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FUNCTIONAL EXPRESSION OF A CLONED *DROSOPHILA* MUSCARINIC ACETYLCHOLINE RECEPTOR IN A STABLE *DROSOPHILA* CELL LINE

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Summary

A cloned *Drosophila* muscarinic acetylcholine receptor (mAChR) has been stably expressed in a *Drosophila* cell line (S2) under the control of an inducible *Drosophila* metallothionein promoter. A clonal cell line (S2–Dm1-1) has been isolated which, after induction of mAChR expression with $CuSO_4$, exhibits high-affinity, saturable, specific binding of the muscarinic antagonist *N*-methyl scopolamine (NMS). The apparent molecular mass of the expressed protein, calculated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), is in good agreement with the apparent molecular mass of mAChRs purified from *Drosophila* brain. Functional expression of the cloned mAChR in this stable cell line has been demonstrated by quantitative fluorescence ratio-imaging of Fura-2-loaded cells. We have observed transient, agonist-induced elevations in intracellular Ca^{2+} levels which can be completely blocked by atropine, whereas AFDX-116, a muscarinic antagonist which binds preferentially to the vertebrate mAChR M_2 subtype, has little effect at 100 $\mu mol\,l^{-1}$. The suitability of this stable *Drosophila* expression system for the characterization of neurotransmitter receptors is discussed.

Key words: muscarinic acetylcholine receptor, stable expression, calcium imaging, *Drosophila melanogaster*.

Introduction

Acetylcholine is the predominant neurotransmitter in the nervous system of insects (Pitman, 1971; Sattelle, 1980) where it binds to several pharmacologically distinct types of acetylcholine receptors (AChRs). In addition to the classical muscarinic and nicotinic AChRs, there is evidence for receptors with mixed (muscarinic and nicotinic) properties in insects (Breer and Sattelle, 1987). Although AChRs are expressed abundantly in both vertebrate and invertebrate species (Sattelle, 1980; Caulfield, 1993; Hannan and Hall, 1993; Trimmer, 1995), AChRs in insects appear to be expressed exclusively in the nervous system (Sattelle, 1980; Breer and Sattelle, 1987). As has been well documented in vertebrates, there is evidence for pharmacological heterogeneity of both muscarinic and nicotinic AChRs in insects (Sattelle, 1980; Hannan and Hall, 1993). In order to obtain a more abundant, homogeneous and more readily available source of a functional muscarinic AChR (mAChR) than can be obtained from native insect neuronal preparations, we have stably expressed a cloned *Drosophila* mAChR cDNA in a cultured *Drosophila* cell line.

Muscarinic AChRs are members of the extensive superfamily of cell surface receptors which contain seven putative transmembrane domains and which activate several distinct signal transduction pathways by interacting with guanine nucleotide binding proteins (G proteins) (Caulfield, 1993). Five vertebrate mAChRs have been cloned, and a similar number of mAChR subtypes have been identified pharmacologically (Nathanson, 1987; Hulme *et al*. 1990; Caulfield, 1993). Binding of agonists to the vertebrate M_1, M_3 and M_5 mAChR subtypes stimulates phospholipase C and the synthesis of inositol phosphates, whereas agonist binding to the M_2 and M_4 subtypes inhibits adenylate cyclase.

There is also pharmacological evidence for the existence of distinct mAChR subtypes in insects (Breer and Sattelle, 1987; Hannan and Hall, 1993). However, only one insect mAChR subtype has been cloned to date (Onai *et al.* 1989; Shapiro *et al.* 1989). This mAChR (Dm1), cloned from the fruit fly *Drosophila melanogaster*, has a high degree of sequence homology with mammalian mAChRs and has structural features typical of other G-protein-coupled receptors. The

cloned Dm1 cDNA has been expressed in *Xenopus* oocytes and in cultured mammalian cells (Shapiro *et al*. 1989; Blake *et al.* 1993). Expression of Dm1 in these cells revealed pharmacological properties of the receptor which broadly resemble those of the vertebrate M_1 and M_3 mAChR subtypes. Expression studies have also demonstrated the ability of this receptor to stimulate the accumulation of intracellular inositol phosphates in response to the application of muscarinic agonists.

The principal aim of the present study was to investigate the suitability of a *Drosophila* expression system (which employs an inducible *Drosophila* metallothionein promoter) for the functional and pharmacological characterization of a cloned G-protein-coupled *Drosophila* neurotransmitter receptor. We have recently reported the stable functional expression and electrophysiological characterization of a *Drosophila* γ-aminobutyric acid ionotropic receptor in this expression system (Millar *et al*. 1994). Here we report the successful establishment of a stable cell line expressing the cloned *Drosophila* mAChR (Dm1) in *Drosophila* S2 cells. Expression of Dm1 has been demonstrated by specific radioligand binding and by western blotting with a polyclonal antiserum raised against a synthetic peptide corresponding to the C terminus of the protein. Functional expression has been demonstrated by agonist-induced changes in intracellular Ca^{2+} levels, measured by quantitative fluorescence ratio-imaging.

