Vol. 213, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 4, 1995 Pages 44-51

HIGH- AND LOW-AFFINITY CCK_A RECEPTOR STATES MEDIATE SPECIFIC GROWTH INHIBITORY EFFECTS ON CHO CELLS

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Received June 28, 1995

To relate specific effects on growth and transformation to activation of specific affinity states of the CCK_A receptor stably expressed in CHO cells we compared responses to the CCK analogues JMV-180 and CCK_8. CCK_8 led to an inhibition of both cell proliferation and transformation. Effects on proliferation were indicated by a reduction of DNA synthesis and cell numbers. Effects on transformation were indicated by a reduction of colony formation in soft-agar. JMV-180 did not inhibit cell proliferation although a small inhibitory effect on DNA synthesis was observed. JMV-180 inhibited the maximal effects of CCK_8 on cell proliferation and DNA synthesis. In contrast, JMV-180 substantially inhibited cell colony formation in soft-agar and did not inhibit the effects of CCK_8 on this parameter. Collectively these data with receptor affinity state specific analogues indicated that inhibition of cell proliferation and growth in soft-agar can be attributed to activation of distinct affinity states. Thus, different second messengers are likely responsible for the inhibitory effects on anchorage-dependent and -independent growth.

G protein linked receptors both positively and negatively influence cell growth and transformation. The growth stimulatory effects of G protein linked receptors are well known (1,2). The further suggestion that these receptors can transform cells has been supported by the observations that a variety of receptors including $5HT_{1C}$ (3), α1-adrenergic (4), and m1,m3 and m5 muscarinic cholinergic (mACh) (5) are able to stimulate foci formation when transfected into NIH 3T3 cells. It is less well known that G protein linked receptors can also inhibit cell growth and transformation (6,7). It has recently been shown that activation of m1, m3, and m5 mACh receptors led to a reversal of the transformed phenotype in CHO cells (8,9). The mechanisms involved in the stimulatory and inhibitory effects of these receptors on growth and transformation are unknown.

Cholecystokinin (CCK) is both a hormone and a neurotransmitter with multiple effects and sites of action (10). The effects of CCK are transduced through at least two distinct classes of

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0006-291X/95 $12.00

specific cell-surface receptors, CCK_A (alimentary) and CCK_B (brain), both of which are linked to the activation of phospholipase C. Previous studies indicate that the CCK_A receptor exists in two affinity states and occupancy of high- and low-affinity states is related to the initiation of different intracellular events and resulting biological responses (11-14). JMV-180, a synthetic CCK analogue, is capable of binding CCK receptors and occupying both high and low affinity states (11). In rat pancreas, JMV-180 occupation of the high affinity state leads to a full secretory response similar to that of CCK. Occupation of the high affinity state has no effect on cellular cAMP content or phosphatidylinositol production, whereas these signal transduction pathways are activated by occupation of the low-affinity state by CCK_8. On the contrary, occupation of the low affinity state by JMV-180 does not activate low affinity responses and blocks the effects of CCK_8 on these responses. These facts have lead to the conclusion that JMV-180 is a full agonist on high affinity CCK_A receptor sites but an antagonist on low affinity sites in the rat (11,15-17). Recently the ability of the cloned CCK_A receptor to differentially activate signal transduction pathways upon stimulation by CCK_8 and JMV-180 was confirmed in stably transfected CHO cells (18). Therefore, JMV-180 is a useful tool to differentiate these two receptor states and their functions.

In the current study we have investigated the effects of activating specific CCK_A receptor affinity states on various aspects of CHO cell growth. Activation of both high- and low-affinity receptor states with CCK_8 strongly inhibited cell proliferation. In contrast, exclusive activation of the high-affinity receptor state with JMV-180 did not inhibit cell proliferation. Both CCK_8 and JMV-180 inhibited the formation of colonies in soft-agar. Thus, CCK_A receptor-mediated growth inhibition occurred by interactions with both high- and low-affinity receptor states and activation of specific affinity states predominantly influenced specific aspects of CHO cell growth. Activation of the low-affinity state inhibited cell proliferation, whereas activation of the high-affinity state inhibited anchorage independent growth.

