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RELIEF FROM CONTACT INHIBITION

Early Increase in Phospholipid Turnover

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The viral transformation of normal into tumorigenic cells is a system under active investigation. The fact that transformation is accompanied by loss of contact-inhibition has focused attention on other situations in which confluent cells can be caused to resume movement and cell division. One such system is the serum-stimulated relief of contact-inhibition in cultured fibroblasts (1, 2, 3). Since an alteration of shape by "rounding-up" precedes movement (4), an investigation of biochemical changes known to accompany other alterations in the cell surface is pertinent. Phospholipid turnover, which appears to increase during such processes as phagocytosis (5, 6, 7, 8), secretion (9, 10) or the phytohaemagglutinin-mediated activation of lymphocytes (11, 12, 13), was accordingly examined. By using fibroblasts prelabeled with isotope, uncertainties of interpretation resulting from fluctuations in precursor pools (14, 15, 16) have been avoided.

METHODS

Chick skin fibroblasts were cultured as described by Baker and Humphreys (4). During the medium change after 24 hr, isotope (4 μCi of choline-methyl-^{3}H chloride or myo-inositol-2-^{3}H and 0.8 μCi of thymidine-methyl-^{14}C or L-valine-^{14}C(U) per plate) was added. When the cells had reached confluency the medium was removed, the plates were washed twice, and nonradioactive medium containing 0.5% serum was added. 24 hr later, the serum concentration in the medium of half the plates was raised to 8.5%. Approximately 24 hr later the cell density on such plates had doubled.

Cells were extracted with ice-cold 5% trichloroacetic acid after careful removal of >99% of the medium. The supernatant, after centrifugation of the extract, is taken to contain the acid-soluble constituents. The sediment was extracted with 1 ml $CHCl_3$:methanol (2:1) and centrifuged; >90% of incorporated choline or inositol was in the supernatant; the nonlipid sediment was dissolved in 1.5 ml of biuret reagent (17). Incorporation of valine or thymidine was determined from the sum of the $CHCl_3$:methanol and biuret extracts. Approximately 1% of added inositol-^{3}H, 2% of choline-^{3}H, 4% of valine-^{14}C, and 0.5% of thymidine-^{14}C was incorporated during a 4-day exposure to isotope.

RESULTS AND DISCUSSION

After the removal of isotope from the medium of confluent cells, the label in the acid-soluble inositol or choline pool declines sharply (Fig. 1), suggesting either leakage from the cell or incorporation into phospholipid. The second suggestion is the more likely since (*a*) the acid-soluble pool of valine does not decrease significantly (Fig. 1) and (*b*) labeling of phospholipid actually increases during the first 20 hr after removal of isotope (Fig. 2). Increase in labeled phospholipid does not match the decrease in the acid-soluble pool, since incorporation is accompanied by degradation, i.e. turnover.

Addition of serum results in a greater loss of

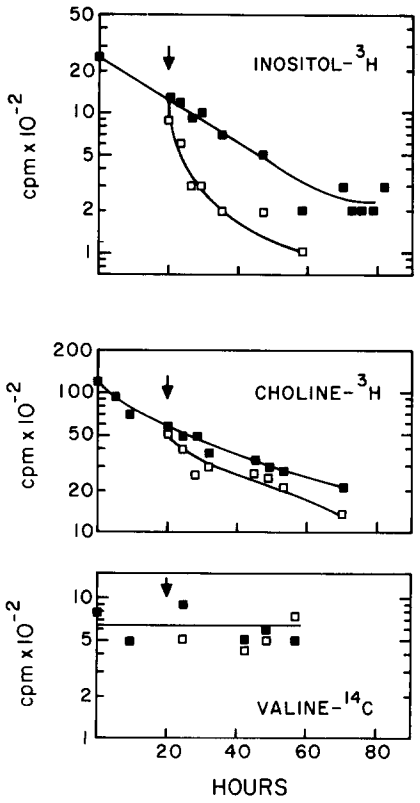

FIGURE 1 Radioactivity in acid-soluble fraction of fibroblasts, after removal of isotope from confluent cells at $t = 0$. ■——■, control; □——□, after serum addition at time indicated by the arrow. Total cpm per plate.

isotope from the acid-soluble pool, especially in the case of inositol (Fig. 1). Again, this is likely to reflect increased synthesis of phospholipid rather than leakage from the cell, since (*a*) labeled valine does not leak out, (*b*) there is a slight increase in labeled phospholipid (Fig. 2) and (*c*) there is indeed an increased incorporation into phospholipid (without significant change in acid-soluble pool) in cells exposed to isotope just before serum (Fig. 3). Nevertheless, addition of serum to confluent 3T3 cells does appear to release a small amount of acid-soluble uridine or phosphate (14).

Inositol- and choline-containing phospholipids of confluent fibroblasts turn over (in nonexponential manner) under conditions in which DNA is stable and protein shows some exponential turnover (Fig. 2). Phospholipids labeled with [1,2-^{14}C]choline behave like [Me-^{3}H]choline-labeled ones. Dividing fibroblasts turn over at the same rate as nondividing cells, as shown by Fig. 2 and by comparing turnover in fibroblasts growing on 60 mm and 100 mm plates; cells continue to divide on the large plates when those on the small plates have stopped, yet turnover is the same. The situation is therefore similar to that of neoplastic mast cells (18) and cannot be reconciled with the view (19) that turnover of membrane constituents occurs only in nondividing cells.

