Routes of delivery of muscarinic acetylcholine receptors to the plasma membrane in NG108-15 cells

Jennifer A. Koenig & ¹J. Michael Edwardson

Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ

1 We have examined the routes of delivery of muscarinic acetylcholine receptors to the plasma membrane in unstimulated and agonist-stimulated NG108-15 cells. Delivery of receptors to the plasma membrane was measured by irreversibly alkylating receptors already at the cell surface with propylbenzylcholine mustard (PrBCM) and then following the recovery of binding of the polar radioligand [³H]-N-methylscopolamine ([³H]-NMS) in intact cells.

2 In unstimulated cells, recovery of [³H]-NMS binding after 2 h of incubation at 37°C was 30% of binding in control cells. Binding affinity of [³H]-NMS was unchanged. In cells that had been pre-exposed to carbachol (0.5 mM) for 30 min, initial [³H]-NMS binding was reduced by 38% but recovery of binding was increased from 30% to 43% of control binding.

3 When the cells were pre-incubated for 1 h with the protein synthesis inhibitor cycloheximide (20 µg ml⁻¹), recovery of [³H]-NMS binding was reduced by similar extents in unstimulated (30% to 9%) and carbachol-stimulated (43% to 19%) cells. Incubation of the cells at 20°C instead of 37°C reduced recovery of [³H]-NMS binding from 30% to 9% in unstimulated cells and from 43% to 23% in carbachol-stimulated cells.

4 Depletion of cellular ATP by addition of antimycin (50 nM) and deoxyglucose (50 mM), reduced recovery of binding to 12% in unstimulated cells and to 6% in carbachol-stimulated cells.

5 These results indicate that in unstimulated NG108-15 cells, delivery of muscarinic receptors to the plasma membrane is almost exclusively through the synthetic pathway. In agonist-stimulated cells, receptor sequestration into an intracellular compartment (probably endosomes) occurs. Under these circumstances, delivery of receptors to the plasma membrane along the synthetic route is unaffected but an additional route of delivery (recycling) now operates.

Keywords: Muscarinic ACh receptor; NG108-15 cells; receptor recycling; sequestration

Introduction

It is well established that exposure of cells to muscarinic cholinergic agonists leads to a selective removal of muscarinic acetylcholine receptors from the plasma membrane (Harden *et al.*, 1985; Feigenbaum & El-Fakahany, 1985; Cioffi & El-Fakahany, 1986; Fisher, 1988; Baumgold *et al.*, 1989; Eva *et al.*, 1990; Thompson & Fisher, 1990; 1991; Thompson *et al.*, 1991). This process occurs in two phases: an initial sequestration, which is not associated with a reduction in the total number of receptors, followed by down-regulation, where receptors are degraded (Galper & Smith, 1980; Harden *et al.*, 1985; Ray *et al.*, 1989; Thompson & Fisher, 1990). Sequestration involves the movement of receptors to a light membrane fraction (Harden *et al.*, 1985; Baumgold *et al.*, 1989), which probably represents endosomes (Helenius *et al.*, 1983). From here, receptors can either be recycled to the plasma membrane on removal of the agonist, or, in the continued presence of agonist, delivered to another compartment (probably the lysosome), where they undergo proteolysis (Ray *et al.*, 1989).

Muscarinic receptors arriving at the plasma membrane, then, follow one of two routes – the ‘synthetic’ route, from the rough endoplasmic reticulum via the Golgi complex (Pfeffer & Rothman, 1987), and the ‘recycling’ route from the endosome (Helenius *et al.*, 1983). While the synthetic route is likely to be constitutive, the recycling route presumably requires a modification of the receptor in order to direct it into the endocytic pathway, and a reversal of this modification to signal its re-insertion into the plasma membrane. We are attempting to investigate the molecular char-

acteristics of these two routes, and to identify the signals that operate on the recycling route. In the present study, we have quantitated and characterized the two routes in both unstimulated and agonist-stimulated NG108-15 cells. In this cell line the muscarinic receptor is of the M₄ sub-type, and agonist stimulation leads to an inhibition of adenylate cyclase (Evans *et al.*, 1984; Peralta *et al.*, 1987). Delivery of muscarinic receptors to the plasma membrane was measured by irreversibly alkylating receptors already at the membrane with propylbenzylcholine mustard (PrBCM; Young *et al.*, 1972) and then following the recovery of the binding of [³H]-N-methylscopolamine, a polar, membrane-impermeant radioligand (Birdsall & Hulme, 1989). A preliminary account of this work has been presented to the British Pharmacological Society (Koenig & Edwardson, 1993).

