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Independent Induction of Morphological Transformation of CHO Cells by Receptor-Activated Cyclic AMP Synthesis or by Receptor-Operated Calcium Influx

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ABSTRACT. Morphological transformation of Chinese hamster ovary (CHO) cells can be induced by exogenous addition of cyclic AMP (cAMP) or through the stimulation of G protein-coupled receptors ectopically expressed in these cells. The morphological transformation has been shown to represent a phenotypic suppression of CHO cell tumorigenic potential. Studies were undertaken to determine which receptor-activated signal transduction pathway initiates the progression from a tumorigenic to a non-tumorigenic phenotype. Stimulation of CHO cells expressing the dopamine D1 receptor (CHOD1) with a D1 selective agonist, SKF38393, resulted in an increase in cAMP accumulation which correlated with morphologic transformation. SKF38393 had no effect on intracellular calcium levels, arguing against a requirement for phospholipase C or calcium mobilization in the D1-stimulated morphology change. In contrast, stimulation of muscarinic m5 (CHOm5) or vasopressin V1a (CHOV1a) receptors expressed in CHO cells with carbachol or arginine vasopressin (AVP), respectively, did result in an increase in intracellular calcium and a morphology change. The time course of carbachol-stimulated calcium influx correlated with the time course of morphological transformation, but not with carbachol-stimulated cAMP or inositol, 1,4,5-trisphosphate (IP_3) accumulation. Furthermore, no increase in cAMP accumulation was observed in AVP-stimulated CHOV1a cells, suggesting a cAMP-independent stimulation of the transformation process. Carbachol-stimulated CHO cells expressing the m2 muscarinic receptor (CHOm2) failed to undergo a morphological transformation, yet released IP_3. Therefore, phospholipase C-mediated signal transduction is not sufficient for the morphological transformation of CHO cells. It appears that receptor-stimulated morphologic transformation of CHO cells can be induced via two independent signaling pathways, mediated by adenylate cyclase or receptor-operated calcium channels. BIOCHEM PHARMACOL 51;4:495–502, 1996.

KEY WORDS. receptor; phospholipase C; adenylate cyclase; receptor-operated calcium channel; morphological transformation of cells; second messengers

G protein-coupled receptors constitute a large family of receptors with diverse biological roles including neurotransmission, mitogenesis, and immune regulation. Several neurotransmitter receptors, including the 5HT1c serotonin, alpha-1 adrenergic, and muscarinic acetylcholine receptors, originally thought to play a role exclusively in synaptic transmission, have been linked recently to the regulation of malignant transformation. Stimulation of the 5HT1c receptor, ectopically expressed in NIH-3T3 cells, resulted in focus formation and ability to proliferate in soft agar, indicators of cell transformation [1]. When inoculated into athymic nude mice, the cells formed solid tumors. Ectopically expressed alpha-1 adrenergic receptors, that were mutated to a constitutively activated form, induced agonist-independent foci in culture and tumors in athymic nude mice [2]. Similar receptor-dependent malignant transformation was demonstrated for muscarinic m1, m3, and m5 receptor subtypes when expressed in NIH-3T3 cells [3]. More recently, muscarinic m5 receptors expressed in CHO† cells were shown to induce both a morphological transformation from a stellate to bipolar shape and a phenotype change characterized by a loss of tumorigenicity in athymic nude mice and by reduction in their ability to colonize in soft agar [4]. It was concluded that the morphology change was a visual index of the regression from a tumorigenic to a non-tumorigenic phenotype.

G protein-coupled receptors bind selectively and with high affinity to agonists and transmit their signals through G protein activation and subsequent stimulation of effector enzymes or ion channels. The initiation of tumor formation or suppres-

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† *Abbreviations:* CHO, Chinese hamster ovary; CHOD1, CHOV1a, CHOm5, and CHOm2, Chinese hamster ovary cells transfected and stably expressing the dopamine D1 receptors, vasopressin V1a receptors, and muscarinic m5 and m2, respectively; AVP, arginine vasopressin; IP_3, inositol 1,4,5-trisphosphate; FURA-2/AM, Fura-2 pentaacetoxymethyl ester; Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; PGE_2, prostaglandin E_2; and cAMP, cyclic AMP.

sion following receptor stimulation is mediated through activation of one or more signaling pathways. Cells expressing receptors with well characterized signaling pathways provide models for investigation of signaling-dependent tumor formation or suppression. Initially, the morphological transformation of CHO cells was shown to be under the regulation of cAMP, by exposing the cells to high concentrations of nonhydrolyzable cAMP analog [5], or cholera toxin [6]. Such morphological transformation was accompanied by changes in cell surface features, cytoskeletal organization, and DNA hypersensitivity to nuclease action [5, 7–9]. It was not known if physiological levels of cAMP generated following receptor-mediated stimulation of adenylate cyclase were capable of inducing the CHO morphological transformation. Subsequently, a cAMP-independent CHO cell morphology change was shown in cells transfected with, and stably expressing, the muscarinic m5 receptor [10]. Muscarinic m1, m3, and m5 receptors couple to the activation of multiple signal transduction effectors including phospholipase A_2, phospholipase C, phospholipase D, adenylate cyclase, tyrosine kinase, and opening of voltage-independent Ca^{2+} channels [4, 11, 12].