Materials and methods

Cells and plasmids

The *Drosophila* mAChR cDNA was kindly provided by Dr Neil Nathanson, University of Washington, Seattle, Washington, USA. The *Drosophila* expression vector pRmHa3 was provided by Dr Thomas Bunch, University of Arizona, USA. Plasmid pRmHa3 is a modification of pRmHa1 (Bunch *et al.* 1988) which contains additional unique restriction enzyme sites (*Eco*RI/*Sac*I/*Kpn*I/*Sma*I/*Bam*HI/*Sal*I) within its multiple cloning site. The selection plasmid pCOHygro (van der Straten *et al*. 1989), which contains the bacterial hygromycin B phosphotransferase gene under the control of the constitutive *Drosophila* COPIA 5′ LTR promoter, was provided by Dr Martin Rosenberg, SmithKline Beecham Pharmaceuticals, Philadelphia, USA. Schneider's line 2 (S2/M3) *Drosophila* cell line (Schneider, 1972), which has been adapted for growth in Shields and Sang M3 growth medium, was obtained from Dr Thomas Bunch, University of Arizona, USA. *Drosophila* S2/M3 cells were maintained at 25 °C in M3 medium (Sigma) containing heat-inactivated foetal calf serum (12.5 %), yeast extract ($2.0\,g\,l^{-1}$), penicillin ($100\,i.u.\,ml^{-1}$) and streptomycin ($100\,\mu g\,ml^{-1}$).

The Dm1 cDNA was excised from its original cloning vector, pBluescript-Dm1 (Shapiro *et al*. 1989), by digestion with *Eco*RI and subcloned into the *Eco*RI site of the *Drosophila* expression vector pRmHa3 to give plasmid pRmHa3–Dm1. The orientation of the Dm1 cDNA insert, with respect to the 5′ *Drosophila* metallothionein promoter and the 3′ alcohol dehydrogenase polyadenylation site, was confirmed by restriction mapping and nucleotide sequencing.

Generation of stable cell lines

Exponentially growing S2 cells (approximately $7\times10^6\,cells\,ml^{-1}$) were diluted 1:10 into a $25\,cm^2$ tissue culture flask and incubated for 12–24 h. Cells were transfected with plasmids pRmHa3–Dm1 ($20\,\mu g$) and pCoHygro ($10\,\mu g$) by a modified calcium phosphate–DNA co-precipitation method (Chen and Okayama, 1987). After an overnight incubation, the medium was changed to remove excess calcium phosphate–DNA precipitate and the cells were incubated for a further 3 days in M3 medium without drug selection (i.e. hygromycin B). In order to isolate clonal cell lines, transfected cells were grown in agar plates containing conditioned M3 medium and hygromycin B. Several dilutions (between 1:8 and 1:800) of transfected cells were prepared in M3 medium (a 1:1 mixture of fresh medium and S2-cell-conditioned M3 medium) containing hygromycin B ($300\,\mu g\,ml^{-1}$). Cells in M3 medium (8 ml) were added to 10 cm tissue culture plates containing 2 ml of molten (45–50 °C) 1.5 % Noble agar (Difco) and the plates were incubated at 25 °C in a humidified chamber. Colonies of transfected cells (approximately 1 mm diameter) were picked after about 6 weeks with a sterile Pasteur pipette and transferred to a 96-well plate containing $100\,\mu l$ of M3 medium with $300\,\mu g\,ml^{-1}$ hygromycin B. When confluent, the colonies were expanded into 6-well plates and then into $25\,cm^2$ flasks.

Radioligand binding

Expression of the *Drosophila* mAChR in clonal S2–Dm1 cell lines was assayed by [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) binding. Expression of the Dm1 cDNA was induced by the addition of $CuSO_4$ to the growth medium of semi-confluent S2–Dm1 cells to a final concentration of $0.6\,mmol\,l^{-1}$ ($CuSO_4$ was prepared as a $30\,mmol\,l^{-1}$ stock). After incubation for 24 h in $CuSO_4$-containing medium, cells were harvested and the cell pellet was washed twice in $10\,mmol\,l^{-1}$ potassium phosphate buffer (pH 7.2) containing EDTA ($1\,mmol\,l^{-1}$) and the protease inhibitor phenylmethylsulphonyl fluoride (PMSF) ($0.5\,mmol\,l^{-1}$). Cell pellets were then resuspended and assayed in $10\,mmol\,l^{-1}$ phosphate buffer containing $5\,mmol\,l^{-1}$ $MgCl_2$ and [^{3}H]NMS. Non-specific binding was determined by the addition of $10\,\mu mol\,l^{-1}$ atropine. Cells were incubated for 90 min at room temperature in a total volume of 0.5 ml, and radioligand binding was assayed by filtration onto presoaked Whatman GF/B filters followed by rapid washing with cold $10\,mmol\,l^{-1}$ phosphate buffer using a Brandel cell harvester. Radioactivity was measured by scintillation counting.

Equilibrium constants for NMS, atropine and 11-({2-[(diethylamino)methyl]-1-piperidinyl}acetyl)-5,11-dihydro-6*H*-pyrido(2,3-*b*)(1,4)] benzodiazepine-6-one (AFDX-116) were calculated by weighted least-squares fitting. Data from [^{3}H]NMS saturation binding experiments were fitted using the Hill equation. The calculated Hill coefficient (n_H) for [^{3}H]NMS binding did not differ significantly from 1, so data

were refitted using the Hill–Langmuir equation (with n_H=1). Data from antagonist competition experiments were normalized to the binding of [^{3}H]NMS observed in the absence of antagonist (=100 % binding). The normalized data were fitted by least-squares methods using the equation:

$$y = y(0)/[1 + (x/IC_{50})]^{n_H}, \quad (1)$$

where y is normalized [^{3}H]NMS binding (%), x is the concentration of antagonist and n_H is the Hill coefficient. With the Hill coefficient constrained to be 1, equilibrium constants (K_i) for atropine and AFDX-116 were estimated, using the normalized concentration of [^{3}H]NMS, from the relationship:

$$K_i = IC_{50}/(1 + [NMS]/K_d). \quad (2)$$

Protein concentrations of membrane preparations used for radioligand binding were determined, after solubilization in 1 % sodium dodecyl sulphate (SDS), by a detergent-compatible protein assay kit (BioRad).