Methods

Cell culture

NG108-15 (mouse neuroblastoma × rat glioma) cells were obtained from Prof. G. Henderson (Department of Pharmacology, University of Bristol) and grown in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum at 37°C in 5% CO₂, 95% humidified air. Cells were detached from the culture flasks for passaging by a brief (<1 min) incubation with trypsin (0.5 mg ml⁻¹) and EDTA (0.2 mg ml⁻¹) in phosphate-buffered saline (PBS). Cells were passaged every 3–5 days and were used at passages 14–41.

¹ Author for correspondence.

[³H]-N-methylscopolamine binding

Cells were seeded into poly-L-lysine-treated (25 mg/well) 24-well plates at approximately 10^5 cells/well in 2 ml culture medium and used when almost confluent (2–3 days). Muscarinic receptors at the plasma membrane of the cells were quantitated through the binding of the polar radioligand [³H]-N-methylscopolamine ([³H]-NMS). Cells were washed with 1 ml PBS containing 0.9 mM CaCl₂ and 0.5 mM MgCl₂ (PBS-Ca,Mg), and [³H]-NMS binding was determined by incubation of the cell monolayers with 0.25 ml/well of [³H]-NMS in the same buffer for 90 min at 4°C. Non-specific binding was defined by N-methylatropine (1 µM). Incubations were terminated by two washes with 1 ml ice-cold PBS-Ca, Mg. The cells were then solubilized in 0.2 ml 0.5% (v/v) Triton X-100 and transferred to vials for liquid scintillation counting. Saturation data were analysed using the programme LIGAND (Munson & Rodbard, 1980). It has been shown previously (Eva *et al.*, 1990) that [³H]-NMS binding to muscarinic receptors reaches equilibrium over 90 min at 4°C, and the linearity of Scatchard plots of the saturation data confirms this observation. At concentrations of [³H]-NMS around the K_D , specific binding represented 90–95% of total binding. Cell density (2×10^5 cells/well) was determined by removing cells from the well with 0.2 ml PBS containing EGTA (1 mM) and counting in a Neubauer haemocytometer.

Treatment of cells with propyl-benzilylcholine mustard

Muscarinic receptors at the plasma membrane of the cells were irreversibly alkylated with propyl-benzilylcholine mustard (PrBCM). The pharmacologically-active species of PrBCM is the aziridinium ion, which was prepared by the method of Young *et al.* (1972). PrBCM (100 µM) was allowed to cyclize in PBS, pH 7.4, at room temperature for 1 h. The reaction was stopped by dilution to 10^{-8} M. This procedure gives a yield of approximately 91% (Young *et al.*, 1972). Cells were washed with 1 ml HEPES-buffered DMEM (DMEM-HEPES) containing 0.2% (w/v) bovine serum albumin (BSA), incubated with PrBCM (10^{-8} M) in PBS-Ca,Mg at 20°C, and then washed with 1 ml PBS-Ca,Mg to remove free PrBCM.

Delivery of muscarinic receptors to the plasma membrane

Following alkylation of muscarinic receptors at the plasma membrane of the cells by PrBCM treatment, delivery of receptors to the plasma membrane was assayed through the recovery of [³H]-NMS binding and expressed as a percentage of binding in control cells (i.e. cells that had been incubated in PBS-Ca,Mg alone instead of buffer plus PrBCM). During recovery, cells were incubated in DMEM-HEPES containing 0.2% (w/v) BSA.

The aim of this study was to examine the effects of various manipulations on the delivery of muscarinic receptors to the plasma membrane. These manipulations included stimulation of the cells with the muscarinic agonist carbachol, pre-incubation of the cells with the protein synthesis inhibitor cycloheximide, reduction of the temperature during recovery from 37°C to 20°C and depletion of cellular ATP by treatment with antimycin plus deoxyglucose. Carbachol stimulation was at 0.5 mM in DMEM-HEPES containing 0.2% (w/v) BSA for 30 min. Carbachol was removed by two washes with 1 ml of the same buffer, and the cells were then treated immediately with PrBCM. Cycloheximide (20 µg ml⁻¹) in DMEM-HEPES containing 0.2% (w/v) BSA was added 1 h before PrBCM treatment (i.e. 30 min before carbachol stimulation, where appropriate) and was present in all buffers, including wash buffers, until determination of [³H]-NMS binding. Pretreatment of cells with both carbachol and cycloheximide was carried out at 37°C. Washing prior to PrBCM treatment and PrBCM treatment itself was at 20°C.

Recovery of [³H]-NMS binding after PrBCM treatment was followed at either 37°C or 20°C. Antimycin and deoxyglucose were added, where appropriate, during washing of the cells before PrBCM treatment, and were present in all buffers up until the [³H]-NMS binding assay.