MATERIALS AND METHODS

Materials Dulbecco's modified Eagle's medium, fetal bovine serum (FBS), penicillin, streptomycin, and amphotericin B were obtained from GIBCO (Grand Island, NY). Tissue culture plastic-ware were obtained from Sarstedt (Newton, NC). CHO-K1 cells were obtained from the American Type Culture Collection (Rockville, MD). CCK_8 sulfated was obtained from Bachem (Torrance, CA): JMV-180 from Research Plus (Bayonne, NJ): [^{3}H]thymidine from Amersham (Arlington Heights, IL); trichloroacetic acid from J.T.Baker (Phillipsburg, NJ).

Cell Proliferation Assays

Development of the CHO-K1 cell line stably expressing rat CCK_A receptors was previously described (18). Cells were routinely cultured in Dulbecco's modified Eagle's media supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (50 μg/ml) and 5% fetal bovine serum (FBS) at 37° C in a humidified atmosphere of 5% CO2. For the proliferation assays the cells were subcultured in 24 well dishes at a density of 25,000 cells per well and allowed to attach overnight.

[^{3}H]thymidine incorporation DNA synthesis was estimated by measurement of [^{3}H]thymidine incorporation into trichloroacetic acid-precipitable material. After a 7h incubation, or at the times indicated, [^{3}H]thymidine was added to each well (final concentration, 0.1 μCi/ml).

Cells were incubated for an additional 1hour, then the media containing [^{3}H]thymidine was removed. Cells were washed twice with ice-cold phosphate buffered saline then twice with trichloroacetic acid (6% final concentration), removed by dissolving in 0.1 N NaOH. Radioactivity was measured by liquid scintillation counting.

Analysis of cell numbers Cells grown in the presence of 5% FBS with or without CCK analogues for 72 hours were removed from the dish with trypsin EDTA solution, diluted in 0.9% saline, and counted using a Coulter counter.

Analysis of colony formation in soft-agar Cells were plated at 1000 cells per dish into 35 mm dishes in agar (0.3%) and cultured in growth media containing 5% FBS and the indicated concentrations of CCK analogues. After two weeks the dishes were examined and all colonies over 0.5 mm in diameter were counted.

Statistical Analysis Statistical analysis was carried out by using a commercial statistical program (InStat; Graphpad Software, San Diego, CA). Differences between individual conditions and controls were tested with ANOVA. In the analysis, differences were considered significant when $P<0.05$.

RESULTS

EFFECTS OF CCK ANALOGUES ON CHO CELL PROLIFERATION

In order to determine the effects of activation of the CCK_A receptor on CHO cell proliferation we first tested effects on DNA synthesis. Cells expressing CCK_A receptors and growing in the presence of 5% FBS were treated with various concentrations of CCK_8 and the effects on incorporation of [^{3}H]thymidine into DNA were evaluated. CCK_8 inhibited DNA synthesis in a dose-dependent manner with significant inhibition observed with 0.1 nM CCK_8 and a maximal inhibition of ~60% occurring with 3 nM CCK_8 (Fig. 1A). No further inhibitory effects were noted with higher concentrations. No effect of CCK_8 treatment was noted on untransfected CHO cells or in the absence of FBS (data not shown). The inhibitory effects of CCK_8 on DNA synthesis were also time-dependent. Cells growing in the presence of 5% FBS were significantly inhibited within 1 hour of treatment (Fig. 1B). Maximal inhibition occurred within 6-8 hours of treatment.

CHO cells bearing CCK_A receptors responded to JMV-180 by a small but significant decrease in the incorporation of [^{3}H]thymidine (Fig. 2A). A maximal inhibition of 20 $\pm$ 5 % of control (n=3, $p<0.05$) was attained at 1 μM JMV-180 . No further effect was seen at higher concentrations of JMV-180 (data not shown). Since JMV-180 alone caused only a small effect on DNA synthesis we examined its ability to influence the response to CCK_8. CHO cells were stimulated with 100 nM CCK_8 and different doses of JMV-180 were added in combination. Reduction of CCK_8 inhibited [^{3}H]thymidine incorporation was noted with 100 nM JMV-180 (n=4, $p<0.01$) and with 1 μM JMV-180 the response to CCK_8 was reduced to the same level as that of 1 μM JMV alone (n=4, $p<0.01$)(Fig. 2A). Thus, the predominant inhibitory effect of CCK_A receptor activation on DNA synthesis occured via the low-affinity CCK receptor state.

Effects observed on thymidine incorporation do not necessarily reflect changes in cell proliferation. Therefore, we directly examined the effect of CCK analogues alone and in combination, on the proliferation of CHO cells (Fig. 2B). CCK_8 caused a large inhibition of CHO cell proliferation. In contrast, JMV-180 had no significant effect on CHO cell numbers.

