Volume 245, number 1,2, 75–79 FEB 06888 March 1989

Carbachol-induced reverse transformation of Chinese hamster ovary cells transfected with and expressing the m5 muscarinic acetylcholine receptor

Christian C. Felder, Alice L. Ma and Bruce R. Conklin

National Institute of Mental Health, Section on Pharmacology, Laboratory of Cell Biology, Bethesda, MD 20892, USA

Received 22 December 1988; revised version received 13 January 1989

Reverse transformation was induced in Chinese hamster ovary (CHO) cells transfected with and stably expressing the m5 subtype of the muscarinic acetylcholine receptor when stimulated with the muscarinic agonist, carbachol. Atropine, a muscarinic antagonist, blocked the carbachol-stimulated reverse transformation. CHO cells not transfected with the muscarinic receptor did not change with added carbachol. PMA induced reverse transformation without increasing cAMP accumulation in CHO cells. Carbachol, prostaglandin E_2, and cholecystokinin increased cAMP accumulation but only carbachol caused reverse transformation. Carbachol-stimulated cAMP accumulation occurred at a higher concentration (EC_{50} 10 μM) than did carbachol-stimulated reverse transformation (EC_{50} 63 nM). Muscarinic m5 acetylcholine receptor transfected into CHO cells can induce reverse transformation which may be independent of cAMP.

Muscarinic acetylcholine receptor; Reverse transformation; Phorbol ester; (CHO cell)

1. INTRODUCTION

Chinese hamster ovary (CHO) cells have been shown to lose their malignant characteristics and revert back to more normal fibroblast-like morphology and growth properties when stimulated with N^6,$O^{2'}$-dibutyryl-cAMP [1] and cholera toxin [2]. This reverse transformation is accompanied by changes in cell surface features, cytoskeletal organization, growth characteristics, and DNA hypersensitivity to nuclease action [3,4]. Although cholera toxin and cAMP induce morphological changes in CHO cells, these changes have not been shown to be mediated through stimulation of a cell surface receptor.

We have used CHO cells transfected with and stably expressing the m5 subtype of the muscarinic acetylcholine receptor to determine whether morphological changes could be seen upon receptor activation. We report that stimulation of the muscarinic m5 acetylcholine receptor transfected into CHO cells can induce reverse transformation. In these cells, the m5 receptor is linked to increases in cAMP and release of inositol phosphates [5], either of which could be involved in the mechanism of reverse transformation. We suggest that reverse transformation may be induced by a mechanism independent of cAMP.

Correspondence address: C.C. Felder, National Institute of Mental Health, Section on Pharmacology, Laboratory of Cell Biology, Bethesda, MD 20892, USA

2. MATERIALS AND METHODS

2.1. *Materials*

Phorbol 12-myristate 13-acetate (PMA) was purchased from Calbiochem (La Jolla, CA). 4α-Phorbol 12-myristate 13-acetate (4αPMA) was purchased from Biomol (Plymouth Meeting, PA). Reagents for the radioimmunoassay of cAMP were supplied by Dr Gary Brooker (Georgetown University, Washington, DC). All other reagents were purchased from Sigma (St. Louis, MO).

2.2. *Cell culture of CHO cells*

CHO cells were maintained in alpha modified Eagle's medium with 10% newborn calf serum (MA Bioproducts, Walkersville, MD) at 37°C, under an atmosphere of 5% CO_2,

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

in plastic petri dishes. CHO cells stably expressing the transfected muscarinic m5 acetylcholine receptor [5] were generously supplied by Drs Mark R. Brann and Noel J. Buckley. The lack of change in basal or carbachol-stimulated levels of cAMP accumulation or inositol phospholipid turnover over the duration of this study suggested stable expression of the m5 muscarinic receptor. CHO cells were subcultured into plastic 6-well clusters (34 mm diameter) using 0.5% trypsin to remove cells from the plastic surface. Morphological transformation (reverse transformation) was observed while cells were grown in alpha modified Eagle's media as described above except that the media contained only 1% newborn calf serum.