Materials

[³H]-NMS (specific activity 85 Ci mmol⁻¹) was from Amersham International plc, Amersham, Bucks. Cell culture medium was from ICN Biomedicals Ltd., High Wycombe, Bucks., and foetal calf serum was from Gibco, Uxbridge. PrBCM was a generous gift of Dr J.M. Young of the Department of Pharmacology, University of Cambridge. All other reagents were from Sigma, Poole, Dorset.

Statistical evaluation of data

Within each experiment, all values were determined in quadruplicate. Standard errors within quadruplicates were typically 5% of the mean. Except for the saturation binding data, all values are expressed as means $\pm$ s.e. mean of values from *n* separate experiments. The statistical significance of differences between groups of data was assessed by Student's *t* test (unpaired). A *P* value of <0.05 was considered to be significant.

*Results**Binding of [³H]-NMS to muscarinic receptors in NG108-15 cells*

Muscarinic receptors at the plasma membrane of NG108-15 cells were quantitated through the binding of the polar radioligand [³H]-NMS (Birdsall & Hulme, 1989). Specific [³H]-NMS binding behaved as a single component of K_D 129 ± 13 pM and B_{max} 3.55 ± 0.22 fmol/well. Since cell density was 2×10^5 /well, this value for B_{max} represents approximately 10^4 sites/cell. In this study [³H]-NMS was routinely used at a concentration of 0.2–0.3 nM. At this concentration, specific binding was 90–95% of the total binding.

Alkylation of muscarinic receptors by propyl-benzilylcholine mustard

As has been shown previously in longitudinal strips of smooth muscle from the guinea-pig ileum (Young *et al.*, 1972), PrBCM, at a concentration of 10^{-8} M, rapidly and irreversibly alkylated muscarinic receptors at the plasma membrane (Figure 1). The half-time of loss of [³H]-NMS binding was approximately 2 min. A 20 min incubation time was used routinely; at this time, no significant specific binding of [³H]-NMS (at a concentration of 0.2 nM) was detectable.

Recovery of [³H]-NMS binding in PrBCM-treated cells

When the PrBCM was removed and the cells were incubated at 37°C, [³H]-NMS binding recovered (Figure 2), indicating that muscarinic receptors were being delivered to the plasma membrane. Over a 2 h period, the number of muscarinic receptors at the plasma membrane increased significantly from $2 \pm 1\%$ to $32 \pm 2\%$ ($n = 16$; $P < 0.001$) of the number initially present in untreated cells; recovery was therefore $30 \pm 2\%$. In some experiments, intermediate timepoints were included, which show that the recovery of binding was approximately linear with time over the first 2 h. It is reasonable to expect that recovery would eventually proceed to completion; the 2 h time-point was chosen simply for convenience in designing the experiments.

Analysis of [3 H]-NMS binding (Figure 3) showed that the receptors arriving at the plasma membrane after PrBCM treatment had a binding affinity for NMS (K_D 116 ± 10 pM) that was not significantly different from that in control cells (K_D 129 ± 13 pM). In the experiment illustrated, B_{max} after 2 h of recovery was 1.27 ± 0.07 fmol/well, that is, 35% of the value in control cells (B_{max} 3.55 ± 0.22 fmol/well). Since NMS binding affinity does not change after recovery, it is valid to use [3 H]-NMS binding at a single concentration as an index of delivery of receptors to the plasma membrane.

Figure 1 Time-course of alkylation of muscarinic receptors by propyl-benzilylcholine mustard (PrBCM). Cells on 24-well plates were incubated with PrBCM (10^{-8} M) for various times, and washed before determination of [3 H]-N-methylscopolamine ([3 H]-NMS) binding. Cells were incubated with [3 H]-NMS (0.2 nM) for 90 min at 4°C , washed and then solubilized for liquid scintillation counting. Non-specific binding was defined by N-methylatropine ($1 \mu\text{M}$). Results are expressed as a percentage of [3 H]-NMS binding to cells that had not been treated with PrBCM. Values are means $\pm$ s.e.mean from three separate experiments, each performed in quadruplicate.

Figure 2 Time-course of recovery of [3 H]-N-methylscopolamine ([3 H]-NMS) binding after incubation of unstimulated cells with propyl-benzilylcholine mustard (PrBCM, 10^{-8} M, 20 min). Cells were incubated for various times at 37°C , before determination of [3 H]-NMS binding (at a concentration of 0.3 nM). Recovery is expressed as a percentage of binding in cells that had not been incubated with PrBCM, but had otherwise been treated identically. Values are means $\pm$ s.e.mean from several separate experiments (numbers in parentheses above each point); each experiment was performed in quadruplicate.