Muscarinic receptor-stimulated calcium influx through receptor-operated calcium channels was implicated in CHO cell transformation when CAI, an inhibitor of the receptor-mediated Ca^{2+} influx [11], attenuated an m5 receptor-stimulated morphology change [4]. Ca^{2+} has been shown to play a role in growth, proliferation, and differentiation of cells [13, 14]. In this study, we sought to determine if cAMP, calcium, or both are involved in receptor-stimulated morphology transformation. We first showed that transformation could be induced by a receptor linked to increases in cAMP accumulation, independent of calcium, using CHOD1 cells. Correlations were then sought between second messengers and ability to induce a morphology change under the regulation of four different receptors: dopamine D1, vasopressin V1a, and muscarinic m5 and m2, each stably expressed in CHO cells. These receptors were chosen because each of them activates various signal transduction pathways (Table 1). A comparison between the induced second messenger in each cell line and the ability of each expressed receptor to activate a morphology transformation of CHO cells was made (Table 1). In CHOm5 and CHOV1a cells, the morphological change correlated with calcium influx through voltage-insensitive calcium channels, and not cAMP. In CHOD1 cells, the morphology change correlated with receptor-mediated changes in cAMP levels and not Ca^{2+}.

TABLE 1. Ability of four different G protein-coupled receptors to activate signal transduction pathways and morphology change in CHO cells

Cell line	Morphology change	cAMP accumulation	Ca^{2+} influx	IP_3 release
CHOD1	+	+	−	−
CHOm5	+	+	+	+
CHOV1a	+	−	+	+
CHOm2	−	−	−	+

CHO-K1 cells transfected with and stably expressing D1, m5, m2, or V1a receptors were exposed to the agonists SKF38393 (D1), carbachol (m5 and m2), and AVP (V1a). Activation of Ca^{2+} influx and release of IP_3 and cAMP were measured and compared with the ability of the receptor to induce morphology change.

MATERIALS AND METHODS

Materials

myo-[2-^{3}H(N)]Inositol was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO) and adenosine 3′,5′-cyclic phosphoric acid-2′-O-succinyl-[^{125}I]iodotyrosine methyl ester from Du Pont-New England Nuclear (Boston, MA). FURA-2/AM was purchased from Molecular Probes (Eugene, OR) and Vitrogen from Celtrix Laboratories (Palo Alto, CA); poly-L-lysine and forskolin were obtained from the Sigma Chemical Co. (St. Louis, MO). RO 20-1724 and PGE_2 were obtained from Biomol (Plymouth Meeting, PA).

Assay of Muscarinic Receptor-Stimulated [^{3}H]IP_3 and cAMP Release

IP_3 RELEASE. CHOm5 cells were grown in 24-well plates at a density of 200,000 cells/well in α-MEM containing 10% fetal bovine serum, 100 U/mL penicillin, 100 μg streptomycin, and 4 mM L-glutamine. They were labeled overnight with [^{3}H]-inositol (1 uCi/mL). [^{3}H]IP_3 release was measured as previously described [11].

cAMP ACCUMULATION. CHO cells were harvested with calcium-free Eagle's No. 2 medium containing 0.5 mM EDTA, washed with Eagle's No. 2 medium containing 1 mM $CaCl_2$ and 0.2% BSA, and resuspended at a concentration of 1–3 × 10^6 cells/mL. Stimulation was performed for 5 min in the presence of the phosphodiesterase inhibitor RO 20-1724 (0.2 mM). Radioimmunoassay of cAMP was performed as previously described [15]. In both assays, cells were treated with carbachol for the time indicated, and 10 min before starting the assay they were washed with the assay buffer to remove bound carbachol and stimulated again with carbachol. Pretreated cells were compared with untreated cells, and data were normalized by calculating the percentage of IP_3 or cAMP release in each group over the basal release for the same group. Data are the means ± SEM of at least three experiments, each experiment performed in triplicate.

CHO Cell Morphology Change

CHOD1, CHOm5 and CHOV1a were photographed, and the length and width of randomly selected cells were measured as described previously [4].

Measurement of Intracellular Ca^{2+} concentration in Single Fura-2-Loaded CHO Cells

Cells were grown on glass cover slips. To assure the attachment of cells, the glass cover slips were incubated in poly-L-lysine (10 ng/mL) for 1 hr, washed with water, dried, incubated in Vitrogen (300 μg/mL), and allowed to air dry. Cells

were incubated for the times indicated in carbachol before measuring the Ca^{2+} level. They were loaded with 5 μM Fura-2/AM for 30 min in Krebs medium containing 5 mM HEPES. Where indicated, carbachol was included through the loading period. Intracellular Ca^{2+} measurements were performed as described previously [16].

