked by TTX.

Arguing against a significant role for intracellular Ca^{2+} stores, the Ca^{2+} ATPase blocker thapsigargin did not prevent the effect of muscarine. These results suggest that intracellular Ca^{2+} stores, store-operated channels and Ca^{2+}-induced Ca^{2+}-release mechanisms do not play a major role. Considering that M3 receptors activate PLC, leading to IP3 and DAG production, the lack of implication of intracellular Ca^{2+} stores may be somewhat surprising. Nonetheless, our finding that activation of PKC is necessary for the ability of muscarinic receptors to cause Ca^{2+} influx in GABAergic neurones is compatible with the known signalling pathways of the M3 receptor. Non-selective cationic conductances have previously been reported to be activated through a PLC-dependent pathway, but the specific involvement of PKC was not determined (Kawanabe et al., 2002). However, recent work has shown that Ca^{2+} influx through TRPV1, a member of the TRP-family receptor-operated Ca^{2+} channels, requires PKC activation (Premkumar and Ahern, 2000). It is interesting to note that muscarinic receptor-mediated Ca^{2+} influx in DAergic neurones was PKC-independent. This suggests that although the same receptor may mediate similar responses in GABAergic

and DAergic neurones, two different signalling pathways may be involved. However, the ability of PMA to occlude responses to muscarine in both cell types suggests that both possess PKC-dependent receptor-operated Ca^{2+} channels, but only in GABAergic neurones is PKC sufficiently activated by muscarinic receptors to mediate channel activation. Other PKC-independent receptor-operated Ca^{2+} channels are likely to be activated by muscarinic receptors in DAergic neurones. More experiments will be necessary to clarify the PKC-dependent and PKC-independent pathways in GABAergic and DAergic neurones.

Our pharmacological characterization of the Ca^{2+} influx pathway activated by muscarinic receptors in mesencephalic GABAergic neurones points toward the involvement of receptor-operated Ca^{2+} channels. In addition to removal of extracellular Ca^{2+}, the inorganic Ca^{2+} channel blockers Co^{2+} and Gd^{3+} blocked the effect of muscarine. These agents are known to block not only VDCCs, but also non-selective cationic channels (NSCCs) or store-operated channels. In addition, both nifedipine and verapamil were effective antagonists. Because the later two drugs are well known to block L-type VDCCs, a simple initial hypothesis was that muscarinic receptors could modulate L-type Ca^{2+} channels, perhaps shifting their voltage-dependence is a way that would allow them to be activated close to resting potential. Our direct recordings of Ca^{2+} currents in GABAergic neurones, however, allow us to exclude this later hypothesis. Not only were VDCCs not facilitated, but they were also actually inhibited by over 30%. Taken together with our finding that the NSCC blocker SKF 96365 was effective at blocking the action of muscarine, we conclude that some currently unidentified receptor-operated Ca^{2+}-permeable channels must be regulated by muscarinic receptors in GABAergic neurones, leading to Ca^{2+} influx.

4.3. Possible involvement of TRPC channels

Inorganic blockers like Gd^{3+} are effective antagonists of NSCCs, but they are clearly not selective inasmuch as they also block VDCCs and a variety of other cationic channels. Much recent work suggests that a subset of NSCCs may be assembled from TRP-family proteins. The identification of TRP channels derived from studies on "transient receptor potential" *Drosophila M.* mutants (Hardie and Minke, 1992). Mammalian TRPC proteins, one of the three subfamilies of TRP, have been cloned recently (Wes et al., 1995; Zhu et al., 1996; Boulay et al., 1997; Philipp et al., 1996, 1998; Okada et al., 1998) and belong to the superfamily of six transmembrane segments cation channels. They are closely related to voltage-gated Ca^{2+} channels in many respects, one notable exception being their lack of voltage sensitivity (Harteneck et al., 2000). Rather, these channels can be activated via $G_{q/11}$ protein-coupled receptors through signal transduction pathways associated with PLC (Lintschinger et al., 2000; Strubing et al., 2001). In particular, a number of studies in heterologous expression systems have shown that muscarinic receptors such as the M3 and M5 can efficiently activate TRPC channels, leading to the idea that TRPCs are molecular entities responsible for the formation of receptor-operated channels (Boulay et al., 1997; Strubing et al., 2001; Vannier et al., 1999; Zhu et al., 1998).

Gd^{3+} is effective at blocking TRPC channels formed by TRPC1, 3 and 6 (Inoue et al., 2001; Zhu et al., 1998; Boulay et al., 1997). There are currently no clearly demonstrated selective blockers of TRPC channels. Nonetheless, a widely used antagonist is SKF 96365, which effectively blocks NSCCs such as TRPC channels (Merritt et al., 1990; Zhu et al., 1998). This drug was very effective at inhibiting the effect of muscarine on Ca^{2+} influx in GABAergic neurones. This finding provides evidence in favour of the hypothesis that TRPC channels are involved. Nifedipine and verapamil also effectively inhibited Ca^{2+} influx elicited by muscarine. These drugs are recognized as L-type VDCC blockers, but they could also target some NSCCs. Although no data are presently available for nifedipine, Zhu et al. (1998) demonstrated that verapamil blocks Ca^{2+} influx through TRPC3 channels with an IC_{50} of 4 μM. This finding lends further support to the potential implication of TRPC channels in the muscarine-induced $[Ca^{2+}]_i$ elevation we observed. We also evaluated Zn^{2+} as a potential blocker of NSCCs. Considering that this cation may be effective at blocking channels containing TRPC6 but not TRPC1 (Kaneko et al., 2002; Liu et al., 2000), one may speculate that muscarinic receptors are coupled to receptor-operated Ca^{2+} channels containing TRPC1 subunits. Our finding that TRPC1 is indeed present in mesencephalic GABAergic neurones makes this hypothesis reasonable. However, because other TRPCs including 3, 5 and 6 are also present, strategies involving selective downregulation of individual TRPCs will be required to determine which is necessary and sufficient to mediate the effects of muscarinic receptor agonists. The potential implication of TRPV (vanilloid) channels should also be investigated considering their sensitivity to PKC (Premkumar and Ahern, 2000).

Flufenamate is another known blocker of Ca^{2+}-activated NSCCs (Partridge and Valenzuela, 2000). We evaluated the ability of this agent to block Ca^{2+} influx induced by muscarine, but we found this drug unsuitable for Ca^{2+} imaging experiments since by itself, it induced significant Ca^{2+} influx. This effect prevented a determination of its ability to interfere with the effect on muscarine (results not shown). Interestingly, Inoue et al. (2001) reported that flufenamate activates TRPC6.

This could explain why this drug initiates Ca^{2+} influx in our preparation.

In conclusion, our findings clarify how ACh acts on GABAergic neurones of the ventral mesencephalon. In particular, we provide a mechanism explaining the Ca^{2+}-dependency of the excitatory effect of muscarinic agonists on mesencephalic GABAergic neurones. We conclude that muscarine induces Ca^{2+} influx through some receptor-operated channels. PKC-dependent activation of TRP-family channels, selectively in GABAergic neurones, provides a likely molecular mechanism explaining our observations. Finally, our findings provide a first step toward an evaluation of the physiological role of muscarinic regulation of mesencephalic GABAergic neurones.

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