Modulation of recovery of [3 H]-NMS binding

When the cells were exposed for 30 min to a concentration of carbachol sufficient to saturate the muscarinic receptors (0.5 mM), [3 H]-NMS binding was reduced to $62 \pm 3\%$ ($n = 16$) of the control value. This result is in close agreement with those reported by others for NG108-15 cells (Baumgold *et al.*, 1989), N1E-115 cells (Feigenbaum & El-Fakahany, 1985; Cioffi & El-Fakahany, 1986), 1321N1 cells (Harden *et al.*, 1985), SK-N-SH cells (Baumgold *et al.*, 1989; Thompson & Fisher, 1990; 1991; Thompson *et al.*, 1991) and corticostriatal cells (Eva *et al.*, 1990).

In carbachol-stimulated cells, recovery of [3 H]-NMS binding in the 2 h period following PrBCM treatment was increased significantly from $30 \pm 2\%$ ($n = 16$) to $43 \pm 3\%$ ($n = 10$; $P < 0.001$, Figure 4). This increase in recovery is likely to be a consequence of recycling of receptors to the plasma membrane from an endosomal pool created by agonist-induced sequestration. In order to confirm this, we tested the effects on recovery of [3 H]-NMS binding in unstimulated and agonist-stimulated cells of two perturbations that should discriminate between delivery to the plasma membrane of newly-synthesized and recycled receptors. These perturbations were inhibition of protein synthesis and incubation at 20°C , which has been reported to block transport of proteins from the *trans*-Golgi network to the plasma membrane (Matlin & Simons, 1983) but not to affect recycling through endosomes (Helenius *et al.*, 1983).

Incubation of the cells with the protein synthesis inhibitor, cycloheximide ($20 \mu\text{g ml}^{-1}$) for 1 h before PrBCM treatment significantly reduced the subsequent recovery of [3 H]-NMS binding from $30 \pm 2\%$ to $9 \pm 2\%$ ($n = 5$; $P < 0.001$) in unstimulated cells and by a similar amount ($43 \pm 3\%$ to $19 \pm 1\%$, $n = 5$; $P < 0.001$) in carbachol-stimulated cells (Figure 4). When unstimulated cells were incubated at 20°C instead of at

Figure 3 [3 H]-N-methylscopolamine ([3 H]-NMS) binding characteristics before and after recovery from propyl-benzilylcholine mustard (PrBCM)-treatment. [3 H]-NMS binding was determined either in control cells (O) or in cells that had been treated with PrBCM (10^{-8} M for 20 min at 20°C), washed and incubated for 2 h at 37°C (●). Data were analysed using the programme LIGAND (Munson & Rodbard, 1980), and fitted to a single site with the binding parameters: K_D 129 ± 13 pM; B_{max} 3.55 ± 0.22 fmol/well (control cells), and K_D 116 ± 10 pM; B_{max} 1.27 ± 0.07 fmol/well (cells after recovery). Data are presented in the form of a Scatchard plot; the lines have been drawn using the parameters obtained from the LIGAND analysis. The result shown is typical of those from three separate experiments, each performed in quadruplicate. Values on the ordinate scale represent fmol bound [3 H]-NMS/fmol free [3 H]-NMS in 0.25 ml incubation volume.

Figure 4 The effect of inhibition of protein synthesis and of incubation at 20°C on recovery of [³H]-N-methylscopolamine ([³H]-NMS) binding following propyl-benzylcholine mustard (PrBCM) treatment of unstimulated or carbachol-stimulated cells. Cycloheximide (CHX, 20 µg ml⁻¹) was added 1 h before PrBCM treatment and was present thereafter. Carbachol pre-incubation was for 30 min before PrBCM treatment. [³H]-NMS binding (at a concentration of 0.3 nM) was determined after a 2 h incubation at either 37°C or 20°C. Recovery is expressed as a percentage of binding in control cells (i.e. cells that had not been treated with either cycloheximide or carbachol, and that had been incubated in PBS-Ca,Mg alone instead of buffer containing PrBCM). Values are corrected for the small amount of binding remaining after PrBCM treatment. Results shown are means ± s.e.mean from several separate experiments (numbers in parentheses); each experiment was performed in quadruplicate.

37°C, recovery of [³H]-NMS binding over 2 h was reduced from 30 ± 2% to 9 ± 2% ($n = 3$; $P < 0.001$). In carbachol-stimulated cells, recovery was reduced by a similar amount (43 ± 3% to 23 ± 2%, $n = 3$; $P < 0.01$). Recovery at 20°C in carbachol-stimulated cells was not significantly affected by pretreatment with cycloheximide (11 ± 4%; $n = 3$).