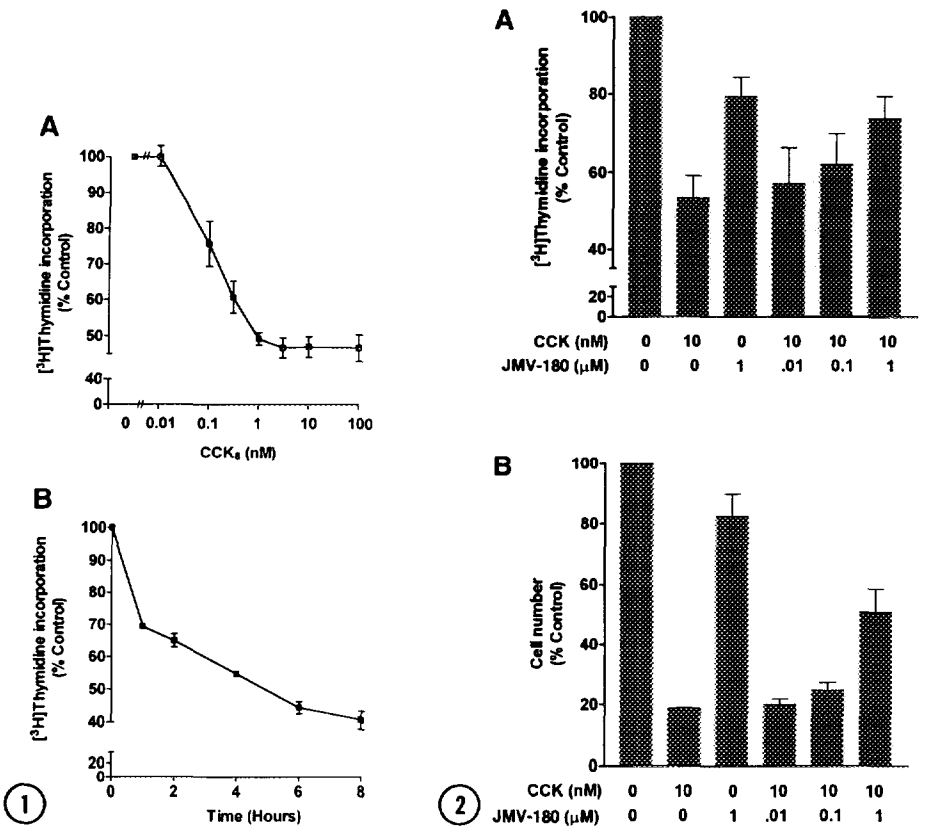


Fig. 1. Effects of CCK_8 on CHO cell DNA synthesis. **A.** Dose-dependence of CCK_8 induced inhibition of CHO cell DNA synthesis. Cells were grown in the presence of 5% FBS and were treated with the indicated concentrations of CCK_8 for 7 hours. Cells were then pulsed for 1 hour with [^{3}H]thymidine and the incorporation into DNA was analyzed. Results are expressed as the percentage of control non-treated cell DNA synthesis and each data point is the mean ± SE for 4 experiments. **B.** Time-course of CCK_8 induced inhibition of CHO cell DNA synthesis. Cells growing in the presence of 5% FBS were treated with 100 nM CCK_8 for the indicated times then pulsed for 1 hour with [^{3}H]thymidine and the incorporation into DNA was analyzed. Each data point is the mean ± SE for 3 experiments.

Fig. 2. Effects of JMV-180 alone and in combination with CCK_8 on CHO cell growth parameters. **A.** Effects on DNA synthesis. Cells were grown in the presence of 5% FBS and were treated with the indicated concentrations of JMV-180 or CCK_8 for 7 hours. Cells were then pulsed for 1 hour with [^{3}H]thymidine and the incorporation into DNA was analyzed. Results are expressed as the percentage of control non-treated cell DNA synthesis and each data point is the mean ± SE for 4 experiments. **B.** Effects on CHO cell proliferation. Cells were grown in the presence of 5% FBS and the presence of the indicated concentrations of JMV-180 and/or CCK_8. At the indicated times cells were taken up and counted using a Coulter counter. Results are expressed as the number of cells for a representative of 3 separate experiments conducted in triplicate.

Furthermore, JMV-180 reduced the ability of CCK_8 to inhibit cell proliferation. Collectively these results on the effects of JMV-180 on DNA synthesis and cell proliferation suggest that the major actions of CCK_8 on CHO cell proliferation are mediated by the low-affinity receptor.