Western blotting

Proteins were extracted from both uninduced and $CuSO_4$-induced S2–Dm1 cells in phosphate-buffered saline (composition: phosphate buffer 10 mmol l^{-1}, NaCl 137 mmol l^{-1}, KCl 2.7 mmol l^{-1}, pH 7.4) containing EDTA 5 mmol l^{-1}, EGTA 3 mmol l^{-1}, PMSF 0.1 mmol l^{-1}, sodium azide 0.2 % and Chaps 1 %. After incubation on ice for 30 min, debris was removed by centrifugation (13 000 ***g*** for 10 min at 4 °C). The pellet was resuspended in SDS loading buffer containing 5 % 2-mercaptoethanol and loaded onto a 7.5 % SDS polyacrylamide gel. Samples were not boiled before loading in an attempt to avoid mAChR aggregation (Haga *et al*. 1990). After electrophoresis, proteins were transferred to nitrocellulose and incubated with a 1:500 dilution of polyclonal antiserum (Ab12A3) raised against a synthetic peptide corresponding to the C-terminal region of the Dm1 protein (Blake *et al.* 1993) by standard methods (Harlow and Lane, 1988). Antibody bound to the nitrocellulose was detected by incubation with an alkaline-phosphatase-conjugated goat anti-rabbit antibody (Sigma) and then incubation in a bromochloroindolyl phosphate/nitro blue tetrazolium substrate solution (Harlow and Lane, 1988).

Calcium imaging

After induction of S2–Dm1 cells in $CuSO_4$-containing medium for 24 h, as described above, cells were dislodged from the tissue culture flask by gentle pipetting, and a drop of cells was placed on a 22 mm diameter glass coverslip. Cells were allowed to settle onto the coverslip and to adhere for at least 1 h in M3 medium (without $CuSO_4$). Cells were washed gently in *Drosophila* saline (composition: NaCl 120 mmol l^{-1}, KCl 5 mmol l^{-1}, $MgCl_2$ 8 mmol l^{-1}, $CaCl_2$ 2 mmol l^{-1}, sucrose 32.2 mmol l^{-1} and Tes 10 mmol l^{-1}, pH 7.2) and then loaded with the calcium-sensitive dye Fura-2 AM (4 μmol l^{-1}) in *Drosophila* saline for 30 min at room temperature in the dark. After loading, cells were washed three times in *Drosophila* saline and stored at room temperature for no more than 30 min before Ca^{2+} imaging.

Fura-2-loaded cells were visualised with a Nikon quartz objective lens (CF-Fluor ×40 NA 1.3, oil immersion) mounted on a Nikon Diaphot microscope. The MagiCal system with TARDIS software (Applied Imaging, Gateshead, UK) was used for dynamic Ca^{2+} imaging and image processing. Excitation wavelengths (340 nm and 380 nm) were selected by means of a computer-controlled rotating filter wheel located between a xenon lamp and the microscope. Emitted light at 510 nm (40 nm half-bandwidth) was passed to an image-intensifying charge-coupled device (CCD) camera (Photonic Science, UK). The resulting images at each wavelength were averaged in real time, typically over eight frames, and digitized at eight bits accuracy to yield 256 grey levels, captured as a 256×256 pixel image, and stored in the 32 Mbyte dynamic random access memory of an image-processing unit. The time resolution in these experiments was 2.5 s between ratio frames. Background images (at 340 nm and 380 nm), taken from an area of the coverslip containing no cells, were captured at the start of each experiment to allow for correction by pixel-by-pixel subtraction of background fluorescence.

The ratio of emitted fluorescence at the two excitation wavelengths (340 nm and 380 nm) was calculated for each frame, following background subtraction, and computed against a table to yield a ratio grey level representative of a calibrated intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. The fluorescence ratios were converted to $[Ca^{2+}]_i$ using the following equation (Grynkiewicz *et al.* 1985):

$$[Ca^{2+}]_i = K_d \times \beta \times [(R - R_{min})/(R_{max} - R)], \quad (3)$$

where K_d is the dissociation constant for Fura-2/Ca^{2+} (225 nmol l^{-1}), R is the ratio at any pixel point, R_{min} and R_{max} are the ratio values of Fura-2 at zero and saturating $[Ca^{2+}]_i$, respectively, and β is the ratio of fluorescence at 380 nm for the dye in saturating and zero $[Ca^{2+}]_i$. The calibration constants R_{min}, R_{max} and β were empirically determined from Fura-2-loaded cells with the experimental method previously described using ionomycin permeabilisation (1 μmol l^{-1}) of the cells in the presence of either 10 mmol l^{-1} EGTA or 10 mmol l^{-1} Ca^{2+} in *Drosophila* saline.

To produce a continuous trace of mean $[Ca^{2+}]_i$ against time, the area around a cell was graphically defined with a light pen and the mean $[Ca^{2+}]_i$ level within the area computed. This could be achieved for up to 20 cells at a time. The computed $[Ca^{2+}]_i$ was calculated on an individual pixel basis. The mean of all values in the pixel 'set' was then taken as mean $[Ca^{2+}]_i$. Individual data was exported to an ASCII file, which was incorporated into a Lotus 123 spreadsheet for further calculations.