2.3. *Measurement of reverse transformation*

Cells were photographed at 800 × magnification with a density of approx. 25 cells per field. The ratios of length to width of the cells were calculated for 10 cells randomly selected from the field. Broken or superimposed cells were excluded from measurement.

2.4. *Measurement of cAMP accumulation*

CHO cells were plated in plastic 24-well clusters at a density of 1×10^6 cells per well and grown to confluence. The growth medium was replaced with 250 μl serum-free media containing the experimental agents to be tested and 1 mM IBMX. The reaction was stopped at the times indicated with 250 μl ice-cold solution containing 0.1 N HCl and 1 mM $CaCl_2$. The accumulation of cAMP was measured by radioimmunoassay as described [6]. No cAMP accumulation was observed in the absence of IBMX.

3. RESULTS

Carbachol, a muscarinic receptor agonist, caused reverse transformation in CHO cells stably expressing the transfected m5 receptor in a similar manner (fig.1) to that described by Hsie and Puck [7] using dibutyryl cAMP. The cells elongated to about twice their width and lined up in a parallel pattern resembling typical fibroblasts. In the absence of carbachol the cells were compact, well separated, and lacked defined orientation (fig.1). The carbachol-induced change was concentration

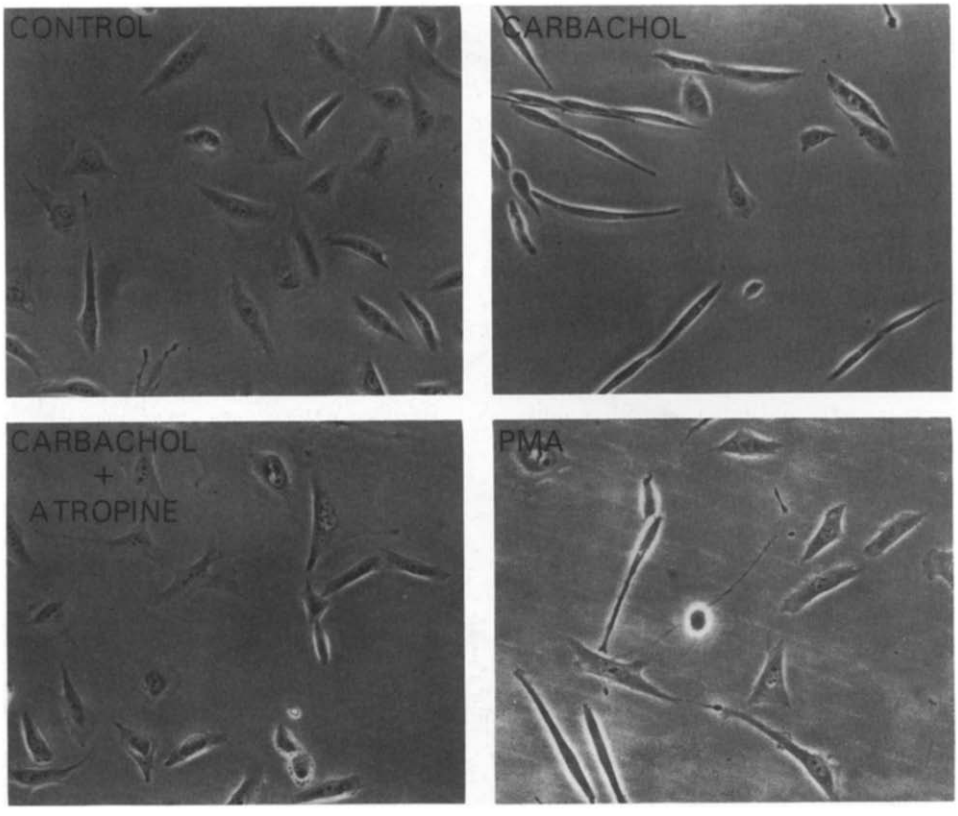

Fig.1. Carbachol- and PMA-induced reverse transformation in CHO cells transfected with and stably expressing the muscarinic m5 receptor. CHO cells transfected with the muscarinic m5 receptor were grown in continuous culture, stimulated with carbachol (10^{-4} M), carbachol (10^{-4} M) + atropine (10^{-5} M), or PMA (10^{-8} M) as indicated for 24 h. Panels were randomly selected and are representative fields.

