Direct Cytochemical Localization of Catalytic Subunits Dissociated from cAMP-dependent Protein Kinase in Reuber H-35 Hepatoma Cells. II. Temporal and Spatial Kinetics

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ABSTRACT The activation of cyclic AMP-dependent protein kinase has been found to be the predominant mode by which cyclic AMP (cAMP) leads to alterations of a large variety of cellular functions. The activation of the kinase results in the release of the catalytic subunit which as the free enzyme possesses phosphotransferase activity for a variety of specific protein substrates. Using a sensitive and specific cytofluorometric technique we monitored the appearance of free catalytic subunit in Reuber H35 hepatoma cells in culture after incubation with N^6-1′-O-dibutyryl-cyclic AMP (DBcAMP), 8-bromoadenosine-3′:5′-cyclic monophosphate (8-BrcAMP), and glucagon. The cytochemical method employs the heat-stable inhibitor of the free catalytic subunit which has been conjugated to fluorescein isothiocyanate (F:PKI) and was validated as described in the companion paper (Fletcher and Byus. 1982. *J. Cell Biol.* 93:719–726). Here we studied the temporal and spatial kinetics of the free catalytic subunit following activation of cAMP-dependent protein kinase by increasing concentrations of DBcAMP,8-BrcAMP, and glucagon. Under similar conditions protein kinase activation was also assessed biochemically in H35 cell supernatants by assaying the protein kinase activity ratio. Incubation of the hepatoma cells with DBcAMP (0.1 mM) led to an increase in the activity ratio from 0.2 in control cultures to a value of nearly 1.0 within a 1- to 2-h period. During this same period using the F:PKI probe, a significant increase in cytoplasmic and nucleolar fluorescence indicative of the release of the free catalytic subunit was coincidentally observed. In contrast to the rapid appearance of catalytic subunit in the cytoplasm and nucleolus of the cell within 5–15 min of the addition of DBcAMP, discernible nucleoplasmic fluorescence did not occur until after 1 h.

H35 cell cultures incubated with 8-BrcAMP (0.01–1.0 mM) exhibited a more rapid activation of the protein kinase measured cytochemically compared to the cells treated with DBcAMP. Cultures incubated with 8-BrcAMP had significantly increased cytoplasmic and nucleolar fluorescence compared to unstimulated cells within 1 min of the addition of the analogue and reached a maximal level within 15 min. By employing a microspectrophotometer a distinct dose-dependent increase in cellular fluorescence (i.e., free catalytic subunit) was observed as the concentration of 8-BrcAMP was increased from 0.01 to 1.0 mM at 1, 5, 15, and 60 min following stimulation. The addition of glucagon (10^{-6} M) to the culture also led to the activation of cAMP-dependent protein kinase as determined by an increase in the activity ratio. This increase was paralleled throughout the incubation period by a marked elevation in cytoplasmic and nucleolar fluorescence. The results reported herein suggest that both cyclic nucleotide analogues and a polypeptide hormone lead to the activation of cAMP-dependent

protein kinase in similar intracellular compartments in Reuber H35 hepatoma cells. The appearance of protein kinase catalytic subunit in the nucleolus also serves to support earlier reports which suggest a biological role for this enzyme in the nucleus.

Many if not all of the effects of adenosine:3′:5′ cyclic monophosphate (cAMP) upon the cell are thought to be mediated by the activation of cAMP-dependent protein kinase (1, 2). The holoenzyme of this protein kinase is composed of regulatory (R) and catalytic (C) subunits which reversibly dissociate in the presence of cAMP in the following manner: R_2C_2 + 4cAMP $R_2 \cdot cAMP_4$ + 2C. The free catalytic subunit (C) is the active form of the enzyme and possesses a phosphotransferase activity for many specific substrates which upon phosphorylation have altered functions. Essentially, it is the degree of dissociation of the protein kinase, and the availability of the free catalytic subunit relative to its protein substrates, that proscribes the manner in which cAMP and many hormones alter cellular functions. For this reason the measurement of the state of activation of cAMP-dependent protein kinase (i.e., the determination of the protein kinase activity ratio [3]) has in many instances proven to be a more accurate and sensitive measurement of the involvement of cAMP in a particular cell process than has assessment of intracellular concentration of the nucleotide. The accurate determination of the protein kinase activity ratio has proven to be difficult, however, due to the rapid equilibrium which exists between the holoenzyme and the free catalytic and regulatory subunits in cell supernatants (4).