Agonists and antagonist were applied to the cells on coverslips using bolus delivery into a 1 ml bath volume, and a rapid aspirator was used to remove liquid and to maintain a constant volume.

Results

Establishment of stable cell lines

The *Drosophila* mAChR (Dm1) cDNA was subcloned into

the *Drosophila* expression vector pRmHa3 (Bunch *et al.* 1988), between the vector's 5′ *Drosophila* metallothionein promoter and the 3′ *Drosophila* alcohol dehydrogenase polyadenylation signal, to create plasmid pRmHa3–Dm1. *Drosophila* S2 cells (Schneider, 1972) were co-transfected by the calcium phosphate precipitation method (Chen and Okayama, 1987) with both pRmHa3–Dm1 and the selectable marker plasmid pCOHygro (van der Straten *et al.* 1989). Co-transfection with pCOHygro, which expresses the bacterial hygromycin B phosphotransferase gene (*hph*), enabled selection of transfected cells on the basis of their resistance to the protein synthesis inhibitor hygromycin B. Cells resistant to hygromycin B were selected by plating transfected cells in soft agar containing conditioned M3 medium and hygromycin B (see Materials and methods for details). No cell growth was seen after treatment of untransfected S2 cells with hygromycin B. After about 6 weeks, colonies of hygromycin-B-resistant cells were picked with a sterile Pasteur pipette and grown into clonal cell lines.

Expression of Dm1 in S2 cells

Expression of the Dm1 cDNA from the metallothionein promoter of pRmHa3–Dm1 was induced by the addition of $0.6\,\text{mmol}\,l^{-1}$ $CuSO_4$ (for 24 h) to the cell culture medium. Twelve clonal cell lines (S2–Dm1-1 to S2–Dm1-12) were screened for mAChR expression by [^{3}H]NMS binding. Specific binding of [^{3}H]NMS was detected on membrane preparations prepared from all twelve clonal cell lines. Non-specific binding, determined by the inclusion of atropine ($10\,\mu\text{mol}\,l^{-1}$), was less than 5% of total binding. No significant specific binding of [^{3}H]NMS was detected on either untransfected S2 cells or on S2–Dm1 cell lines in the absence of induction with $CuSO_4$. One of the highest-expressing cell lines, S2–Dm1-1, was used for all further radioligand binding and Ca^{2+}-imaging experiments.

High-affinity, specific binding of [^{3}H]NMS to S2–Dm1-1 cell membrane preparations is illustrated in Fig. 1A. The data in Fig. 1A are from a single saturation binding experiment, but are typical of three similar experiments. Data points are the mean of duplicate samples after subtracting non-specific binding in the presence of $10\,\mu\text{mol}\,l^{-1}$ atropine (non-specific binding was also calculated as the mean of duplicate samples). Data from [^{3}H]NMS saturation binding experiments were fitted by least-squares methods using the Hill equation. The calculated Hill coefficient (n_H) for [^{3}H]NMS binding did not differ significantly from 1, so the data were refitted using the Hill–Langmuir equation (with n_H=1). The equilibrium dissociation constant (K_d) for the binding of [^{3}H]NMS to S2–Dm1 cell membrane preparations was K_d=0.7±0.3 $\text{nmol}\,l^{-1}$ (mean ± S.E.M. for three determinations). Maximum specific binding (B_{max}) of [^{3}H]NMS was 2.4±0.8 $\text{pmol}\,\text{mg}^{-1}$ membrane protein. The S2–Dm1-1 clonal cell line has shown similarly high levels of [^{3}H]NMS binding over a period of 5 months in continuous culture. Estimated values for the equilibrium dissociation constant and B_{max} of [^{3}H]NMS binding to S2–Dm1 cell membranes are similar to those determined by Blake *et al.* (1993) on transiently transfected mammalian COS-7 cells.