One potential drawback of the use of recovery of [³H]-NMS binding following PrBCM treatment to measure delivery of muscarinic receptors to the plasma membrane is the possibility that receptors are arriving at the cell surface (and being alkylated) during incubation of the cells with PrBCM (20 min at 20°C). One would expect the delivery of newly-synthesized receptors to the plasma membrane to occur at a constant rate (see Figure 2), so that any delivery during alkylation would not interfere with the measurement of delivery over the subsequent 2 h incubation period. Recycling, on the other hand, involves a limited intracellular pool of receptors and would be expected to be an exponential process. Consequently, any delivery during alkylation would result in an underestimation of the extent of recycling. In order to establish whether this was a significant problem, we measured [³H]-NMS binding immediately after treatment of the cells for 30 min at 37°C with carbachol (0.5 mM), and after a further 20 min incubation at 20°C in the absence of both carbachol and PrBCM. Cells were pretreated for 1 h with cycloheximide to block delivery of newly-synthesized receptors to the plasma membrane. The values obtained for [³H]-NMS binding before and after the 20 min period at 20°C were respectively 49 ± 2% ($n = 4$) and 47 ± 2% ($n = 4$) of binding in untreated cells. Hence, no delivery of receptors to the plasma membrane will occur during treatment of the cells with PrBCM.

Figure 5 shows that incubation of cells with a combination of antimycin (50 nM) and deoxyglucose (50 mM) immediately before PrBCM treatment, in order to inhibit both mitochondrial and glycolytic generation of ATP (Hertel *et al.*, 1986), substantially reduced recovery of [³H]-NMS binding in both

Figure 5 Effect of inhibition of protein synthesis and of ATP depletion on recovery of [³H]-N-methylscopolamine ([³H]-NMS) binding following propyl-benzylcholine mustard (PrBCM) treatment of unstimulated or carbachol-stimulated cells. Carbachol pre-incubation was for 30 min before PrBCM treatment. Antimycin (AM, 50 nM) and deoxyglucose (DG, 50 mM) were added immediately before PrBCM treatment and were present thereafter. [³H]-NMS binding (at a concentration of 0.3 nM) was determined either immediately after PrBCM treatments or after 2 h at 37°C. Recovery is expressed as a percentage of binding in control cells (see caption to Figure 4), corrected for the small amount of binding remaining after PrBCM treatment. Values are means ± s.e.mean from several separate experiments (numbers in parentheses); each experiment was performed in quadruplicate.

unstimulated and carbachol-stimulated cells. Recovery fell significantly from 30 ± 2% ($n = 16$) to 12 ± 1% ($n = 4$; $P < 0.001$) in unstimulated cells and from 43 ± 3% ($n = 10$) to 6 ± 1% ($n = 3$; $P < 0.001$) in carbachol-stimulated cells. The residual recoveries were not significantly different from that seen after cycloheximide treatment of unstimulated cells (9 ± 2%, $n = 3$). This result indicates that delivery to the plasma membrane of both newly synthesized and recycled receptors requires the presence of ATP in the cell cytosol.

Discussion

Our aim was to dissect the two routes by which muscarinic receptors are delivered to the plasma membrane in NG108-15 cells – the ‘synthetic’ route and the ‘recycling’ route – and to examine how these routes are affected by agonist stimulation of the cells. In order to achieve this aim, we used two perturbations, cycloheximide treatment and 20°C incubation, that we hoped would block synthetic delivery but not recycling. In unstimulated cells the cycloheximide-sensitive component of recovery of [³H]-NMS binding following PrBCM treatment was 21 ± 3%. The cycloheximide-resistant component was 9 ± 2%, indicating that most of the recovery in these cells is due to new synthesis of receptors. The residual recovery may represent delivery of receptors from a depleted synthetic pool, since it has been shown that the half-time of transport of a membrane protein along the synthetic pathway is typically of the order of 1 h (Daniels & Edwardson, 1988), so that a 1 h preincubation with cycloheximide will not completely deplete this pathway of receptors. In unstimulated cells, 20°C incubation caused a reduction in recovery (21 ± 3%) that was not significantly different from that caused by cycloheximide. It is likely, therefore, that the two perturbations are affecting the same population of receptors.

In carbachol-stimulated cells, the recovery at 37°C after PrBCM treatment was greater than that in unstimulated cells. This enhancement was entirely a consequence of an increased cycloheximide-insensitive component (from 9 ± 2% to 19 ± 1%; $P < 0.01$), which is likely to represent recycling

of receptors from endosomes. The cycloheximide-sensitive component of recovery, on the other hand ($24 \pm 3\%$), was not significantly different from that in unstimulated cells, indicating that short-term (30 min) agonist stimulation does not affect the synthetic pathway. It has been shown previously, in fact (Habecker & Nathanson, 1992), that long-term (up to 24 h) stimulation of cells with muscarinic agonists causes a progressive reduction in muscarinic receptor mRNA levels, most likely through a decrease in the rate of transcription. However, this effect is unlikely to feed through to a fall in the rate of receptor synthesis during the brief agonist exposure used here. As in unstimulated cells, reduction in temperature to 20°C caused the same fall in recovery as cycloheximide ($20 \pm 4\%$). Cycloheximide pretreatment did not cause a further significant reduction in recovery, supporting our argument that cycloheximide and low temperature are having similar effects on receptor traffic.