As stated previously, the specific localization of the free catalytic subunit relative to its protein substrate inside the cell is of great importance in understanding the regulation by cAMP of a variety of specific cell processes. Intracellular compartmentalization of cAMP and protein kinase has been suggested for many years to play a major role in the specificity of a cAMP response. The localized accumulation of cAMP or catalytic subunit in the nucleus, mitochondria, endoplasmic reticulum, or membranes has been postulated to mediate the specific actions of cAMP in gene regulation (5), growth regulation (6), steroid biosynthesis (7), protein synthesis (8), and membrane permeability (9).

We have attempted to determined the temporal and spatial kinetics of the free catalytic subunit upon activation of cAMP-dependent protein kinase. We have employed the fluorescence cytochemical procedure described previously (10), which makes use of the fluorescein isothiocyanate-conjugated protein kinase inhibitor (F:PKI) specific for the catalytic subunit. The activation of protein kinase has been studied in Reuber H35 cells, a minimal deviation hepatoma cell line, incubated with a variety of concentrations of cyclic nucleotide analogues and glucagon. For the first time the intracellular localization of the free catalytic subunit in discrete cellular compartments has been measured concomitant with the cAMP-dependent protein kinase activity ratio measured biochemically.

MATERIALS AND METHODS

Cell Culture Procedures

A cloned cell line (#7) derived from Reuber H35 hepatoma was kindly provided by Dr. Wesley D. Wicks (University of Tennessee). Cells were grown in Dulbecco's modified Eagle's medium (DME) with 5% calf serum and 5% fetal calf serum (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) and were routinely subcultured once a week by trypsinization (0.05% trypsin and 0.02% EDTA). Cells not exceeding twenty passages were used in all experiments.

Assay for cAMP-dependent Protein Kinase Activity

The cAMP-dependent protein kinase activity was assayed according to a modification of the procedure of Cherrington et al. (11). Approximately 3×10^6 H35 cells were scraped from the culture dishes in cold Hanks' balanced salt solution (HBSS) and pelleted at 100 *g* for 30 s. The pellet was sonicated for 2–3 s in 100–200 μl of ice-cold 0.01 M sodium phosphate buffer, pH 6.9, containing 5.0 mM EDTA, 0.5 mM isobutyl-methylxanthine (MIX), 75 mM NaCl, and centrifuged at 20,000 *g* for 2 min. The supernatant solution was removed and immediately used as the source of enzyme.

The cAMP-dependent protein kinase activity was determined by measuring the activity in the presence and absence of exogenous cAMP. The assay mixture contained 100 μg of mixed calf thymus histone, 0.01 M phosphate buffer, pH 6.8, 0.025 M $Mg(Ac)_2$, 20 mM NaF, r-[^{32}P]-ATP and sufficient unlabeled ATP for a final ATP concentration of 0.15 mM, 0.025 ml of a 1:1 dilution of the original enzyme extract, and ± cAMP to a final concentration of 5 μM. The reaction was allowed to proceed for 5 min at 30°C and was terminated by pipetting 0.05 ml of the reaction medium onto Whatman 3 MM filters (2.3 cm) (Whatman Inc., Paper Div., Clifton, NJ) that were immediately placed into 10% cold trichloroacetic acid and then rinsed two times for 20 min in 5% trichloroacetic acid, two times in 95% ethanol, dried, and counted in an Omnifluor/toluene liquid scintillation cocktail.