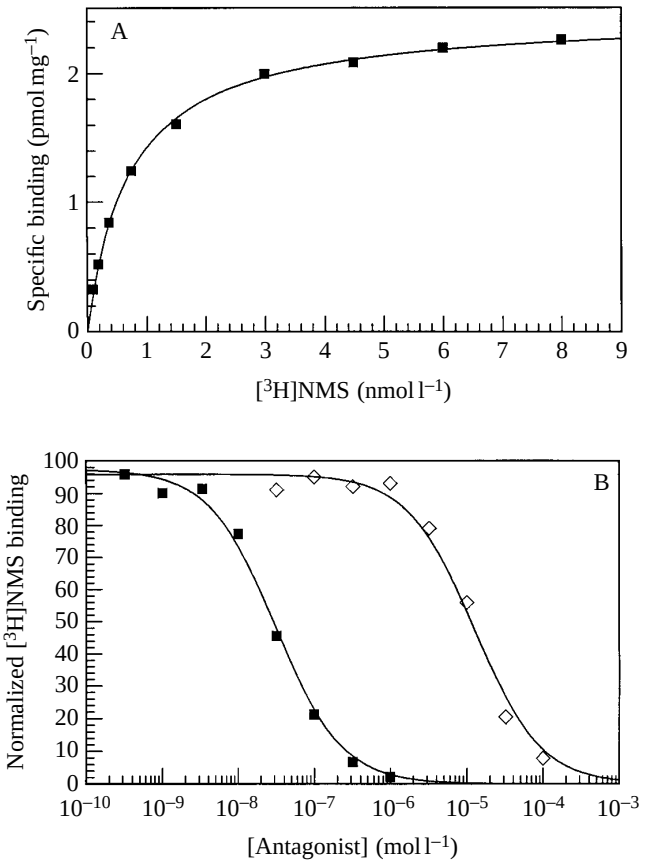

Fig. 1. Pharmacological characterization of a *Drosophila* mAChR (Dm1) stably expressed in *Drosophila* S2 cells. (A) Specific binding of [^{3}H]NMS to S2–Dm1 cell membranes. Non-specific binding, measured in the presence of $10\,\mu\text{mol}\,l^{-1}$ atropine, has been subtracted. Data points are the means of duplicate samples and the data are typical of three separate determinations which gave a mean K_d of 0.73±0.3 $\text{nmol}\,l^{-1}$ and B_{max} of 2.4±0.8 $\text{pmol}\,\text{mg}^{-1}$ membrane protein. (B) Competitive displacement of [^{3}H]NMS by atropine (filled squares) and AFDX-116 (open diamonds). A single set of data is shown for atropine and AFDX-116 in which the data points are the means of triplicate samples. At least three separate competitive displacement experiments were performed for each antagonist. The concentration of [^{3}H]NMS was approximately $500\,\text{pmol}\,l^{-1}$ and the mean calculated K_i values for atropine and AFDX-116 were 8.3±4.8 $\text{nmol}\,l^{-1}$ and 11.1±3.3 $\mu\text{mol}\,l^{-1}$, respectively. Both saturation binding and competitive displacement data were fitted by weighted least-squares methods.

The ability of two competitive muscarinic antagonists to inhibit the equilibrium binding of [^{3}H]NMS was investigated by competitive displacement binding assays (Fig. 1B). Atropine, a nonspecific antagonist of vertebrate mAChRs, inhibited binding of [^{3}H]NMS with relatively high affinity. In contrast, AFDX-116, which binds preferentially to the vertebrate M_2 mAChR subtype, showed significant inhibition of [^{3}H]NMS binding only at much higher concentrations (Fig. 1B). Data from antagonist competition experiments were

normalized to the binding of [^{3}H]NMS observed in the absence of antagonist. The normalized data were fitted by least-squares methods as described in the Materials and methods section. Hill coefficients for the competitive displacement of [^{3}H]NMS by atropine and AFDX-116 were close to 1. With the Hill coefficient constrained to be 1, equilibrium constants (K_i) for atropine and AFDX-116 were calculated using the normalized concentration of [^{3}H]NMS. Estimated equilibrium constants for atropine and AFDX-116 were 8.3±4.8 nmol l^{-1} and 11.1±3.3 μmol l^{-1}, respectively (both are means ± S.E.M. for three determinations). These estimates are similar to the values determined in studies with the cloned *Drosophila* mAChR transiently expressed in mammalian COS-7 cells (Blake *et al.* 1993) and with native mAChRs expressed in *Drosophila* (Haim *et al.* 1979).

Expression of the cloned mAChR has also been demonstrated by western blotting with a polyclonal antiserum raised against a synthetic peptide corresponding to the C terminus of the polypeptide. Cell protein was isolated from both $CuSO_4$-induced and uninduced S2–Dm1 cells by detergent extraction. After electrophoresis of the extracted proteins on a 7.5 % SDS–polyacrylamide gel and transfer to nitrocellulose, the expressed mAChR protein was detected by immunoblotting (Fig. 2). The anti-Dm1 antiserum detected two bands with apparent molecular masses of approximately 90 kDa and >200 kDa in induced, but not in uninduced, S2–Dm1 cells. As discussed later, we believe that the 90 kDa band corresponds to the glycosylated monomeric form of the Dm1 polypeptide. The band with a higher molecular mass is presumably an aggregated form of the mAChR, as has been observed with other mAChRs (Haga *et al.* 1990). Bands with lower molecular masses were observed in both induced and uninduced cells, which we presume to be due to non-specific binding of the antiserum.

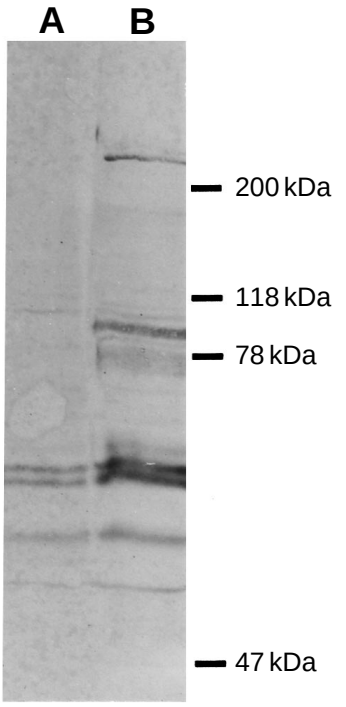

Fig. 2. Western blot showing expression of Dm1 protein in $CuSO_4$-induced, but not in uninduced, S2–Dm1 cells. Proteins extracted from uninduced (lane A) and $CuSO_4$-induced (lane B) S2–Dm1 cells were separated by SDS–PAGE and then transferred to nitrocellulose. The Dm1 polypeptide was detected by means of a polyclonal antibody raised against a synthetic peptide corresponding to the C terminus of Dm1 together with an alkaline-phosphatase-conjugated second antibody. Bands of approximately 90 kDa and >200 kDa are present only in induced cells and presumably correspond to the glycosylated form of the Dm1 monomer and to an aggregated form of Dm1. In addition, bands with a lower molecular mass are visible in both induced and uninduced cells, which we assume to be due to non-specific binding of the polyclonal antibody.