Is it possible, then, to use the data presented here to estimate the numbers of receptors moving along these various intracellular routes and quantitate the relative importance of the recycling and synthetic routes? The measurement of [^3H]-NMS binding after carbachol stimulation of the cells indicates that of 100 receptors initially at the plasma membrane, 38 are internalized during a 30 min treatment with agonist. The difference between the recoveries of [^3H]-NMS binding at 37°C in unstimulated and carbachol-stimulated cells (13%) suggests that, of these 38 receptors, 13 are recycled to the plasma membrane within 2 h. Since, it has been shown previously (Thompson & Fisher, 1990) that recycling occurs rapidly at 37°C ($t_{1/2}$ 7 min in SK-N-SH cells), the remaining 25 receptors must be degraded intracellularly. For every 100 receptors initially present at the plasma membrane, 30 are delivered to the plasma membrane over 2 h in unstimulated cells. Since the cycloheximide-resistant recovery of [^3H]-NMS binding at 20°C in unstimulated cells was 7%, an upper limit of 7 receptors can be placed on the contribution of recycling to this figure. At least 23 receptors must therefore be delivered along the synthetic pathway in 2 h. The true figure for recycling in unstimulated cells is likely to be lower than 7%, however, since a cycloheximide-resistant component of delivery along the synthetic route probably persists even at this reduced temperature.

It is unlikely that there is a significant amount of receptor recycling occurring during the PrBCM incubation at 20°C , since we have demonstrated that no recycling to the plasma membrane occurs during the first 20 min of incubation of the cells at 20°C after carbachol stimulation in the absence of PrBCM. This may indicate the presence of a lag period before delivery of receptors to the plasma membrane begins. This lag may represent the time required for receptors to be mobilised from an endosomal compartment and transported back to the cell surface, and may include a period for the removal of an 'internalization signal' from the receptors. The temperature-dependence of recovery of [^3H]-NMS binding

following carbachol stimulation has been investigated previously in SK-N-SH cells (Thompson & Fisher, 1991), although in this study surface receptors were not pre-alkylated, so that recycling would have been occurring in parallel with internalization. Recovery was seen at 25°C but not at 15°C , which suggests that there might be a threshold temperature for recycling at around 20°C . The apparent lag in recovery might also be seen only at this reduced temperature, since there is no evidence for such a phenomenon at 37°C (Harden *et al.*, 1985; Thompson & Fisher, 1990).

Many steps in intracellular protein transport, including transport between the Golgi complex and the plasma membrane (Gravotta *et al.*, 1990; Miller & Moore, 1991) and recycling through endosomes (Goda & Pfeffer, 1988), are known to require ATP. The fact that treatment with a combination of antimycin and deoxyglucose substantially inhibited recovery of [^3H]-NMS binding in both unstimulated and agonist-stimulated cells, therefore, confirms our suggestion that both cycloheximide-sensitive and cycloheximide-insensitive components of delivery of receptors to the plasma membrane represent *bona fide* ATP-dependent intracellular protein transport processes.

The molecular signalling involved in the movement of receptors into the recycling route following agonist stimulation is poorly understood. In the case of the better-characterized β -adrenoceptor, agonist stimulation leads to phosphorylation of the receptor by both protein kinase A (PKA) and β -adrenoceptor kinase (β ARK) (Lefkowitz *et al.*, 1990). Muscarinic receptors are thought to undergo similar processes (Kwatra *et al.*, 1989; Haga *et al.*, 1993; Tobin & Nahorski, 1993). Furthermore, a serine/threonine-rich domain of the third cytoplasmic loop of the m1 receptor has been shown to be required for sequestration (Lameh *et al.*, 1992; Moro *et al.*, 1993). It is tempting to speculate, therefore, that agonist-stimulated phosphorylation of this region of the receptor triggers sequestration. In support of this idea, there have been reports of a stimulation of sequestration/down-regulation by activation of protein kinase C (PKC; Liles *et al.*, 1986; Cioffi & Fisher, 1990). On the other hand, Hoover & Toews (1990) found that PKC activation inhibited sequestration/down-regulation in 1321N1 cells, and Thompson *et al.* (1991) have provided evidence that in SK-N-SH cells sequestration of the m3 sub-type of receptor can occur without the production of phosphoinositide-derived second messengers. The picture, therefore, is still confused, and it is reasonable to suppose that the molecular mechanisms of sequestration might be different in cell types expressing different sub-types of the receptor.