The protein kinase activity ratio is expressed as the ratio of kinase activity measured in the absence of exogenous cAMP (-cAMP) relative to the activity measured in the presence of additional cAMP (+cAMP). Changes in the activity ratio occurred without changes in the total protein kinase activity, and the addition of activated charcoal at a concentration of 5 mg/ml in the homogenizing buffer did not alter the control or stimulated activity ratio. In addition to the assays being performed plus and minus cAMP, the protein kinase activity for each sample was determined with and without saturating amounts of protein kinase inhibitor (i.e., -I, -cAMP; +I, -cAMP; -I, +cAMP; and +I, +cAMP). The protein kinase activity ratio was based upon only kinase activity which was blocked by the addition of the inhibitor, i.e., due to the catalytic subunit of cAMP-dependent protein kinase. In this regard ~25–30% of the protein kinase activity in the H35 supernatants assayed in the absence of cAMP was not inhibited by a large excess of kinase inhibitor and thus not due to the catalytic subunit of cAMP-dependent protein kinase.

Fluorescent Localization of Protein Kinase

Reuber H35 cells were cultured on #1 glass cover slips (12 × 12 mm) in plastic culture dishes as described above. The cultures were incubated with DBcAMP, 8-BrcAMP, or glucagon, or appropriate diluent for the times indicated and prepared for cytochemistry, as described in our companion article (preceding this one) (10). Briefly, cultures were rinsed for 3 min in 4°C phosphate-buffered saline (PBS), pH 7.4, then fixed for 15 min in anhydrous acetone at −30°C. Cover slips were then rinsed in PBS and covered with 100 μl of FITC-conjugated affinity-purified protein kinase inhibitor (F:PKI; 1.25 μg/ml). After 48-h incubation at 4°C with the F:PKI, preparations were washed twice in cold PBS, then mounted on glass slides with glycerol:PBS or dehydrated with −30°C acetone and embedded in entellan (EM Laboratories Inc., Elmsford, NY).

Pictures were taken with a Leitz-Vario-orthomat with a select field integrating photometer using a 75W HBO UV source and a specific excitation/barrier (Leitz K2) Kodak Tri-X film was exposed at ASA 800 developed with D-76 (Kodak) for 12 min. The exposure time was based on the average of 10 exposures of the most fluorescent preparation in each protocol. For >20 protocols, 15-s exposures proved appropriate and was thus used to obtain all of the images presented here.

A Leitz MPV compact microspectrophotometer was used to quantify fluorescence intensity. The size of the rectangular probe was calibrated with an eyepiece reticle. For all of the readings done in this article the probe size was set at 5 × 5 μM. The MPV was coupled to a HP97 SI-0 computer which was programmed to control the shutter sequencer to obtain standardized readings of 250 ms each. The computer also recorded all data. For each data point, no fewer than 50 readings were obtained. Fluorescence quantification was done by one of us or by students that were not told the nature of the preparations. Random readings were obtained by following an *x-y* grid pattern using the microscope stage drive. Whenever the probe aperture overlapped the nucleus and cytoplasm of a cell the shutter sequencer was activated. All data were analyzed by Duncan's one-way analysis of variance.

RESULTS

Determination of the protein kinase activity ratio in hepatocytes has been used by numerous investigators (11–15) to measure the state of activation of cAMP-dependent protein kinase after stimulation of the cells by cyclic nucleotide analogues and hormones (see Materials and Methods). When Reuber H35 hepatoma cells at ~50% confluence were incubated with 0.1 mM DBcAMP, cAMP-dependent protein kinase was activated maximally within 1 h (Fig. 1). The addition of DBcAMP to the H35 cell cultures resulted in an increase in the activity ratio from 0.2 in control cultures to a value of nearly 1.0, indicating total activation of the cAMP-dependent protein kinase within 1 h. The protein kinase remained activated for up to 3 h, whereupon the activity ratio began to return to control levels by 4 h. This decrease in the activity ratio between 3 and 4 h is indicative of reassociation of the regulatory and catalytic subunits due to cellular metabolism of the cyclic nucleotide analogue and a subsequent fall in intracellular cAMP levels. In parallel preparations the localization of the catalytic subunit was observed employing the direct cytochemical procedure described in the accompanying paper (10).