Functional characterization by calcium imaging

Muscarinic-agonist-induced changes in intracellular Ca^{2+} concentration $[Ca^{2+}]_i$, mediated by the second messenger inositol trisphosphate, have been demonstrated in cells transfected with vertebrate mAChRs (Lambert *et al.* 1991). Since agonist-induced accumulation of inositol phosphates has been demonstrated previously in mammalian cells transfected with this cloned *Drosophila* mAChR (Shapiro *et al.* 1989; Blake *et al.* 1993), we have investigated whether agonist-induced changes in $[Ca^{2+}]_i$ could be detected in S2–Dm1 cells.

S2–Dm1 cells were loaded with the Ca^{2+}-sensitive dye Fura-2 AM (4 μmol l^{-1}) for 30 min, and $[Ca^{2+}]_i$ was measured by quantitative fluorescence ratio-imaging, as described in Materials and methods. Cells which appeared to have non-uniform loading of Fura-2 dye were often observed, particularly when they had been loaded for longer than 30 min. Groups of cells showing uniform loading of Fura-2 (typically 20–30 cells) were selected for intracellular Ca^{2+} imaging. Bath application of the muscarinic agonist carbamylcholine (final concentration 100 μmol l^{-1}) resulted in the transient elevation of $[Ca^{2+}]_i$ (Figs 3, 4). Intracellular $[Ca^{2+}]_i$ responses of similar amplitude were observed upon repeated applications of carbamylcholine. Transient elevations of $[Ca^{2+}]_i$ of varying magnitude were observed in most cells which had been adequately loaded with Fura-2.

Fig. 4A shows the ability of the muscarinic antagonist atropine (100 μmol l^{-1}) to completely block the response of S2–Dm1 cells to carbamylcholine. In contrast, the more selective antagonist AFDX-116, which shows lower-affinity binding to S2–Dm1 cell membranes (Fig. 1B), had little or no effect on the response to carbamylcholine when it was co-applied at 100 μmol l^{-1} (Fig. 4B). No increase in $[Ca^{2+}]_i$ was observed on untransfected S2 cells, indicating the absence of endogenous AChRs (Fig. 4C).

Discussion

The expression of a cloned *Drosophila* mAChR in a stable *Drosophila* cell line has been demonstrated by specific, high-affinity binding of the muscarinic antagonist [^{3}H]NMS. A protein with the expected molecular mass of Dm1 was detected

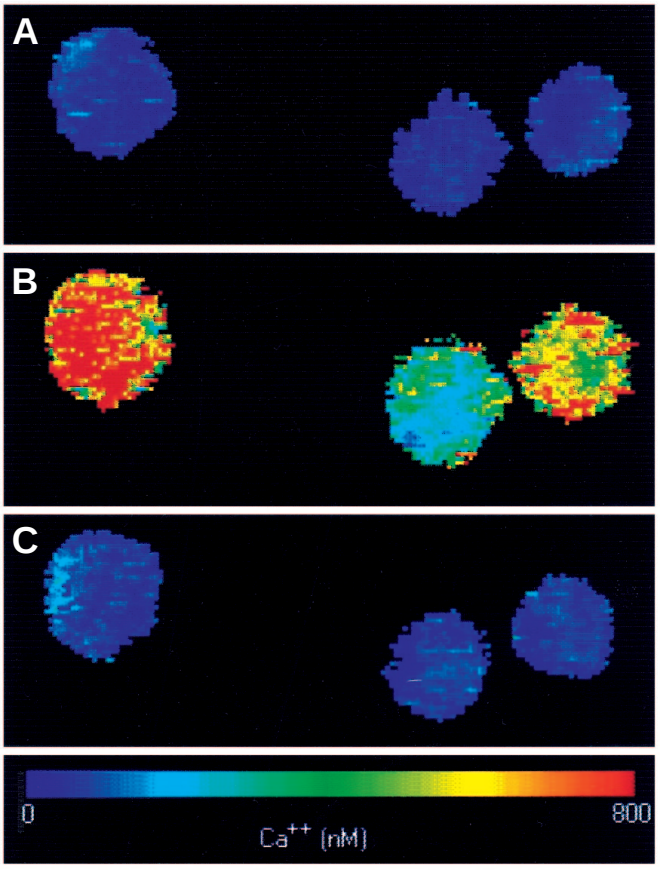

Fig. 3. Transient carbamylcholine-induced elevation of intracellular Ca^{2+} levels in Fura-2-loaded S2–Dm1 cells. Cells were excited alternately at 340 nm and 380 nm and emitted fluorescence was detected at 510 nm. The ratio of fluorescence emitted in response to the two excitation wavelengths, calculated on a pixel-by-pixel basis, is displayed as a pseudocolour scale which is calibrated to intracellular Ca^{2+} concentrations (see scale bar). (A) A group of three Fura-2-loaded S2–Dm1 cells prior to the application of carbamylcholine. (B) The same cells after the application of 100 μmol l^{-1} carbamylcholine. (C) Recovery of cells to initial Ca^{2+} levels after carbamylcholine was washed off. The response to application of carbamylcholine was repeatable (not shown in this figure, but illustrated in Fig. 4). Typically, data were recorded simultaneously from about 30 cells. The three cells illustrated in this figure are from approximately one-tenth of the field recorded during this experiment.

in S2–Dm1 cells by western blotting with an anti-Dm1 polyclonal antiserum. Little or no expression of Dm1 was detected in S2–Dm1 cells, either by radioligand binding or by immunoblotting, in the absence of the induction of Dm1 expression with $CuSO_4$. Expression of Dm1 from the metallothionein promoter of plasmid pRmHa3 is, therefore, tightly regulated by the presence of the inducer $CuSO_4$. Similar results, showing little or no expression from this promoter in S2 cells in the absence of $CuSO_4$, have been reported previously by us (Millar *et al.* 1994) and by others (Bunch *et al.* 1988).