RESULTS

Morphology Change in CHO-K1 Cells Associated with Dopamine D1, Vasopressin V1a, and Muscarinic m5 Receptors

CHO-K1 cells transfected with and stably expressing the muscarinic m5 receptor (CHOm5 cells), in the absence of an agonist, were characterized by a stellate morphology with a high nuclear to cytoplasm ratio (Fig. 1A). The same stellate morphology shape was shared by CHOV1a (see Fig. 3), CHOD1, and CHOm2 receptors in the absence of agonists (data not shown for the latter two receptors). Stimulation of CHOm5 (Fig. 1, E–F), CHOV1a (see Fig. 3), and CHOD1 cells with carbachol, AVP, and SKF38393, respectively, resulted in a morphological transformation characterized by a bi-polar shape and a reduction in the nuclear to cytoplasmic ratio. The morphological transformation was quantified by measurement of the change in the length to width ratio of randomly selected cells. The carbachol-induced transformation in CHOm5 cells was first observed at 3.5 hr, was maximal by 6.5 hr, and persisted for 17 hr, the last time point tested (Figs. 1, B–F and 2A). The time course of the SKF38393-induced morphology change in CHOD1 cells was very similar to the time course of transformation observed in CHOm5 cells in the presence of carbachol (Fig. 2A); a morphology change was observed first after 4 hr and was completed after 6 hr. The time course of the AVP-induced morphology change in CHOV1a cells was similar to that of carbachol and after 17 hr, the last time point tested, the length to width ratio was 5 ± 0.97 (N = 15) in the presence of 1 μM AVP and 0.37 ± 0.02 (N = 17) in the absence of AVP (Fig. 3) (data are representative of at least five experiments, each performed in triplicate). Treatment of CHOm2 cells with carbachol (100 μM) had no effect on CHO cell morphology within 24 hr of exposure (data not shown).

Correlation Between Muscarinic m5 Receptor-Induced Morphology Change and Ca^{2+} Influx in CHO Cells

The change in intracellular calcium concentration stimulated by carbachol was monitored in CHOm5 cells (Fig. 2B) and was compared with the time course for the carbachol-induced morphological transformation (Fig. 2A). The cells were pretreated with carbachol and, at indicated times, loaded with the calcium-sensitive fluorescence dye, Fura-2/AM. The level of intracellular Ca^{2+} was measured in the continued presence of carbachol. There was a rapid rise and slow decline in $[Ca^{2+}]_{in}$ after carbachol stimulation. $[Ca^{2+}]_{in}$ remained elevated for at least 17 hr. CHOV1a cells showed a similar intracellular Ca^{2+}