Cells of cultures that were treated for 2–4 h with PBS or with 0.10 mM DBcAMP in PBS but were not stained with the F:PKI probe had negligible fluorescence. Therefore, neither the fixation method nor the nucleotide analog engendered a nonspecific fluorescence (10). When unstimulated cultures were stained with F:PKI, <5% of the cells exhibited a slight fluorescence which was restricted to cytoplasm (Fig. 1). This corresponds to the basal state of activation of cAMP-dependent

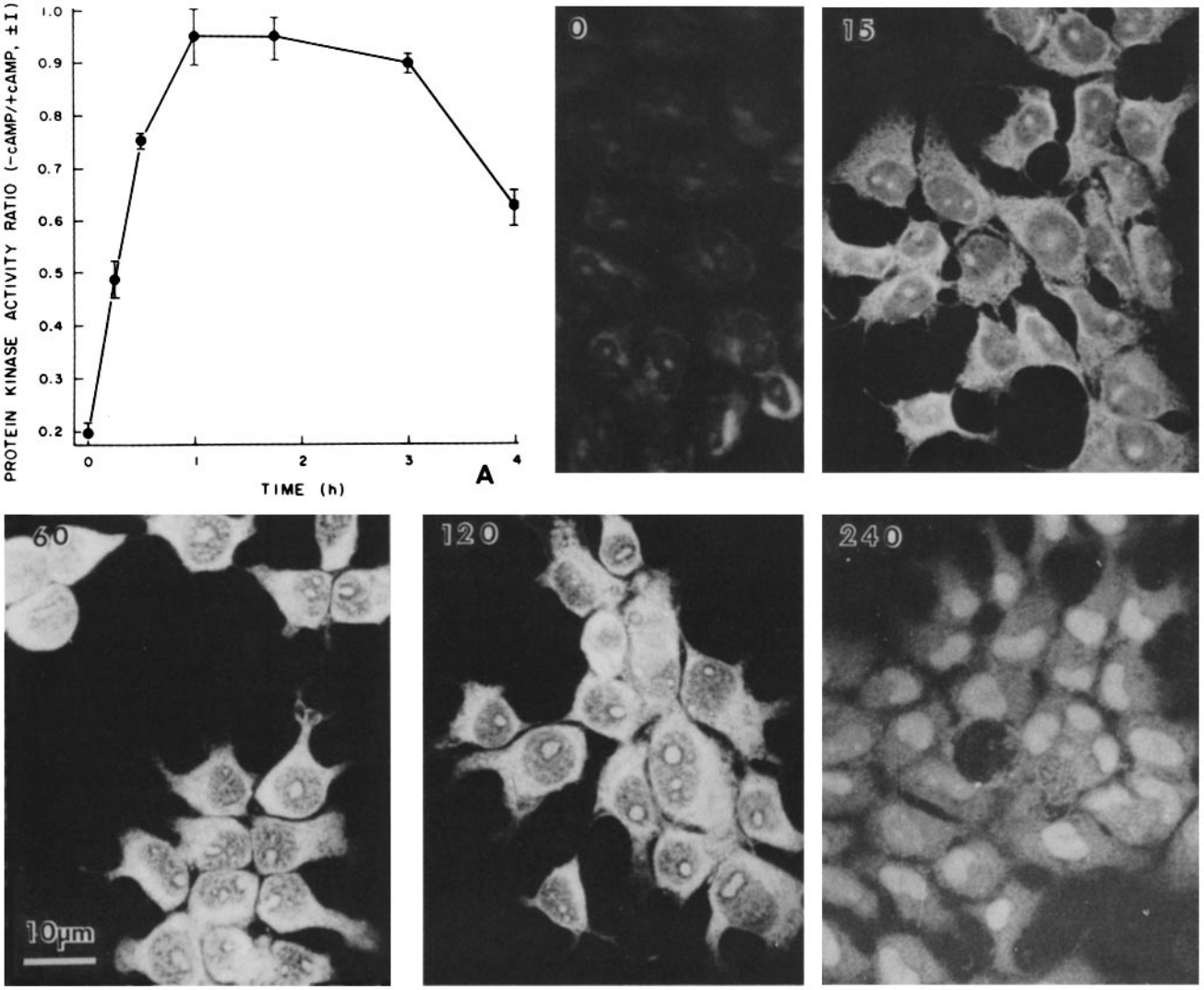

FIGURE 1 Activation of cAMP-dependent protein kinase and localization of free catalytic subunit in Reuber H35 hepatoma cells incubated with DBcAMP. (A) The effect of DBcAMP upon the activation of cAMP-dependent protein kinase in Reuber H35 hepatoma cells. At time-zero, DBcAMP dissolved in Hanks' balanced salt solution was added to the cultures to a final concentration of 0.1 mM. The cells were scraped from the culture dishes at the times indicated and the protein kinase activity ratio was determined in cellular supernatants as described in Materials and Methods. The data are represented as the mean ± SE of the activity ratio determined from five replicate culture dishes at each time following the addition of DBcAMP. Reuber H35 hepatoma cells were cultured on glass cover slips to ~50% confluence and DBcAMP was added in Hanks' balanced salt solution to a final concentration of 0.10 mM for up to 240 min. For photography the exposure time (15 s) was based on preparations incubated for 240 min with DBcAMP. The cultures were fixed in acetone and incubated with 100 μl of F:PKI(1.25 μg/ml) as described in Materials and Methods. The unstimulated cell culture (0) was not incubated with DBcAMP whereas the other preparations were treated with DBcAMP (0.10 mM) for periods of 15, 60, 120, and 240 min.

protein kinase shown which is indicative of the presence of some free catalytic subunit even in unstimulated cells. In addition, a modest nucleolar fluorescence was occasionally observed in these unstimulated, stained preparations.