The anatomical localization of mAChRs in *Drosophila* brain has been examined previously by immunocytochemical labelling of brain sections with an antiserum raised against a synthetic peptide corresponding to the C terminus of the *Drosophila* mAChR amino acid sequence (Blake *et al.* 1993). These studies revealed strong, specific staining in the antennal lobe region, suggesting that mAChRs may play a role in olfactory sensory processing in *Drosophila*. We have used this antiserum to detect the expression of the Dm1 protein in our S2–Dm1 cell line (Fig. 2).

The apparent molecular mass of Dm1 expressed in S2–Dm1 cells, determined by SDS–PAGE analysis (90 kDa), is in good agreement with the previously reported molecular mass of an mAChR purified from *Drosophila* brain, also determined by SDS–PAGE analysis (Venter *et al.* 1984). Both of these molecular mass estimates are larger than the molecular mass of Dm1 (78 kDa), calculated from the deduced amino acid sequence of the cloned gene (Shapiro *et al.* 1989). The larger apparent molecular mass of the expressed mAChR is presumably due to post-translational glycosylation of the polypeptide. A similar discrepancy between apparent and calculated molecular masses has been described for vertebrate mAChRs expressed in mammalian cell lines (Liang *et al.* 1987). The similarity between the apparent molecular mass of the cloned mAChR expressed in S2 cells with that of the native mAChR expressed in *Drosophila* (Venter *et al.* 1984) suggests that this protein is glycosylated to a similar extent in the *Drosophila* nervous system and when expressed heterologously in *Drosophila* S2 cells.

The band with a higher molecular mass in Fig. 2 (>200 kDa), which is visible only in induced cells, is presumably an aggregated form of the mAChR, as has been observed with vertebrate mAChRs (Haga et al. 1990). The bands with lower molecular masses detected by western blotting (which we interpret as being due to non-specific antibody binding) appear to be more abundant in the induced cells. We do not believe that this can be explained by differences in the amount of the two samples loaded. It is possible, however, that the induction of mAChR expression (from the metallothionein promoter, by $CuSO_4$) affects the level of expression of some endogenous proteins in the S2 cell. Indeed, we have observed morphological changes in S2–Dm1 cells, after 24 h of exposure to $CuSO_4$, which may be correlated with altered expression of endogenous proteins.

To date, most heterologous expression of cloned insect neurotransmitter receptors has been carried out either in *Xenopus* oocytes or in cultured mammalian cells. We were motivated to investigate the expression of cloned *Drosophila* receptors in a *Drosophila* cell line (Millar *et al.* 1994, and the work reported here) by the expectation that post-translational processing of *Drosophila* proteins might mimic more closely the post-translational processing that occurs in the native cell. In the case of *Drosophila* G-protein-coupled receptors, the intracellular machinery necessary for signal transduction events in the native cell may also be mimicked more closely by a *Drosophila* cell than by a non-*Drosophila* cell expression system.