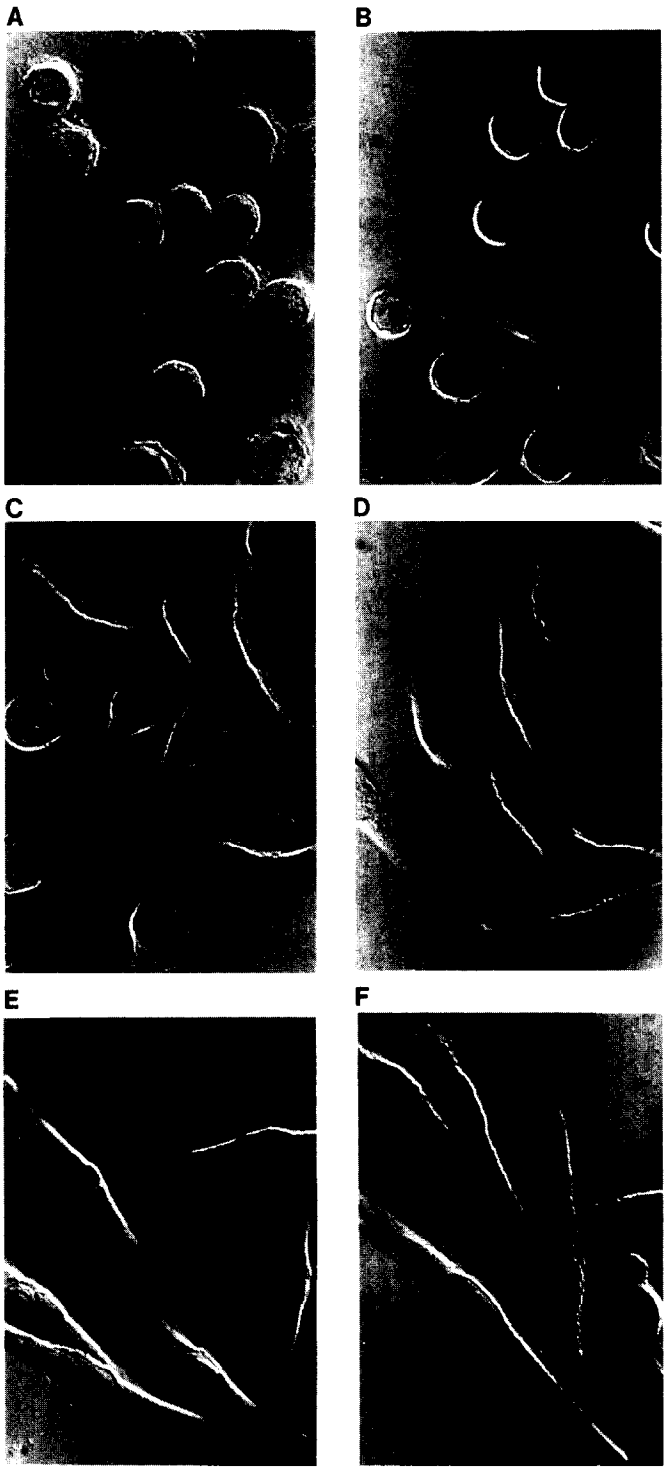

FIG. 1. Carbachol-induced morphology change in CHOm5 cells. CHOm5 cells were plated on glass cover slips, treated in monolayer with carbachol for the indicated hours, and then photographed using differential interference contrast optics. (A) control CHOm5 cells. (B–F) CHOm5 cells treated with 100 μM carbachol for: (B) 2.5 hr, (C) 3.5 hr, (D) 4.5 hr, (E) 6.5 hr, and (F) 17 hr. Cells were randomly selected from at least three separate experiments.

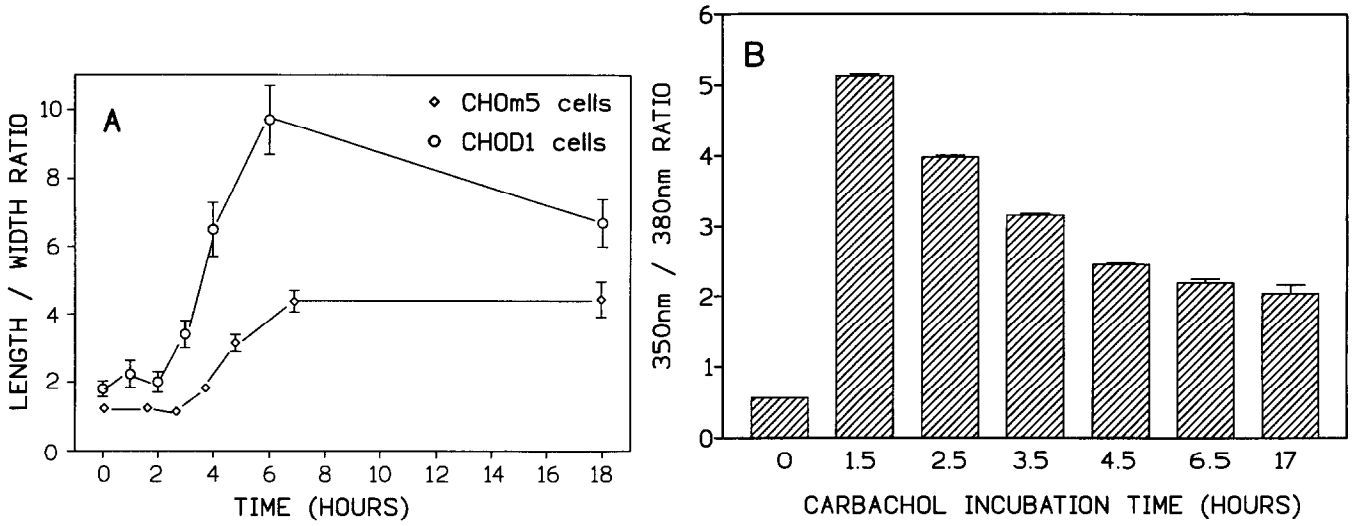

FIG. 2. Time course for carbachol-induced morphology change and carbachol-induced Ca^{2+} influx in CHOm5 cells. (A) The morphology change of CHOm5 cells was measured as a length to width ratio in the presence of carbachol (100 μM, ◇) and CHOD1 cells in the presence of SKF38393 (1 μM, ○). Data are the means ± SEM of at least 15 cells. (B) CHOm5 cells were plated on cover slips and incubated in carbachol (100 μM) for the indicated hours. At the end of the incubation time, intracellular Ca^{2+} levels were measured. The cells were illuminated alternately with wavelength light of 350 and 380 nm, and the ratio from light emitted at 510 nm was an index of intracellular calcium levels. Each column represents the mean ± SEM of at least 10 cells.

