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Inhibition of Demecolcin-Induced DNA Synthesis by Inhibitors of Phospholipase C and Protein Kinase C

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Exposing normal human breast epithelial (HBE) cells, which were growth arrested by a 3-day culture in EGF-deprived medium, to the microtubule disrupting agent, demecolcin (N-deacetyl-N-methyl-colchicine), for 2 hr significantly stimulated the initiation of DNA synthesis 22 hr later. The demecolcin-induced DNA synthesis was not accompanied by activation of the EGF receptor and it was inhibited by calphostin C, an inhibitor of protein kinase C (PKC), and U-73122, an inhibitor of phospholipase C (PLC). Contrary to this, the EGF-induced DNA synthesis was inhibited by tyrphostin A25, a specific inhibitor of the EGF receptor tyrosine kinase, and calphostin C. The results suggested that the involvement of PLC and PKC in the demecolcin-induced signal transduction pathway leads to the initiation of DNA synthesis.

A line of studies has suggested the involvement of the cytoskeleton as a modulator of cell-surface events and cellular responses to these events in activating cell growth. For example, colchicine and other agents that promote microtubule disassembly enhance EGF stimulated DNA synthesis in 3T3 cells (1, 2) and the insulin- or serum-stimulated entry of chick embryo cells into the S phase of the cell cycle (3). Furthermore, agents that disrupt microtubules themselves, release confluent chick embryo cells from density-dependent growth inhibition (4) and induce the initiation of DNA synthesis in serum-free cultures of nonproliferating chick embryo cells (5). The latter two studies suggest that the binding of growth factor to its receptor is not necessarily required for growth stimulation and that microtubule depolymerization is sufficient to initiate both DNA synthesis and the events leading to cell division. Nevertheless, the signal transduction pathways following the stimulation of cells with microtubule disrupting agents remain unknown, contrary to those in cells stimulated by growth factors.

In this study, we found that the microtubule disrupting agent demecolcin can stimulate HBE cells to initiate DNA synthesis and that this activity was inhibited by inhibitors of signal transduction-related enzymes that include PLC and PKC.

MATERIALS AND METHODS

Cell culture. Normal human breast epithelial (HBE) cells (Cronetics Inc.) were cultured in serum-free MCDB170 basal medium supplemented with 10 ng/ml EGF, 5 μg/ml insulin, 1.4 μM hydrocortisone, 0.1 mM phosphoethanolamine, 0.1 mM ethanolamine, and 25 μM prostaglandin E1 as described (6, 7). Subconfluent cultures were then incubated without EGF for 3 days (8).

Stimulation of DNA synthesis. Cells which were growth arrested by EGF-deprivation were then stimulated with demecolcin (N-deacetyl-N-methyl-colchicine) (Wako) for 2 hr (5), then the medium was replaced with demecolcin-free medium and the cells were cultured for an additional 22 hr in the presence of 1 μCi/ml [methyl-^{3}H]thymidine (ICN). To measure DNA synthesis, the amount of radioactivity incorporated into the 10% TCA-insoluble materials was determined using a liquid scintillation counter. In some experiments, the cells were incubated with enzyme inhibitors that include tyrphostin A25, U-73122 (Calbiochem), calphostin C, wortmannin, methyl 2,5-dihydroxycinnamate (2,5-Mec) (Kyowa Medex), together with 10 μM demecolcin for 2 hr or with EGF for 4 hr. In the latter experiments, cells were then incubated without inhibitors but with EGF and [^{3}H]thymidine for an additional 20 hr.

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Immunohistochemistry. Cells on glass slides (Lab-Tek chamber, Nunc) were stimulated with demecolcin or EGF, then fixed in 50% methanol and 50% acetone for 2 min and air dried. Microtubule assembly was examined under a fluorescent microscope (Olympus) after reacting the fixed cells with anti-tubulin antibody (Chemicon) and FITC-conjugated secondary antibody.

Autophosphorylation of EGF receptor. The protein-tyrosine kinase activity of EGF receptor was determined by measuring the autophosphorylation activity of the receptor (9, 10). Cells were lysed on dishes with RIPA buffer containing 1 mM sodium orthovanadate (11). The EGF receptor was immunoprecipitated with anti-EGF receptor antibody (Oncogene Science) and Protein A-Sepharose (Pharmacia), separated by 8% polyacrylamide-SDS gel electrophoresis, then transferred onto membranes (Millipore). The Western blots were probed with the anti-phosphotyrosine antibody PY20 (ICN) and anti-EGF receptor antibody (Transduction Labs.).