dependent (EC_{50} 63 nM) and was blocked by the addition of a saturating dose of atropine (10^{-5} M), a muscarinic receptor antagonist (figs 1,2), suggesting that the morphological change was associated with the stimulation of a membrane-bound receptor. Cells, which were not transfected with the m5 muscarinic receptor, did not undergo reverse transformation when treated with carbachol (10^{-4} M), further suggesting that the change was due to the presence of the muscarinic receptor (table 1). The reverse transformation induced by carbachol had the same appearance as that induced by dibutyryl cAMP (10^{-4} M), 8-bromo cAMP (10^{-4} M), and cholera toxin (100 ng/ml) measured at 24 h (not shown). Butyrate is therefore probably not involved in stimulating reverse transformation.

Carbachol stimulates cAMP accumulation and inositol phospholipid turnover in CHO cells transfected with the m5 receptor [5]. Either of

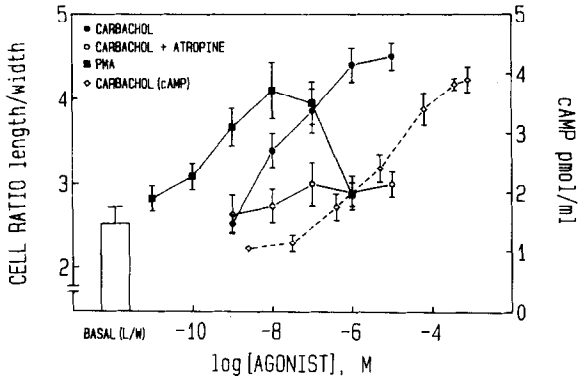

Fig.2. Carbachol-stimulated cAMP accumulation, and carbachol- and PMA-stimulated reverse transformation in CHO cells transfected with and stably expressing the muscarinic m5 receptor. (Left axis and solid lines) Reverse transformation measured as the ratio of cell length/width was stimulated in CHO cells transfected with the muscarinic m5 receptor by PMA and the muscarinic agonist carbachol which was blocked by the muscarinic antagonist atropine (10^{-5} M). Each point is the mean ± SE cell ratio of four experiments (n = 10 cells per experiment). Cell ratios at 10^{-8} M carbachol or greater were significantly different from basal ratios ($p < 0.01$ ANOVA, Dunnett's test). Cells treated with atropine were not significantly different from basal ratios ($p < 0.01$ ANOVA, Dunnett's test). PMA at 10^{-10}–10^{-7} M was significantly different from basal cell ratios ($p < 0.01$ ANOVA, Dunnett's test). (Right axis and dotted line) cAMP accumulation stimulated over 5 min by carbachol as described in section 2. Concentrations of carbachol above 10^{-7} M were significantly different from basal levels (basal value same as level at 10^{-9} M) ($p < 0.01$ ANOVA, Dunnett's test).

Table 1

Length/width ratios and cAMP accumulation in control and m5 receptor-transfected CHO cells

Cells		L/W ratio
m5-transfected CHO cells	(cAMP pmol/ml (24 h))	
Basal	13.1 ± 0.3	2.5 ± 0.2
Prostaglandin E_2 (10^{-5} M)	7.5 ± 0.3[a]	2.5 ± 0.2
Cholecystokinin (10^{-6} M)	21.8 ± 0.6[a]	2.6 ± 0.3
PMA (10^{-8} M)	12.6 ± 0.6	4.1 ± 0.3[a]
Carbachol (10^{-4} M)	31.2 ± 1.2[a]	4.3 ± 0.3[a]
Control CHO cells	(cAMP pmol/ml (5 min))	
Basal	12.3 ± 0.3	2.9 ± 0.6
Carbachol (10^{-4} M)	13.3 ± 0.5	3.0 ± 0.5