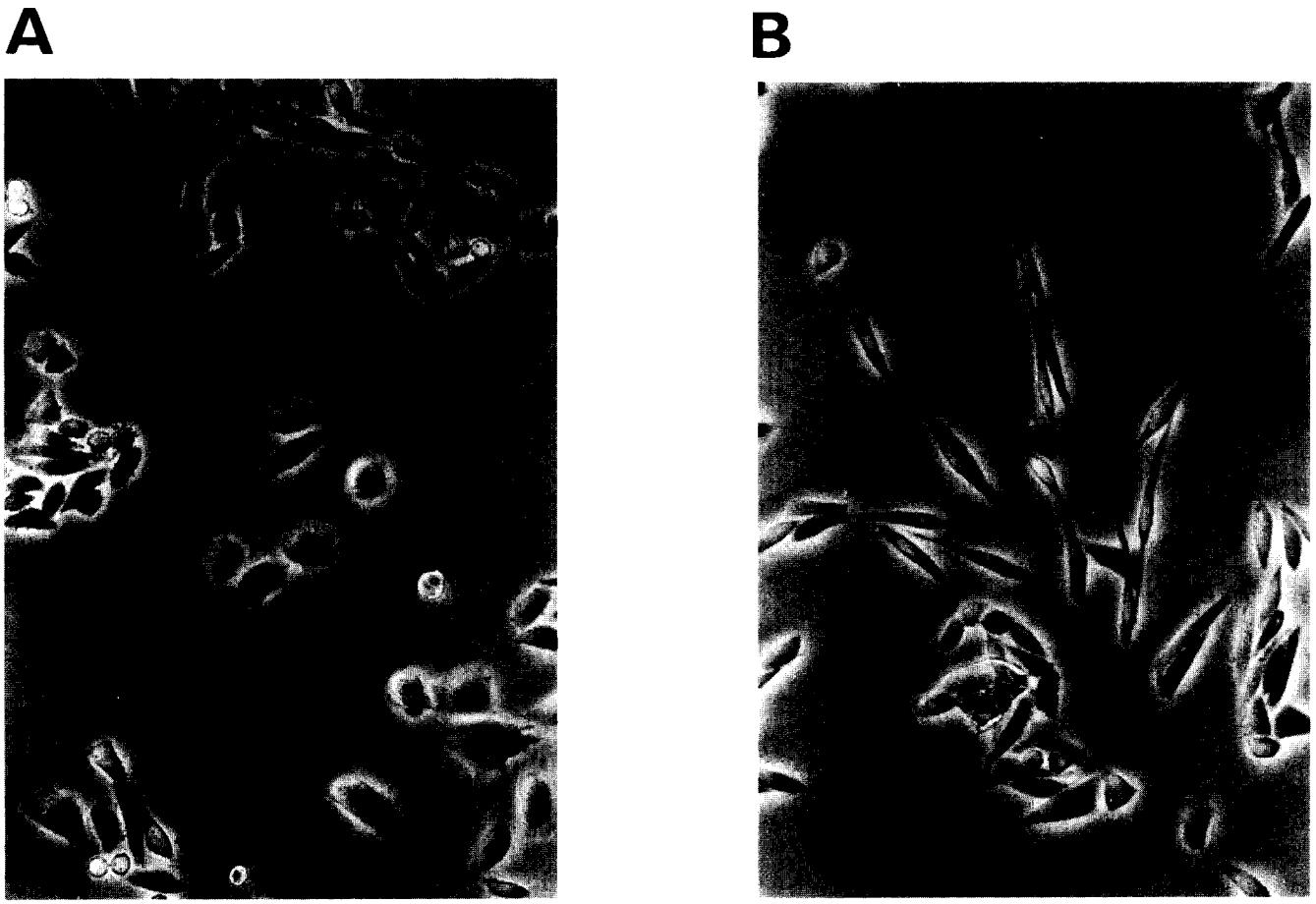

FIG. 3. AVP-induced morphology change in CHOV1a cells. CHOV1a cells were plated on glass cover slips, treated in monolayer with AVP for the indicated hours, and then photographed using differential interference contrast optics. (A) control CHOV1a cells. (B) CHOV1a cells treated with 1 μM AVP for 17 hr.

behavior in response to AVP (data not shown). In contrast, in SKF38393-stimulated CHOD1 cells there was no change in intracellular Ca^{2+} levels. Exposure of CHOm2 cells to carbachol resulted with just a rapid and transient rise in $[Ca^{2+}]_{in}$, which was a result of Ca^{2+} release from intracellular stores (data not shown).

Receptor-Regulated CHO Cell Morphological Transformation and cAMP Levels

The effect of receptor stimulation on cAMP levels was measured in CHOD1, CHOV1a, and CHOm5 cells. The CHO cells were pre-exposed to agonist for the times indicated and then washed free of agonist just prior to the 5-min cAMP accumulation assay performed in the presence of the phosphodiesterase inhibitor RO 20-1724. The physiologic potential to accumulate cAMP could then be compared between the CHO cell lines over time while the cells were in various stages of transformation. This approach was used because RO 20-1724 was found to be cytotoxic upon long-term exposure to CHO cells.

Long-term SKF38393 treatment stimulated cAMP accumulation with a slightly lower EC_{50} than the CHOD1 cell morphological transformation (Fig. 4), suggesting that cAMP elevation may regulate the transformation in this CHO cell. Long-term pre-exposure of CHOm5 cells to carbachol resulted in a >75% reduction in the potential of the m5 receptor to stimulate cAMP accumulation (Fig. 5). These data suggest that cAMP is not involved in the process of carbachol-induced morphologic transformation of CHOm5 cells. This inference was supported by the finding that AVP did not stimulate cAMP accumulation in CHOV1a cells (Fig. 6) despite inducing a morphology change comparable to that induced by carbachol in CHOm5 cells.