RESULTS AND DISCUSSION

Stimulation of DNA synthesis by demecolcin. When arrested HBE cells after culture in EGF-deprived medium for 3 days were incubated with 0.1 μM demecolcin for 2 hr, the thymidine incorporation was stimulated to 2.7-fold the level in cells not exposed to demecolcin 22 hr later (Fig. 1). The DNA synthesis stimulating activity of demecolcin was dose-dependent in a range from 1 to 50 μM and the activity between 5 and 50 μM was comparable with that of EGF at 10 ng/ml. While stimulation of HBE cells with EGF resulted in the synchronous elevation of DNA synthesis 20 hr later (8), demecolcin caused a gradual increase in the thymidine incorporation until 20 hr after stimulation (data not shown). The results showed that exposing the cells for 2 hr to demecolcin can itself stimulate HBE cells to initiate DNA synthesis like EGF and thus is a agreement with the results reported by others (4,5).

Effect of demecolcin on microtubule assembly. To examine the status of the microtubule assembly after exposure to demecolcin, cells were immunostained with antitubulin antibody. Figure 2a shows the fibrous assembly of microtubules in cells which were growth arrested in medium without EGF. Exposing cells to 10 μM demecolcin for 5 min resulted in a slight, but insignificant decrease in the fibrous structures of microtubules, especially in the peripheral region of the cells (Fig. 2b). Incubating the cells with demecolcin for 2 hr resulted in a complete loss of the fibrous architecture of microtubules and produced an even staining profile of tubulin (Fig. 2c). Contrary to

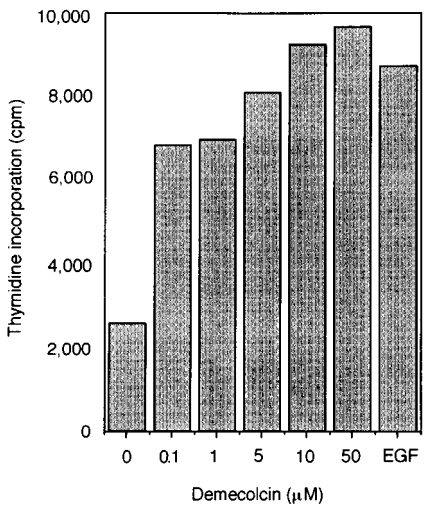

FIG. 1. Stimulation of HBE cells with demecolcin. Subconfluent cultures of cells were incubated in medium without EGF for 3 days. Arrested cells were then incubated with demecolcin at the indicated concentrations for 2 hr, followed by an incubation in demecolcin-free medium for an additional 22 hr with [^{3}H]thymidine. EGF at 10 ng/ml was present throughout the incubation for the total 24 hr. The amount of radioactivity incorporated into 10% TCA-insoluble materials was determined and the mean of duplicate cultures is given.