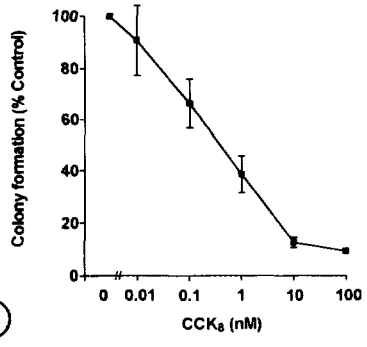
EFFECTS OF CCK ANALOGUES ON CHO CELL TRANSFORMATION

Previous studies suggested that several G protein linked receptors can inhibit the ability of CHO cells to form colonies in soft-agar, an effect referred to as "reverse transformation" (8,9). To determine whether activation of CCK_A receptors also caused reverse transformation we tested the effects of CCK_8 on the growth of cells in soft-agar. CCK_8 treatment inhibited the anchorage independent growth of CHO cells in a dose-dependent manner (Fig. 3). Significant effects of CCK_8 on colony formation were noted with 0.1 nM CCK_8 and maximal effects were noted at 10 nM CCK_8. Maximal concentrations of CCK_8 inhibited colony formation by 93 $\pm$ 1%, n=7.

JMV-180 substantially inhibited the formation of CHO cell colonies in soft-agar (Fig. 4). The effects of CCK_8 were somewhat greater than those of JMV-180, indicating the possible involvement of low-affinity state effects. However, JMV-180 did not significantly reduce the inhibitory effects of CCK_8 treatment on colony formation. Thus, the high affinity receptor state appears to play a predominant role in causing CHO cell reverse transformation.

DISCUSSION

It has previously been shown that activation of mACh receptors can induce growth inhibitory responses in A9 L cells (6), small lung cancer cells (7), and CHO cells (unpublished observation). However, the mechanisms responsible for these growth inhibitory responses are unknown. In the current study we have investigated the growth responses to activation of the CCK_A receptor. The signal transduction mechanisms activated by the CCK_A receptor are similar to those activated by mACh receptors and have been previously characterized in both pancreatic acinar



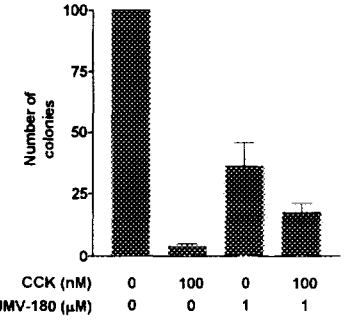


Fig. 3. Dose-dependence of CCK_8 induced inhibition of CHO cell colony formation in soft-agar. Cells were plated at 1000 cells per dish into 35-mm dishes in agar and treated with the indicated concentrations of CCK_8. After two weeks the dishes were examined and all colonies over 0.5 mm in diameter were counted. Results were expressed as a percentage of colonies formed in control dishes and represent the means $\pm$ SE for three separate experiments.

Fig. 4. Effects of CCK analogues on CHO cell colony formation in soft-agar. Cells were plated at 1000 cells per dish into 35-mm dishes in agar and treated with the indicated concentrations of JMV-180 and CCK_8. After two weeks the dishes were examined and all colonies over 0.5 mm in diameter were counted. Results were expressed as a percentage of colonies formed in control dishes and represent the means $\pm$ SE for three separate experiments.

(19) and CHO cells (18). We demonstrate for the first time that activation of CCK_A receptors also leads to inhibition of growth and transformation of CHO cells. Furthermore, in the current study JMV-180 was utilized to elucidate the potential second messengers involved in the inhibitory actions.

In CHO cells, similar to normal pancreatic acinar cells, the CCK_A receptor exists in at least two affinity states and JMV-180 is only able to activate the high affinity state. These affinity states activate specific intracellular second messenger systems. Thus, in CHO cells CCK_8 but not JMV-180 was able to stimulate increases in cAMP generation and arachidonate release (18). Also, in acinar (19) and CHO cells (18) CCK_8 generated large increases in phosphotidylinositol (PI) hydrolysis. In contrast, JMV-180 caused no or only a minor increase in PI hydrolysis in pancreatic acinar cells (15,20,21) and a small increase in CHO cells (18). Both CCK_8 and JMV-180 stimulated Ca^{2+} responses in acinar (15,16) and CHO (18) cells.