Within 15 min of the addition of DBcAMP most of the cells exhibited a cytoplasmic fluorescence which was greater than that of the F:PKI control cultures (Fig. 1). Although the intensity of cytoplasmic fluorescence was above that of the control or zero-time group there was a definite variation among cells of any given culture and often between cells of a single cluster. (This heterogeneity of response in protein kinase activation to DBcAMP is not entirely apparent in Fig. 1, due to the small number of cells shown in the image.) Nucleoli also bound the F:PKI probe and, on a single cell basis, their fluorescence was equivalent to that of cytoplasm after 15 min in the presence of DBcAMP. The nucleoplasm, however, was only slightly fluorescent even in those cells in which the cytoplasm and nucleoli were heavily stained.

With exposure to DBcAMP for 60 min, appreciable amounts of cAMP-dependent protein kinase were activated in nearly all of the cells (Fig. 1). The variation in fluorescence intensity among cells was much reduced from the earlier time periods but was still a feature. Cytoplasmic and nucleolar fluorescence remained equivalent but both compartments bound more F:PKI than cells of the 15-min treatment group. Some cells seemed to release slightly from the substrate and round up. In those cases it was difficult to determine which cell compartments were labeled with F:PKI, due to the covering of cytoplasm overlying the nucleus.

2 h of exposure to DBcAMP induced cAMP-dependent protein kinase dissociation in all of the cells, resulting in cellular fluorescence which was somewhat greater than the maximum observed after 1 h of stimulation (Fig. 1). While cytoplasmic and nucleolar staining were intense after the 2-h treatment with DBcAMP, nucleoplasmic binding of F:PKI appeared much greater than at any earlier time.