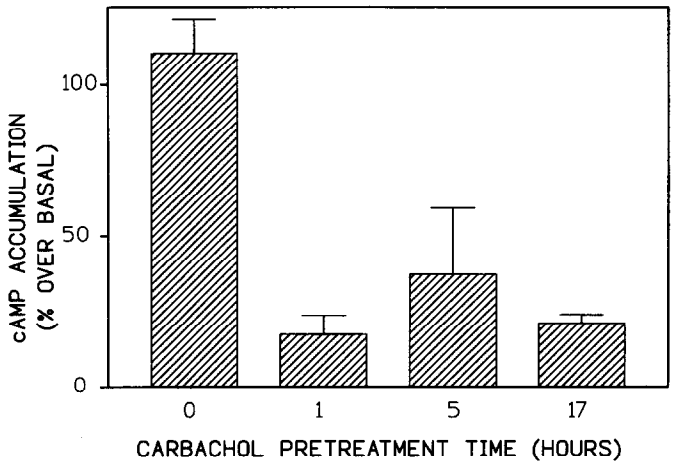

FIG. 5. Time course of carbachol-induced cAMP generation in CHOm5 cells. Cells were plated, incubated in carbachol (100 μM) for the indicated hours, and then were washed free of carbachol. Carbachol was reapplied, and cAMP accumulation was measured over 5 min. Data are the means ± SEM of seven experiments performed in triplicates and are represented as percentage over basal release. The averaged basal values of cAMP release from cells that were preincubated in carbachol for 0, 1, 5, and 17 hr were: 1.4 ± 0.2, 1.6 ± 0.3, 1.8 ± 0.2, and 2.9 ± 1.5 pmol/mL, respectively. Carbachol-induced cAMP release from cells that were not preincubated in carbachol was 2.6 ± 0.7 pmol/mL.

The levels of cAMP observed after activation of the D1 receptor (Fig. 4) are much higher than those observed with activation of the endogenous PGE_2 receptor (Fig. 6). This difference may result from a different level of expression of the endogenous receptors.

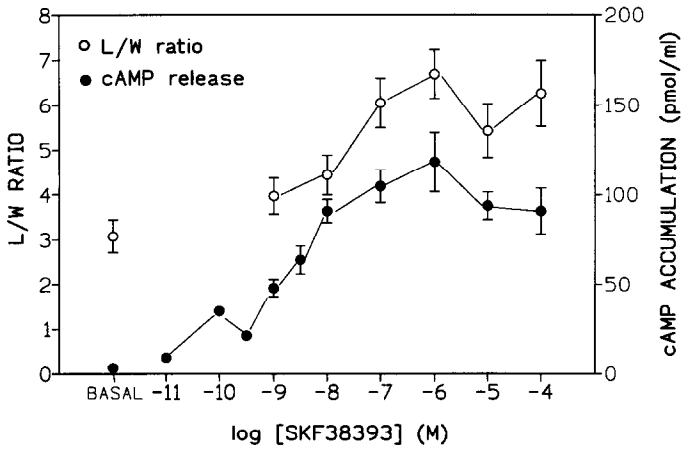

FIG. 4. Comparison between concentration-response curves of SKF38393-induced morphology change and cAMP release in CHOD1 cells. CHOD1 cells were stimulated with various concentrations of SKF38393 for 17 hr, and the cAMP release was measured (●). Data are the means ± SEM of triplicate determinations from one of four experiments. The SKF39383-induced morphology change (○) was measured as a length/width ratio of at least 15 cells.

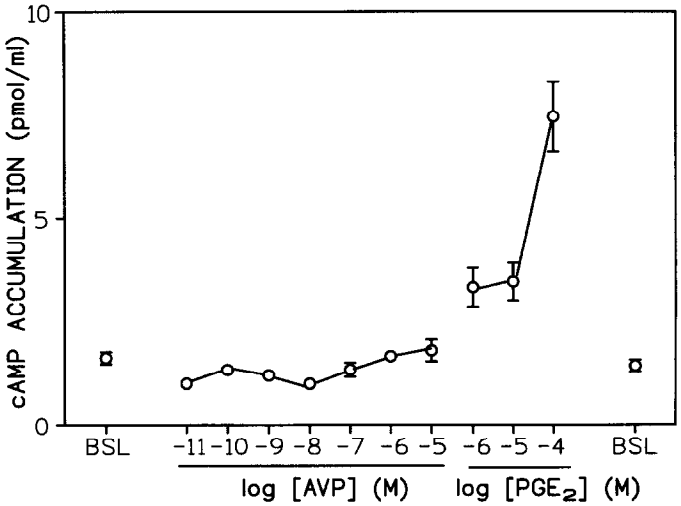

FIG. 6. Concentration-response curve for AVP and PGE_2-induced cAMP release in CHOV1a cells. CHOV1a cells were stimulated with various concentrations of AVP and PGE_2, and the cAMP release was measured. Data are the means ± SEM of four experiments performed in triplicate. PGE_2 served as a control for cAMP release in these cells.