We are grateful to Dr J.M. Young (Department of Pharmacology, University of Cambridge) for providing us with supplies of propylbenzilylcholine mustard and advice about how to use it.

References

- BAUMGOLD, J., COOPERMAN, B.B. & WHITE, T.M. (1989). Relationship between desensitization and sequestration of muscarinic receptors in two neuronal cell lines. *Neuropharmacology*, **28**, 1253–1261.
- BIRDSALL, N.J.M. & HULME, E.C. (1989). The binding properties of muscarinic receptors. In *The Muscarinic Receptors*. ed. Brown, J.H. pp. 31–92. New Jersey: Humana Press.
- CIOFFI, C.L. & EL-FAKAHANY, E.S. (1986). Short-term desensitization of muscarinic cholinergic receptors in mouse neuroblastoma cells: Selective loss of agonist low-affinity and pirenzepine high-affinity binding sites. *J. Pharmacol. Exp. Ther.*, **238**, 916–923.
- CIOFFI, C.L. & FISHER, S.K. (1990). Reduction of muscarinic receptor density and of guanine nucleotide-stimulated phosphoinositide hydrolysis in human SH-SY5Y neuroblastoma cells following long-term treatment with 12-*O*-tetradecanoylphorbol 13-acetate or mezerein. *J. Neurochem.*, **54**, 1725–1734.
- DANIELS, P.U. & EDWARDSON, J.M. (1988). Intracellular processing and transport of influenza-virus envelope proteins in Madin-Darby canine kidney cells. Effects of the carboxylic ionophores monensin and nigericin. *Biochem. J.*, **252**, 693–700.
- EVA, C., GAMALERO, S.R., GENAZZANI, E. & COSTA, E. (1990). Molecular mechanisms of homologous desensitization and internalization of muscarinic receptors in primary cultures of neonatal corticostriatal neurons. *J. Pharmacol. Exp. Ther.*, **253**, 257–265.
- EVANS, T., SMITH, M.M., TANNER, L.I. & HARDEN, T.K. (1984). Muscarinic cholinergic receptors of two cell lines that regulate cyclic AMP metabolism by different molecular mechanisms. *Mol. Pharmacol.*, **26**, 395–404.
- FEIGENBAUM, P. & EL-FAKAHANY, E.S. (1985). Regulation of muscarinic receptor density in neuroblastoma cells by brief exposure to agonist: Possible involvement in desensitization of receptor function. *J. Pharmacol. Exp. Ther.*, **233**, 134–141.