Stimulation of confluent fibroblasts by serum has little *apparent* effect on phospholipid turnover (Fig. 2). However, a slight but significant difference is to be noted, especially in the case of inositol. This is consistent with the view that turnover *is* stimulated by serum; since incorporation of precursor (labeled even after 20 hr in "cold" medium [Fig. 1]) is accompanied by degradation, the effect is minimal. If isotope is added to confluent cells just before serum, incorporation into phospholipids is stimulated rapidly (Fig. 3). This confirms the above interpretation of Figs. 1 and 2 and makes it likely that the serum-stimulated incorporation of phosphate into the phospholipids of 3T3 cells (14*a*) is also due to increased turnover.

It is interesting to note that valine incorporation into protein (20) increases in serum-stimulated fibroblasts (Fig. 2). The additional isotope that is incorporated comes not from the acid-soluble pool (Fig. 1) but from the medium (which contains some labeled valine that is "trapped" when the fibroblasts are washed but is released during the subsequent 20 hr as cells move [even at confluency] about the plate). Since serum is known to stimulate phagocytosis (5), it is possible that labeled valine is incorporated without equilibrating with the acid-soluble pool. The fact that choline and inositol (but not thymidine) are similarly "trapped" and subsequently released, yet do *not* accumulate in the phospholipids of serum-stimulated cells, is again compatible with the view that any increased synthesis (Fig. 3) is offset by degradation.

SUMMARY

Taken together with the observations on 3T3 cells (14*a*), the present experiments show that phospholipid turnover (of phosphatidyl inositol in particular) is stimulated when contact inhibition of confluent cells is relieved by serum. Since the biochemical and morphological (4) changes occur equally rapidly, the hypothesis[1] that phospholipid

[1] Pasternak, C. A. Unpublished observations.

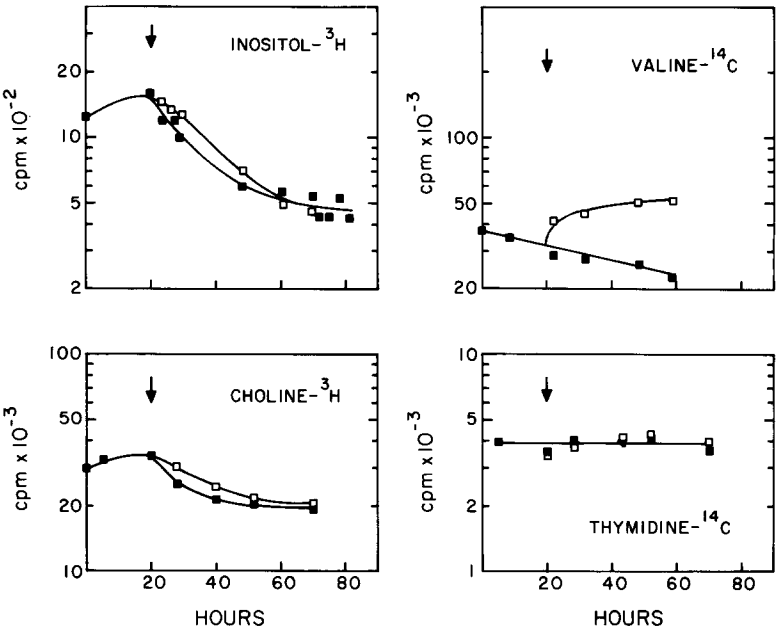

FIGURE 2 Radioactivity incorporated by fibroblasts, after removal of isotope from confluent cells at $t = 0$. ■——■, control; □——□, after serum addition at time indicated by the arrow. Total cpm per plate.

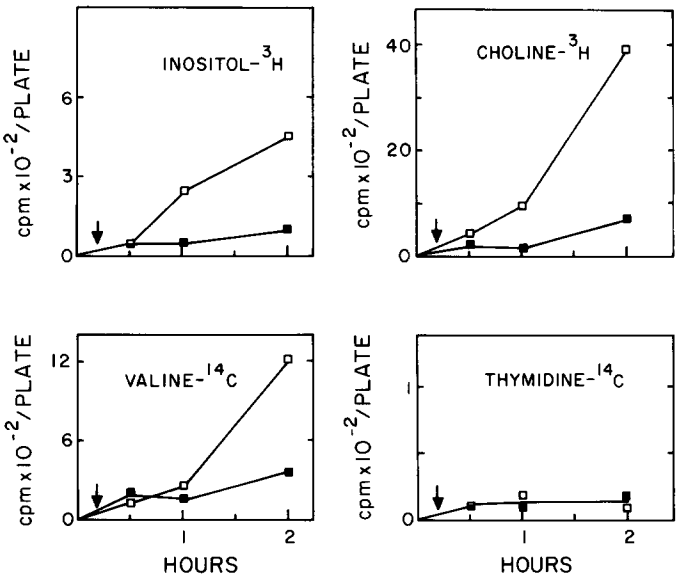

FIGURE 3 Radioactivity incorporated by fibroblasts, after addition of isotope (10 μCi of choline-^{3}H or inositol-^{3}H and 1 μCi of valine-^{14}C or thymidine-^{14}C per plate) to confluent cells at $t = 0$. ■——■, control; □——□, after serum addition at time indicated by the arrow.

turnover is associated with a *movement of membranes* receives support.

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