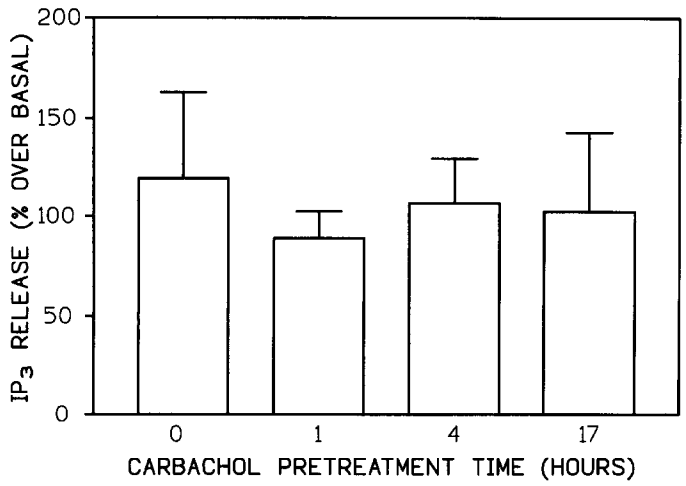

FIG. 7. Time course of carbachol-induced IP_3 release in CHOm5 cells. Cells were plated, incubated in carbachol (100 μM) for the indicated hours, and then washed free of carbachol. Carbachol was reapplied, and IP_3 accumulation was measured over 5 min. Data are the means ± SEM of five experiments performed in triplicate and are represented as percentage over basal release. The averaged basal values of IP_3 release from cells that were preincubated in carbachol for 0, 1, 4, and 17 hr were: 144 ± 19, 137 ± 31, 140 ± 32, and 93 ± 15 cpm, respectively. Carbachol-induced IP_3 release from cells that were not preincubated in carbachol was 320 ± 80 cpm.

Receptor-Regulated CHO Cell Morphological Transformation and IP_3 Release

The effect of receptor stimulation on IP_3 levels was measured in CHOm5 cells. There was essentially no reduction in the potential of the m5 receptor to stimulate IP_3 release following pre-exposure to carbachol for the times indicated (Fig. 7). Figure 8A demonstrates a typical change in intracellular calcium levels in CHOm5 cells after stimulation with carbachol. We demonstrated previously that the initial rapid rise is due to carbachol-stimulated IP_3 formation and release of calcium from intracellular stores, and the sustained phase is maintained by influx of extracellular calcium [16]. In contrast, in CHOm2 cells, carbachol stimulated a similar IP_3-dependent rapid rise in intracellular calcium after a delay of about 5 min which decayed back to basal levels within several minutes (Fig. 8B). The m2-stimulated calcium transient was insensitive to removal of extracellular calcium, suggesting that its source was from an intracellular compartment (data not shown). Carbachol failed to stimulate a morphological transformation in CHOm2 cells [4].

DISCUSSION

We have established previously that the transformation of CHO cells from a stellate to bipolar morphology is a visual index of an associated phenotypic regression from a tumorigenic phenotype to one lacking the ability to proliferate in either soft agar or *in vivo* in athymic nude mice [4]. Induction of the morphological transformation by exogenous addition of cAMP analog [5] suggested that this process was under the regulation of G protein-coupled receptors and their associated signaling pathways. Receptors with differential coupling to signaling effectors were tested to distinguish among signaling pathways that might be involved in CHO cell transformation. In this manuscript, we have established that a D1 receptor-stimulated increase in cAMP accumulation will result in a morphological transformation (Fig. 4). D1 receptors do not couple to mobilization of intracellular calcium in the CHOD1 cell, ruling out a requirement for IP_3-dependent calcium release in the transformation process. This is further supported by the observation that stimulation of CHOm2 cells with carbachol failed to induce a morphology change, yet activated an IP_3-dependent intracellular calcium transient (Fig. 8B).