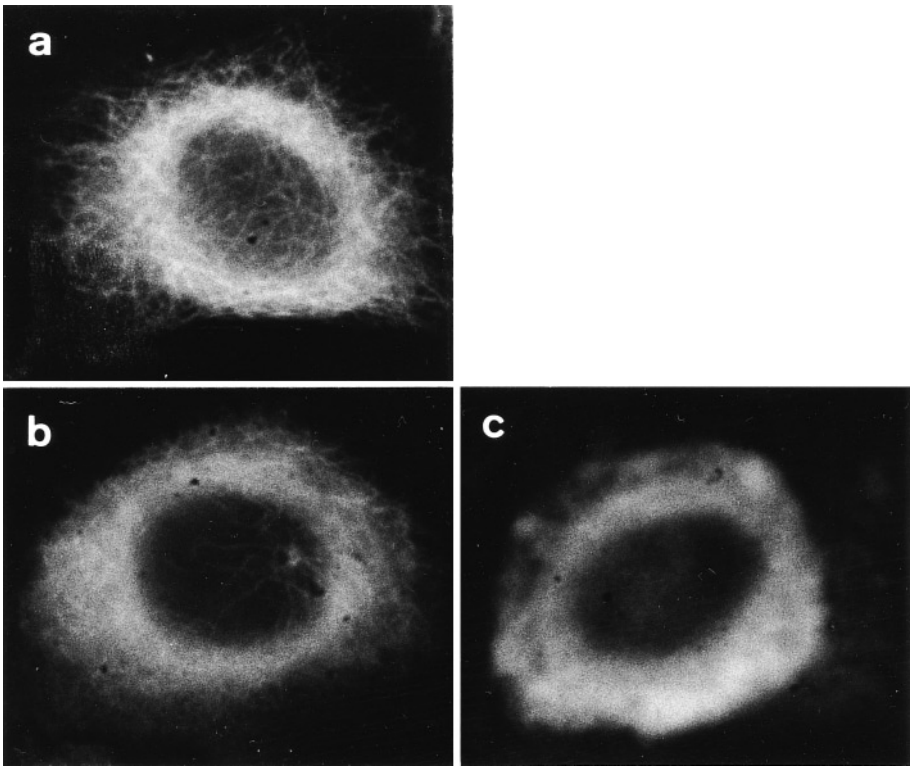

FIG. 2. Effect of demecolcin on microtubule assembly. HBE cells which were growth arrested (*a*) were incubated with 10 mM demecolcin at 37°C for 5 min (*b*) or 2 hr (*c*). The cells were then fixed and reacted with anti-tubulin antibody followed by FITC-conjugated secondary antibody.

demecolcin, treating the cells with 10 ng/ml of EGF caused no apparent alterations in the microtubule assembly 5 min or 2 hr later (data not shown).

Activation of the EGF receptor. The EGF receptor is a glycoprotein having an apparent molecular mass of 170 kDa, and it posesses intrinsic protein tyrosine kinase activity (12,13). The binding of EGF to the receptor induces the activation of the tyrosine kinase, leading to autophosphorylation (9,10). Since transmembrane surface receptors such as the EGF receptor are indirectly linked to microtubules (14), agents that modulate them are thought to affect the association of enzymatic subunits or aggregation of the receptors necessary for mitogenesis (3). To examine whether stimulation of cells with demecolcin is accompanied by the activation of EGF receptor without ligand binding, the tyrosine phosphorylation level of the receptor was monitored after adding demecolcin or EGF. EGF rapidly induced receptor autophosphorylation (5 min later), and the elevated level continued for 30 min (Fig. 3). Contrary to this, adding 10 μM demecolcin to the cells caused no increase in the phosphorylation level of EGF receptor up to 30 min later.

These results showed that the demecolcin-induced initiation of DNA synthesis was not accompanied by activation of the EGF receptor and suggested that the growth stimulating signal triggered by demecolcin was transduced through the EGF receptor-independent pathway.

Effects of enzyme inhibitors on demecolcin- or EGF-induced DNA synthesis. To understand the signal transduction pathway following the stimulation of cells with demecolcin, the effects of inhibitors of signal transduction-related enzymes on demecolcin-induced DNA synthesis were examined and compared to those on EGF-induced DNA synthesis. Tyrphostin A25, a specific inhibitor of the EGF receptor tyrosine kinase (15), did not significantly inhibit demecolcin-induced DNA synthesis, with the half maximal inhibitory concentration (IC50) of 1 mM (Table I). As the

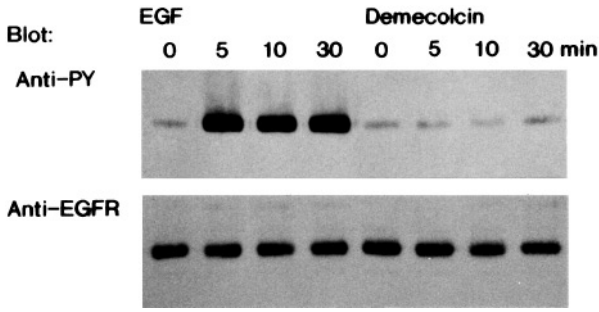

FIG. 3. Activation of the EGF receptor. Cells in growth arrest (0 min) were incubated with 10 ng/ml EGF or 10 μM demecolcin for the indicated periods (min). The EGF receptor immunoprecipitated with an anti-EGF receptor antibody was separated on 8% polyacrylamide-SDS gels, and reacted with an anti-phosphotyrosine antibody (anti-PY) or an anti-EGF receptor antibody (anti-EGFR).