In the current study, we found that CCK_8 activation of CCK_A receptors led to a substantial inhibition of CHO cell proliferation as assessed by measurements of DNA synthesis or cell numbers. In contrast, JMV-180 had a minor effect on DNA synthesis and no significant effect on CHO cell proliferation. Thus, the second messenger pathways activated primarily by the low-affinity CCK receptor state are most likely the important pathways mediating the inhibition of proliferation. The second messenger pathways that have currently been identified as being exclusively activated by the low affinity receptor state include activation of adenylate cyclase and release of arachidonic acid. Of these, activation of adenylate cyclase is an attractive candidate for a growth inhibitory mechanism because CHO cells are growth inhibited by cAMP (22). However, it is unclear whether the concentrations of cAMP induced by CCK_A receptor activation are sufficient to account for growth inhibition. Further studies will be necessary to determine the role of cAMP in CCK_8 mediated growth inhibition.

Activation of CCK_A receptors also caused reverse transformation of CHO cells as indicated by the inhibition of colony formation in soft-agar. The ability of CCK_A receptors to mediate this effect on transformed cells has not been previously reported. Interestingly, JMV-180 had an extensive effect in this assay of anchorage-independent growth while it had no effect on anchorage-dependent growth. That JMV-180 substantially reduced colony formation indicated that the high-affinity CCK_A receptor state generated signals mediating this response. However, CCK_8 had a somewhat greater effect than JMV-180. This suggested that either additional second messengers were generated by the low-affinity state of the CCK_A receptor, or activation of the low-affinity state was capable of further increasing the level of a shared second messenger. Second messengers induced by both CCK_A receptor high- and low-affinity states include PI hydrolysis and increased intracellular Ca^{2+}. However, the extent of PI hydrolysis induced by JMV-180 does not exceed ten percent of that generated by CCK_8 (18). In contrast, JMV-180 induced increases in cytoplasmic Ca^{2+} concentrations approach those generated by CCK_8 (16,18). Thus, effects on Ca^{2+} concentrations more closely correlated with the effects on reverse transformation. Ca^{2+} influx has been suggested to be responsible for reverse transformation induced by activation of m3 mACh receptors in CHO cells (9). Therefore, effects on Ca^{2+} are likely responsible for the effects of CCK_A receptor activation on CHO cell reverse transformation. Further investigations will be needed to test this hypothesis.

CCK_A receptor activation stimulates the growth of pancreas *in vivo* (23-25) and in primary cultures of pancreatic acinar cells (26,27). Our current data show for the first time that CCK_A receptors are also capable of mediating growth inhibitory effects when activated in the context of the CHO cell. Thus, the growth effects of activation of CCK_A receptors depend upon the cellular context. Cellular context has previously been shown to determine the growth effects of activation of other receptors. Activation of m1, m3 and m5 mACh receptors (5) stimulate growth and transformation of NIH3T3 cells. In contrast, activation of m1 receptors inhibits the growth of A9 L cells (6) and activation of m3 receptors inhibits the growth of small lung cancer cells (7) and CHO cells (unpublished observation). These seemingly disparate observations emphasize the complexities of cellular growth regulation. Cellular growth regulation involves interactions between various second messenger systems and therefore, depends upon the presence, relative abundance, and isoform specific characteristics of a variety of growth regulatory molecules. Thus, increases in a specific second messenger, for example cAMP, may be inhibitory in some cells and stimulatory in others (28). Growth responses to receptor activation are even more complex because the specific second messengers generated, as well as the extent and duration of the signal produced by receptor activation, depend upon the properties of both the receptor and the cell. Therefore, the observation that receptors, such as those for CCK, can stimulate, inhibit, or have no effect on growth of various cells may be due to differences in signals transduced, in responses to the transduced signals, or to the rate of signal dissipation due to desensitization or stimulus removal. The specific factors that explain the opposite growth responses to CCK_A receptor activation in pancreatic acinar cells versus CHO cells are as yet unknown.

In conclusion, this study demonstrated that rat CCK_A receptors expressed in CHO cells inhibited proliferation predominantly by effects mediated by the low-affinity state, and caused a reversal of transformation predominantly by effects mediated by the high-affinity state. Because CCK_A receptor affinity states activate separate sets of second messengers, these observations should help direct further investigations into the cellular mechanism responsible for these growth effects.

ACKNOWLEDGMENTS

This work was supported by National Institue of Diabetes and Digestive and Kidney Disease Grants DK-35912 and the GI peptide center DK-34933.

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