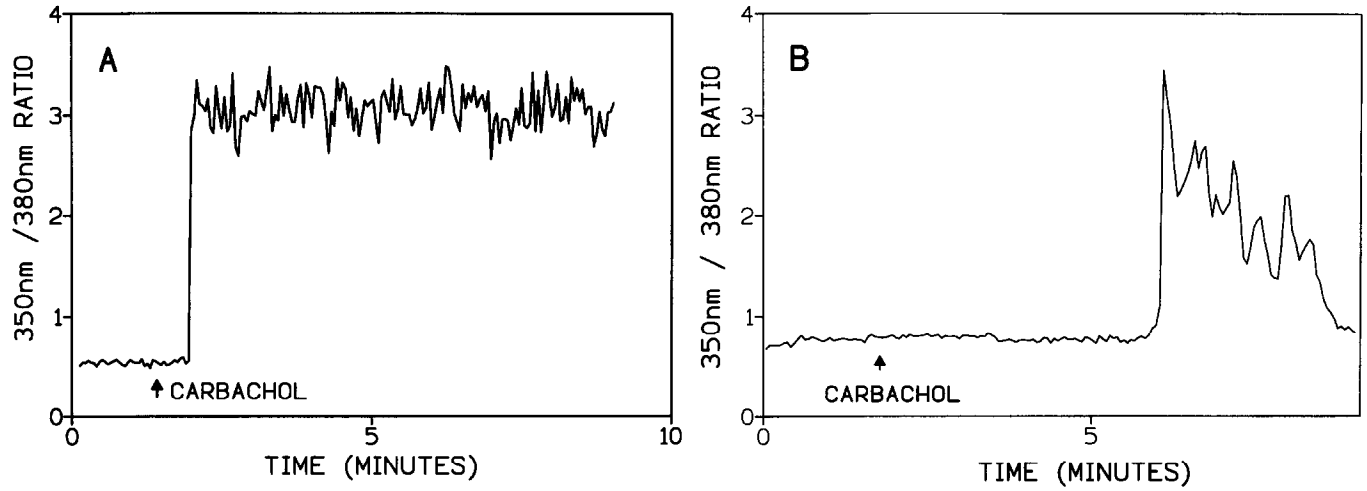

FIG. 8. Carbachol-induced Ca^{2+} release from intracellular stores in CHOm5 and CHOm2 cells loaded with Fura-2. Cells were plated on glass cover slips, and the intracellular Ca^{2+} levels were measured by single cell fluorescence imaging. The cells were illuminated alternately with wavelength light of 350 and 380 nm, and the ratio of the light emitted at 510 nm was an index of intracellular calcium levels. Carbachol was added at the time indicated. In the CHOm5 cells (A), data are from one representative cell out of 10. In CHOm2 cells (B), data are from one representative cell out of 4.

Therefore, it is unlikely that phospholipase C or associated calcium transients are responsible for CHO cell transformation.

The m5 receptor is linked to stimulation of multiple signal transduction pathways including calcium influx through second messenger-independent receptor-operated Ca^{2+} channels [16]. Activation of phospholipase D [4], phospholipase A_2 [17], phospholipase C-γ [4], and tyrosine kinase [18] by the muscarinic receptor depends on calcium influx, and activation of adenylate cyclase [15] requires release of Ca^{2+} from intracellular stores. However, in a recent work it was shown that muscarinic m1 receptor activates adenylate cyclase in the absence of Ca^{2+} [19]. Earlier studies suggested that m5 receptor stimulation led to a morphological transformation that is independent of cAMP and dependent on calcium influx [4, 10]. To further rule out the involvement of cAMP in the transformation change, CHOV1a cells were chosen because they stimulate similar signal transduction pathways as m5 receptors [20, 21], yet lack functional coupling to adenylate cyclase. Under identical assay conditions as used for the CHOD1 cells, stimulation of the V1a receptor failed to activate cAMP accumulation, yet did stimulate a morphology change, suggesting that an alternate signaling pathway may exist. The possibility of a cAMP-independent pathway was also evident in CHOm5 cells, where the morphology change was stimulated at a concentration of carbachol two orders of magnitude below that required for any detectable levels of cAMP accumulation [4]. It was also shown that a calcium channel inhibitor (CAI) blocks the morphology change but has no effect on receptor-stimulated cAMP accumulation [4, 11]. Taken together, this evidence rules out the involvement of adenylate cyclase stimulation in morphology change of CHO cells expressing calcium-mobilizing receptors such as m5 or V1a. However, a role for calcium influx in m5-stimulated morphological transformation was supported by the strong correlation between sustained m5 receptor-operated Ca^{2+} influx and morphology change.

Selection of receptors displaying differential signal transduction allowed us to conclude that two independent pathways seem sufficient for the initiation of the morphology change. cAMP can induce the morphology change whether applied exogenously or increased by stimulation of adenylate cyclase through receptor activation. It is apparent that calcium mobilizing receptors, such as m5 or V1a, do not utilize the activation of adenylate cyclase to induce the morphology change. Previous pharmacological experiments designed to specifically block Ca^{2+} influx [4], and the correlation study presented in this work of the transformation time course, suggested that calcium influx may play a role in morphological transformation. The stimulation of phospholipase C and the subsequent release of IP_3-sensitive calcium stores were ruled out as mediators in morphological transformation in the experiments with CHOm2 cells. Receptor-operated calcium channels are poorly characterized [22]. Further support for their involvement in cell transformation will require less cytotoxic and more selective channel inhibitors as well as the cloning of the voltage-independent calcium channel. CHO cells transfected with various G protein-coupled receptors (m5, V1a, m2, and D1) may serve as model systems for the study of the molecular mechanism leading to tumor formation and suppression.

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