At the longest time of DBcAMP exposure, 4 h, nearly all cells exhibited some degree of cAMP-dependent protein kinase activation. Cytoplasmic fluorescence was markedly reduced from that of the 1- or 2-h groups but, as seen in Fig. 1 (240 min) nuclear fluorescence was most striking. Some nuclear content of free catalytic units had been observed at earlier times but it was generally minor relative to that of cytoplasm or nucleoli. At 4 h the reverse was the case. Nucleoli also avidly bound the F:PKI probe but this was more difficult to discern due to the intensity of the total nuclear fluorescence. Sodium butyrate alone (1.0 mM) resulted in no increase in intracellular fluorescence during this 4-h period of incubation.

The intracellular localization of the free catalytic subunit was also determined after incubation of the Reuber H35 cells with another cyclic nucleotide analogue, 8-BrcAMP (Fig. 2). Cultures treated with 0.1 mM 8-BrcAMP exhibited a significant increase in cytoplasmic and nucleolar fluorescence compared to the unstimulated cultures (zero-time) within 1 min of the addition of the analogue (Fig. 2). A marked time-dependent increase in cytoplasmic and nucleolar fluorescence was observed which reached a maximal level 15 min after treatment with 8-BrcAMP. The protein kinase activity ratio determined in parallel preparations also increased to a peak value of 0.85 within 15 min of 0.1 mM 8-BrcAMP addition (data not shown).

Total cellular fluorescence was measured in Reuber H35 cell cultures treated with 0.01, 0.10, and 1.0 mM 8-BrcAMP employing the microspectrofluorometer as described in Materials and Methods. With this procedure both a time- and dose-dependent increase in total cellular fluorescence can be observed in the F:PKI-stained H35 cells (Fig. 2). A distinct elevation in fluorescence can be detected within 1 min of the addition of either of the three concentrations of 8-BrcAMP. As the concentration of 8-BrcAMP was increased from 0.01 to 1.0 mM, a dose-dependent difference in the fluorescence of the F:PKI-treated hepatoma cells could also be observed at 1, 5, 15, and 60 min (Fig. 2). In these preparations, cytoplasmic and nucleolar fluorescence increased coincidentally as the concentration of 8-BrcAMP became greater.

In addition to the cyclic nucleotide analogues DBcAMP and 8-BRcAMP, the localization of protein kinase was studied further in H35 cells incubated with glucagon (Fig. 3). The addition of glucagon (10^{-6} M) to the culture medium resulted in the activation of cAMP-dependent protein kinase as determined by an increase in the protein kinase activity ratio (Fig. 3). The activity ratio reached its maximal value within 10 min and remained at this level for >60 min.

The intracellular location of the free catalytic subunit dissociated after glucagon stimulation was also determined in these cells by the F:PKI-procedure (Fig. 3). Glucagon treatment led to a marked increase in both cytoplasmic and nucleolar fluorescence compared to the unstimulated cells within 1 min of the addition of the hormone. Cytoplasmic and nucleolar fluorescence continued to increase in the H35 cells to its peak value after <10 min of incubation with glucagon. Nucleoplasmic fluorescence could also be observed during this period. In addition, a distinct dose-dependent appearance of catalytic subunit after glucagon treatment indicative of protein kinase activation was observed and quantified with the F:PKI procedure. At the lower doses of glucagon a similar subcellular distribution of catalytic subunit in the cytoplasm and nucleolus was observed as in cells stimulated with the higher concentration of hormone (data not shown).