[a] $p < 0.01$ vs basal levels ANOVA (Dunnett's test)

Reverse transformation was measured in CHO cells which were incubated with agonists for 24 h as described in the text. Data are the mean length/width ratios ± SE measured on 10 cells per experiment for four experiments. cAMP in transfected cells was measured after 24 h under idential conditions in the presence of 1 mM IBMX and is the mean ± SE of three experiments performed in triplicate. cAMP was measured at 5 min in control cells

these second messenger systems could be involved in the initiation of reverse transformation. Carbachol-stimulated cAMP accumulation at 5 min occurred at a higher concentration (EC_{50} 10 μM) than did carbachol-stimulated reverse transformation measured at 24 h (EC_{50} 63 nM) (fig.2). PMA, which stimulates protein kinase C, also caused concentration-dependent reverse transformation (figs 1,2). PMA demonstrated decreased stimulation above 100 nM. The inactive 4α isomer of PMA at 10^{-8} M did not cause reverse transformation (not shown).

The time course of cAMP accumulation for carbachol, cholecystokinin, prostaglandin E_2 and PMA was measured in the presence of IBMX (fig.3, table 1). Carbachol stimulated cAMP accumulation above basal levels which plateaued at 30 min and was elevated 1-fold over basal levels at 24 h. PMA did not increase cAMP accumulation over basal levels up to 24 h, suggesting an alternative mechanism to increases in intracellular cAMP for reverse transformation. This was further substantiated when prostaglandin E_2 and

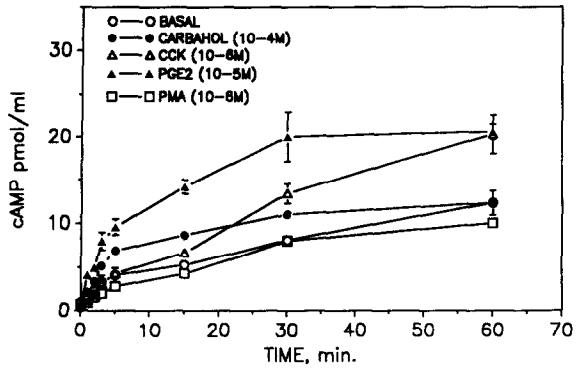

Fig.3. Time course of cAMP accumulation in CHO cells transfected with and stably expressing the muscarinic m5 receptor. CHO cells transfected with the muscarinic m5 receptor were grown in continuous culture, stimulated with carbachol (10^{-4} M), cholecystokinin (CCK) (10^{-6} M), prostaglandin E_2 (PGE_2) (10^{-5} M), and PMA (10^{-8} M) for the times indicated. Data are means ± SE of 3 experiments performed in triplicate.

cholecystokinin increased cAMP accumulation but failed to induce reverse transformation. Cholecystokinin increased and prostaglandin E_2 decreased cAMP accumulation at 24 h.

4. DISCUSSION

Our studies showed that carbachol stimulated reverse transformation in CHO cells transfected with the m5 subtype of the muscarinic acetylcholine receptor but not in cells lacking the receptor. The carbachol-stimulated reverse transformation was blocked with atropine, further suggesting that it was initiated through the activation of a membrane receptor.