We have been able to demonstrate functional expression of

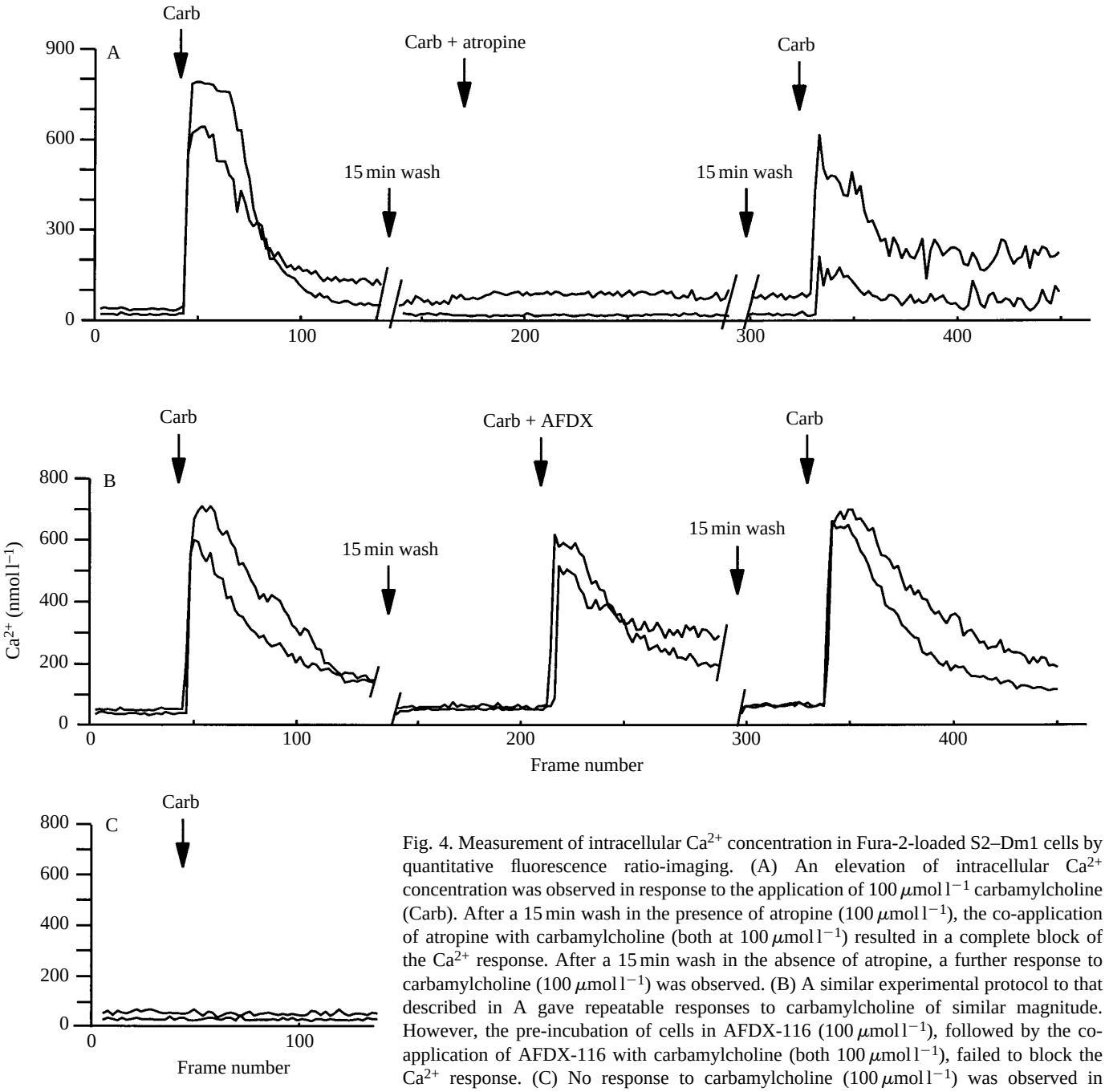

Fig. 4. Measurement of intracellular Ca^{2+} concentration in Fura-2-loaded S2–Dm1 cells by quantitative fluorescence ratio-imaging. (A) An elevation of intracellular Ca^{2+} concentration was observed in response to the application of 100 μmol l^{-1} carbamylcholine (Carb). After a 15 min wash in the presence of atropine (100 μmol l^{-1}), the co-application of atropine with carbamylcholine (both at 100 μmol l^{-1}) resulted in a complete block of the Ca^{2+} response. After a 15 min wash in the absence of atropine, a further response to carbamylcholine (100 μmol l^{-1}) was observed. (B) A similar experimental protocol to that described in A gave repeatable responses to carbamylcholine of similar magnitude. However, the pre-incubation of cells in AFDX-116 (100 μmol l^{-1}), followed by the co-application of AFDX-116 with carbamylcholine (both 100 μmol l^{-1}), failed to block the Ca^{2+} response. (C) No response to carbamylcholine (100 μmol l^{-1}) was observed in untransfected S2 cells. In A, B and C, cells were loaded with 4 μmol l^{-1} Fura-2 for 30 min at room temperature. For each experiment, simultaneous recordings from two different cells are shown. There is 2.5 s between frames.

this receptor by Ca^{2+} imaging of Fura-2-loaded S2–Dm1 cells. Agonist-induced modulation of $[Ca^{2+}]_i$ in S2–Dm1 cells is consistent with earlier reports that, when expressed in cultured mammalian cells, activation of this *Drosophila* receptor causes the accumulation of inositol phosphates (Shapiro *et al.* 1989; Blake *et al.* 1993). The modulation of $[Ca^{2+}]_i$ in S2–Dm1 cells is, therefore, presumably mediated by stimulation of phospholipase C and the subsequent mobilisation of inositol trisphosphate.

Although the establishment of a stable clonal cell line can be a relatively slow process (typically requiring 6–8 weeks after transfection), it presents several advantages over more commonly used transient expression systems such as the *Xenopus* oocyte (Dascal, 1987) or the baculovirus-based insect expression system (Miller, 1988). Once a stable cell line is established, it can provide an abundant and readily available supply of transfected cells. This can be particularly beneficial for the application of techniques such as radioligand binding assays. Also, in contrast to transient transfection methods, the ability to generate a clonal population of cells by stable

transfection can be of particular benefit for the functional characterization of expressed receptors by single-cell techniques such as Ca^{2+} imaging and electrophysiology.

It is anticipated that this *Drosophila* expression system will enable the study of this and other insect receptors in what is likely to be a more native cellular environment than that provided by the hitherto more widely used mammalian stable cell expression systems. In common with other gene expression systems, the ability to express a single homogeneous population of receptors from a single cloned gene provides an opportunity to investigate the molecular basis for the observed diversity in receptor subtype pharmacology. We recently described the stable functional expression and electrophysiological characterization of a *Drosophila* GABA ionotropic receptor in the *Drosophila* S2 cell line (Millar *et al.* 1994). This demonstrates the suitability of the *Drosophila* expression system for the stable functional expression of both a ligand-gated and a G-protein-coupled neurotransmitter receptor. Further characterization of these and other neurotransmitter receptors and ion channels in this expression system is in progress.

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