DISCUSSION

We have been able to measure the time- and dose-dependent alterations in the localization of the free catalytic subunit of cAMP-dependent protein kinase in Reuber H35 cells stimulated with DBcAMP, 8-BrcAMP, or glucagon. The use of FITC-conjugated inhibitor for this study appears to be uniquely suited for measuring the activation of the kinase in that the inhibitor does not bind to the holoenzyme as do all existing antibodies to the catalytic subunit (24, 25). The data presented here show that there is a close correlation between the biochemical and cytochemical procedures used to monitor protein kinase activation. Changes in the protein kinase activity ratio observed following incubation of the cells with DBcAMP and glucagon (Figs. 1 and 3) are quite similar to the time-dependent alterations in total cellular fluorescence seen with the F:PKI-procedure (Figs. 1 and 3). The methods employed for the determination of the activity ratio and for the cytochemistry are not, however, without limitations (see reference 4 for discussion of activity ratio and our accompanying article [10] for F:PKI procedures); yet the high degree of correlation between them further add to the validity of employing the fluorescinated inhibitor for the study of the activation and intracellular compartmentalization of the catalytic subunit. More importantly, however, it is now possible to determine in which discrete cell or compartment of the cell the cAMP-dependent protein kinase becomes activated. Measurement of the protein kinase activity ratio only provides information