IC50 of tyrphostin A25 for the EGF receptor tyrosine kinase is reported as 15 μM (15), the demecolcin-induced DNA synthesis appeared to be independent of the EGF receptor and confirms the result shown in Figure 3. Contrary to this, EGF-induced DNA synthesis was sensitive to tyrphostin A25 and an IC50 estimated was 28 μM (Table I). This IC50 value was comparable with that for the EGF receptor tyrosine kinase, suggesting the involvement of the EGF receptor activation in the EGF-stimulated signal transduction.

Wortmannin and calphostin C were the most potent inhibitors of the demecolcin-induced DNA synthesis tested. The IC50 values of the two inhibitors for the DNA synthesis were 14 and 32 nM, respectively (Table I). When compared with these, U-73122 less potently inhibited the demecolcin-induced stimulation of DNA synthesis, with an IC50 of 4.5 μM (Table I). However, the IC50 values of calphostin C and U-73122 for DNA synthesis were almost equivalent to those for PKC (50 nM) (16) and PLC (1–2 μM) (17), respectively. The IC50 of wortmannin for DNA synthesis (14 nM) was about 5-fold above that for P13K (3 nM) (18). Therefore the results suggested that the stimulation of DNA synthesis by demecolcin is most likely to be mediated through PLC and PKC. The possibility that P13K is involved in the demecolcin-induced signal transduction cannot be excluded by this study. Since activated PLC can activate PKC via production of diacylglycerol (19), it is likely that PLC is activated first in HBE cells after adding demecolcin. However, the presence of 2,5-Mec, a general tyrosine kinase inhibitor (20), was weakly inhibitory, having an IC50 of 10 μM (Table I). This IC50 was 13-fold above that for the EGF receptor tyrosine kinase (0.77 μM) (20), suggesting that the demecolcin signaling is not accompanied by protein-tyrosine phosphorylation. This assumption was confirmed by Western analysis showing that neither PLC nor P13K was tyrosine phosphorylated after stimulation of cells with demecolcin (data not shown).

TABLE 1
Inhibition of Democolcin- or EGF-Induced DNA Synthesis by Enzyme Inhibitors

Inhibitor	IC50 for DNA synthesis (μM)		IC50 for enzyme (μM)	(Ref.)
	Demecolcin	EGF		
Tyrphostin A25	1,000	28	15	EGFR (15)
Calphostin C	0.032	0.054	0.05	PKC (16)
U-73122	4.5	23	1-2	PLC (17)
Wortmannin	0.014	0.1<	0.003	P13K (18)
2,5-Mec	10	ND	0.77	EGFR (20)

ND, not determined; EGFR, EGF receptor tyrosine kinase; PKC, protein kinase C; PLC, phospholipase C; P13K, phosphatidylinositol 3-kinase; 2,5-Mec, methyl 2,5-dihydroxycinnamate.

Therefore, if the signaling from PLC to PKC works in HBE cells, how PLC is activated without activating the EGF receptor or other tyrosine kinases remains to be proven.

On the other hand, EGF-induced stimulation of DNA synthesis was effectively inhibited by tyrphostin A25 and calphostin C. The IC50 values of the two inhibitors for the EGF-induced DNA synthesis were 28 μM and 54 nM, respectively (Table I). These respective values were equivalent to those for the EGF receptor tyrosine kinase (15 μM) and PKC (50 nM). The results suggested the involvement of the EGF receptor and PKC in the EGF signaling in HBE cells. Involvement of PKC in the EGF-induced biological effects, such as membrane ruffling (21) and the EGF receptor mRNA accumulation (22), has been reported. An IC50 value of U-73122 was 23 μM (Table I), and 100 nM wortmannin exhibited no remarkable effect on the EGF-induced DNA synthesis (93% of the control). The IC50 values are more than 10- and 30-fold above those for PLC (1-2 μM) and P13K (3 nM), respectively, suggesting that neither PLC nor P13K is involved in the EGF-stimulated signal transduction. When compared the EGF signaling to the demecolcin signaling, PKC appeared to be involved commonly in the two signaling pathways. This suggested that PKC can be activated by dual pathways, an EGF receptor-dependent pathway and an EGF receptor-independent pathway.

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