- FISHER, S.K. (1988). Recognition of muscarinic cholinergic receptors in human SK-N-SH neuroblastoma cells by quaternary and tertiary ligands is dependent upon temperature, cell integrity, and the presence of agonists. *Mol. Pharmacol.*, **33**, 414–422.
- GALPER, J.B. & SMITH, T.W. (1980). Agonist and guanine nucleotide modulation of muscarinic cholinergic receptors in cultured heart cells. *J. Biol. Chem.*, **255**, 9571–9579.
- GODA, Y. & PFEFFER, S.Y. (1988). Selective recycling of the mannose 6-phosphate/IGF-II receptor to the *trans* Golgi network in vitro. *Cell*, **55**, 309–320.
- GRAVOTTA, D., ADESNIK, M. & SABATINI, D.D. (1990). Transport of influenza HA from the *trans*-Golgi network to the apical surface of MDCK cells permeabilized in their basolateral plasma membranes: energy dependence and involvement of GTP-binding proteins. *J. Cell Biol.*, **111**, 2893–2908.
- HABECKER, B.A. & NATHANSON, N.M. (1992). Regulation of muscarinic acetylcholine receptor mRNA expression by activation of homologous and heterologous receptors. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 5035–5038.
- HAGA, T., HAGA, K., KAMEYAMA, K. & NAKATA, H. (1993). Phosphorylation of muscarinic receptors: regulation by G proteins. *Life Sci.*, **52**, 421–428.
- HARDEN, K.T., PETCH, L.A., TRAYNELIS, S.F. & WALDO, G.L. (1985). Agonist-induced alterations in the membrane form of muscarinic cholinergic receptors. *J. Biol. Chem.*, **260**, 13060–13066.
- HELENIUS, A., MELLMAN, I., WALL, D. & HUBBARD, A. (1983). Endosomes. *Trends Biochem. Sci.*, **8**, 245–250.
- HERTEL, C., COULTER, S.J. & PERKINS, J.P. (1986). The involvement of cellular ATP in receptor-mediated internalization of epidermal growth factor and hormone-induced internalization of β -adrenergic receptors. *J. Biol. Chem.*, **261**, 5974–5980.
- HOOVER, R.K. & TOEWS, M.L. (1990). Activation of protein kinase C inhibits internalization and downregulation of muscarinic receptors in 1321NI human astrocytoma cells. *J. Pharmacol. Exp. Ther.*, **253**, 185–191.
- KOENIG, J.A. & EDWARDSON, J.M. (1993). Delivery of muscarinic ACh receptors to the plasma membrane in unstimulated and agonist-stimulated NG108-15 cells. *Br. J. Pharmacol.*, **110**, 70P.
- KWATRA, M.M., BENOVIĆ, J.L., CARON, M.G., LEFKOWITZ, R.J. & HOSEY, M.M. (1989). Phosphorylation of chick heart muscarinic cholinergic receptors by the β -adrenergic receptor kinase. *Biochemistry*, **28**, 4543–4547.
- LAMEH, J., PHILIP, M., SHARMA, Y.K., MORO, O., RAMACHANDRAN, J. & SADEE, W. (1992). Hml muscarinic cholinergic receptor internalization requires a domain in the third cytoplasmic loop. *J. Biol. Chem.*, **267**, 13406–13412.
- LEFKOWITZ, R.J., HAUSDORFF, W.P. & CARON, M.G. (1990). Role of phosphorylation in desensitization of the β -adrenoceptor. *Trends Pharmacol. Sci.*, **11**, 190–194.
- LILES, W.C., HUNTER, D.D., MEIER, K.E. & NATHANSON, N.M. (1986). Activation of protein kinase C induces rapid internalization and subsequent degradation of muscarinic acetylcholine receptors in neuroblastoma cells. *J. Biol. Chem.*, **261**, 5307–5313.
- MATLIN, K.S. & SIMONS, K. (1983). Reduced temperature prevents transfer of a membrane glycoprotein to the cell surface but does not prevent terminal glycosylation. *Cell*, **34**, 233–243.
- MILLER, S.G. & MOORE, H.-P.H. (1991). Reconstitution of constitutive secretion using semi-intact cells: regulation by GTP but not calcium. *J. Cell Biol.*, **112**, 39–54.
- MORO, O., LAMEH, J. & SADEE, W. (1993). Serine- and threonine-rich domain regulates internalization of muscarinic cholinergic receptors. *J. Biol. Chem.*, **268**, 6862–6865.
- MUNSON, P.J. & RODBARD, D. (1980). Ligand: a versatile computerized approach for characterization of ligand binding systems. *Anal. Biochem.*, **107**, 220–239.
- PERALTA, E.G., ASHKENAZI, A., WINSLOW, J.W., SMITH, D.H., RAMACHANDRAN, J. & CAPON, D.J. (1987). Distinct primary structure, ligand-binding properties and tissue-specific expression of four human muscarinic acetylcholine receptors. *EMBO J.*, **6**, 3923–3929.
- PFEFFER, S.R. & ROTHMAN, J.E. (1987). Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. *Annu. Rev. Biochem.*, **56**, 829–852.
- RAY, P., MIDDLETON, W. & BERMAN, J.D. (1989). Mechanism of agonist-induced down-regulation and subsequent recovery of muscarinic acetylcholine receptors in a clonal neuroblastoma $\times$ glioma hybrid cell line. *J. Neurochem.*, **52**, 402–409.
- THOMPSON, A.K. & FISHER, S.K. (1990). Relationship between agonist-induced muscarinic receptor loss and desensitization of stimulated phosphoinositide turnover in two neuroblastomas: methodological considerations. *J. Pharmacol. Exp. Ther.*, **252**, 744–752.
- THOMPSON, A.K. & FISHER, S.K. (1991). Preferential coupling of cell surface muscarinic receptors to phosphoinositide hydrolysis in human neuroblastoma cells. *J. Biol. Chem.*, **266**, 5004–5010.
- THOMPSON, A.K., MOSTAFAPOUR, S.P., DENLINGER, L.C., BLEASDALE, J.E. & FISHER, S.K. (1991). The aminosteroid U-73122 inhibits muscarinic receptor sequestration and phosphoinositide hydrolysis in SK-N-SH neuroblastoma cells. *J. Biol. Chem.*, **266**, 23856–23862.
- TOBIN, A.B. & NAHORSKI, S.R. (1993). Rapid agonist-mediated phosphorylation of m3-muscarinic receptors revealed by immunoprecipitation. *J. Biol. Chem.*, **268**, 9817–9823.
- YOUNG, J.M., HILEY, C.R. & BURGEN, A.S.V. (1972). Homologues of benzilylcholine mustard. *J. Pharm. Pharmacol.*, **24**, 950–954.

(Received September 16, 1993

Revised November 9, 1993

Accepted November 30, 1993)