Previous studies have demonstrated that reverse transformation in CHO cells is associated with increases in intracellular cAMP. Hsie and Puck [7] stimulated reverse transformation with dibutyryl cAMP which was enhanced with testosterone. The enterotoxins of *Vibrio cholerae, Escherichia coli* and *Salmonella* increased intracellular cAMP and also induced reverse transformation [8]. In our studies, carbachol stimulated cAMP accumulation and induced reverse transformation. Cholecystokinin and prostaglandin E_2 also stimulated cAMP accumulation but did not induce reverse transformation. It is unlikely that carbachol-stimulated cAMP induced the reverse transformation, since carbachol can stimulate reverse transformation at a concentration that has no apparent effect on cAMP. Carbachol maximally stimulated cAMP levels above basal levels at 5 min and declined to 1-fold above basal levels at 24 h. It is also unlikely that this elevation of cAMP levels at 24 h is sufficient to induce reverse transformation, since cholecystokinin-stimulated cAMP accumulation was also elevated at 24 h. It is possible that cholera toxin and dibutyryl cAMP induce a sustained or unusually high level of cAMP formation not possible in a receptor-mediated system, which may be an alternative mechanism for the induction of reverse transformation.

Increased intracellular cAMP levels upon addition of dibutyryl cAMP and bacterial enterotoxins do not appear to be the only mechanism involved in reverse transformation. We have observed a reverse transformation with PMA similar to that seen with the addition of carbachol, yet PMA did not increase cAMP accumulation over basal levels. PMA has been shown to effect a multiplicity of cellular processes including stimulation of protein kinase C [9], inhibition of phospholipase C [10] and stimulation of phospholipase A_2 [11]. We are currently investigating the mechanism of PMA-induced reverse transformation and its relationship to the muscarinic acetylcholine receptor-induced reverse transformation. Of the five muscarinic receptor subtypes now cloned, m1, m3, and m5 are biochemically similar in that they couple to increases in cAMP accumulation and inositol phospholipid turnover, whereas m2 is coupled to inhibition of cAMP accumulation [5,12–14]. In initial studies, carbachol-stimulated m1 and m3 muscarinic receptors transfected into CHO cells induced a reverse transformation similar to that induced by m5. This suggests a common transduction pathway for the induction of reverse transformation in the CHO cell which has yet to be clarified.

Acknowledgement: We thank Julius Axelrod for his encouragement and helpful discussions.

REFERENCES

[1] Ashall, F., Sullivan, N. and Puck, T.T. (1988) Proc. Natl. Acad. Sci. USA 85, 3908–3912.

[2] Guerrant, R.L., Brunton, L.L., Schnaitman, T.C., Rebhun, L.I. and Gilman, A.G. (1974) Infect. Immun. 10, 320–327.
[3] Ashall, F., Sullivan, N. and Puck, T.T. (1988) Proc. Natl. Acad. Sci. USA 85, 3908–3912.
[4] Porter, K., Prescott, D. and Frye, J. (1973) J. Cell Biol. 57, 815–836.
[5] Bonner, T.I., Young, A.C., Brann, M.R. and Buckley, N.J. (1988) Neuron 1, 403–410.
[6] Brooker, G., Harper, J.F. and Terasaki, W.L. (1979) Adv. Cyclic Nucleotide Res. 10, 1–33.
[7] Hsie, A.W. and Puck, T.T. (1971) Proc. Natl. Acad. Sci. USA 68, 358–361.
[8] Sandefur, P.D. and Peterson, J.W. (1977) Infect. Immun. 15, 988–992.
[9] Kikkawa, U. and Nishizuka, Y. (1986) Annu. Rev. Cell Biol. 2, 149–178.
[10] Berridge, M.J. (1987) Annu. Rev. Biochem. 56, 159–193.
[11] Burch, R.M., Ma, A.L. and Axelrod, J. (1988) J. Biol. Chem. 263, 4764–4767.
[12] Conklin, B.R., Brann, M.R., Buckley, N.J., Ma, A.L., Bonner, T.I. and Axelrod, J. (1988) Proc. Natl. Acad. Sci. USA 85, 8698–8702.
[13] Peralta, E.G., Ashkenazi, A., Winslow, J.W., Ramachandran, J. and Capon, D.J. (1988) Nature 334, 434–437.
[14] Jones, S.V.P., Barker, J.L., Buckley, N.J., Bonner, T.I., Collins, R.M. and Brann, M.R. (1988) Mol. Pharmacol. 34, 421–426.