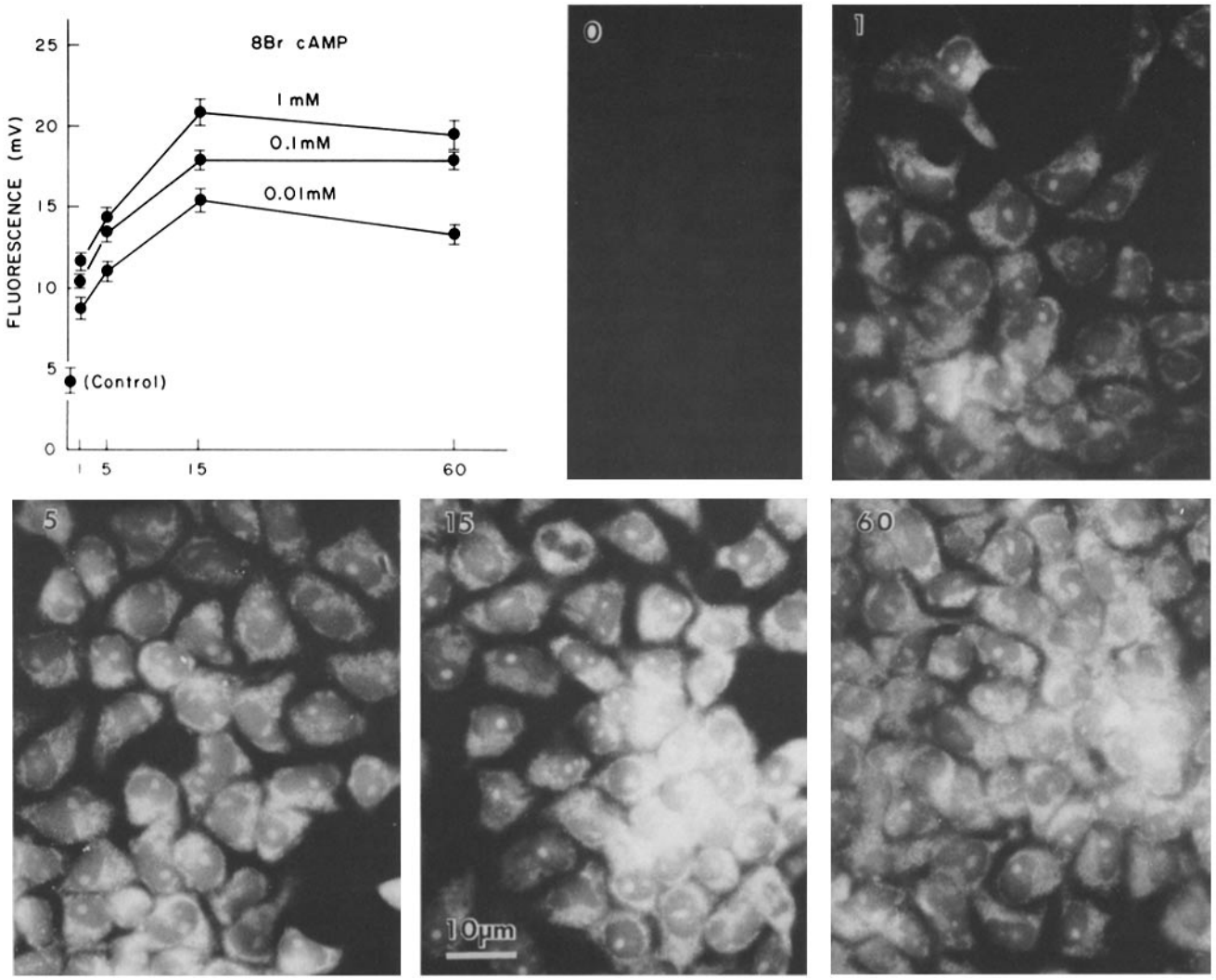

FIGURE 2 Fluorescent localization of the free catalytic subunit in Reuber H35 hepatoma cells incubated with 8-BrcAMP. Reuber H35 cells were cultured on glass cover slips to ~50% confluence and 8-BrcAMP was added to a final concentration of 0.10 mM for up to 60 min. At the times indicated, the cover slips were removed and prepared for cytochemistry as described in Materials and Methods and in reference 10. Photographs were obtained using the 60-min preparation for the standard exposure time (15 s). The unstimulated cell culture (0) was not incubated with 8-BrcAMP whereas the remaining cultures were treated with 8-BrcAMP for periods of 1, 5, 15, and 60 min. (All cultures were stained with 100 μl of the F:PKI (1.25 μg/ml). In addition to 0.10 mM 8-BrcAMP, replicate cultures were incubated with 0.01 or 1.0 mM 8-BrcAMP for 1, 5, 15, and 60 min. These cells were also fixed and were incubated with F:PKI as described in Materials and Methods. The fluorescence intensity (*mv*) of cells was measured using the microspectrophotometer as described in Materials and Methods. The data are presented as the mean ± SE of the determination of total cellular fluorescence from 50 cells in each culture (done in duplicate or triplicate). The "control" in this instance refers to unstimulated cultures (time-zero), fixed, and treated with F:PKI.

concerning the average degree of kinase activation in a population of cells independent of either any variation between individual cells or intracellular compartmentalization.

In terms of the specific localization of the catalytic subunit after treatment of the hepatoma cells with either DBcAMP, 8-BrcAMP, or glucagon, in all cases there appears to be concurrent activation of the kinase in the cytoplasm and in the nucleolus. In the case of glucagon this appearance of cytoplasmic and nucleolar fluorescence was quite rapid and could be observed within 1 min (Fig. 3). The fluorescence data suggest a moderate heterogeneity of response of the cells to the low dose of DBcAMP which is not apparent when the state of kinase activation is measured biochemically (Fig. 1). This is due perhaps to intracellular differences in the level of esterases which must remove the butyrate groups from DBcAMP before the cyclic nucleotide analogue can bind to the regulatory subunit of the holoenzyme. In this regard, 8-BrcAMP (which can activate the kinase directly) (2) and glucagon which raises endogenous cAMP content results in the same general pattern of intracellular fluorescence discussed above, but in a more rapid temporal sequence and with a somewhat greater homogeneity of response between cells. A marked difference between 8-BrcAMP and glucagon in the subcellular distribution of the free catalytic subunit was not routinely observed in the H35 cells. However, in a number of primary cultured cells more responsive to polypeptide hormones, hormone treatment caused a more rapid and intense accumulation of nucleoplasmic catalytic subunit than did cyclic nucleotide analogues (34). These observations support the suggestion that hormones which activate adenylate cyclase may be capable of leading to

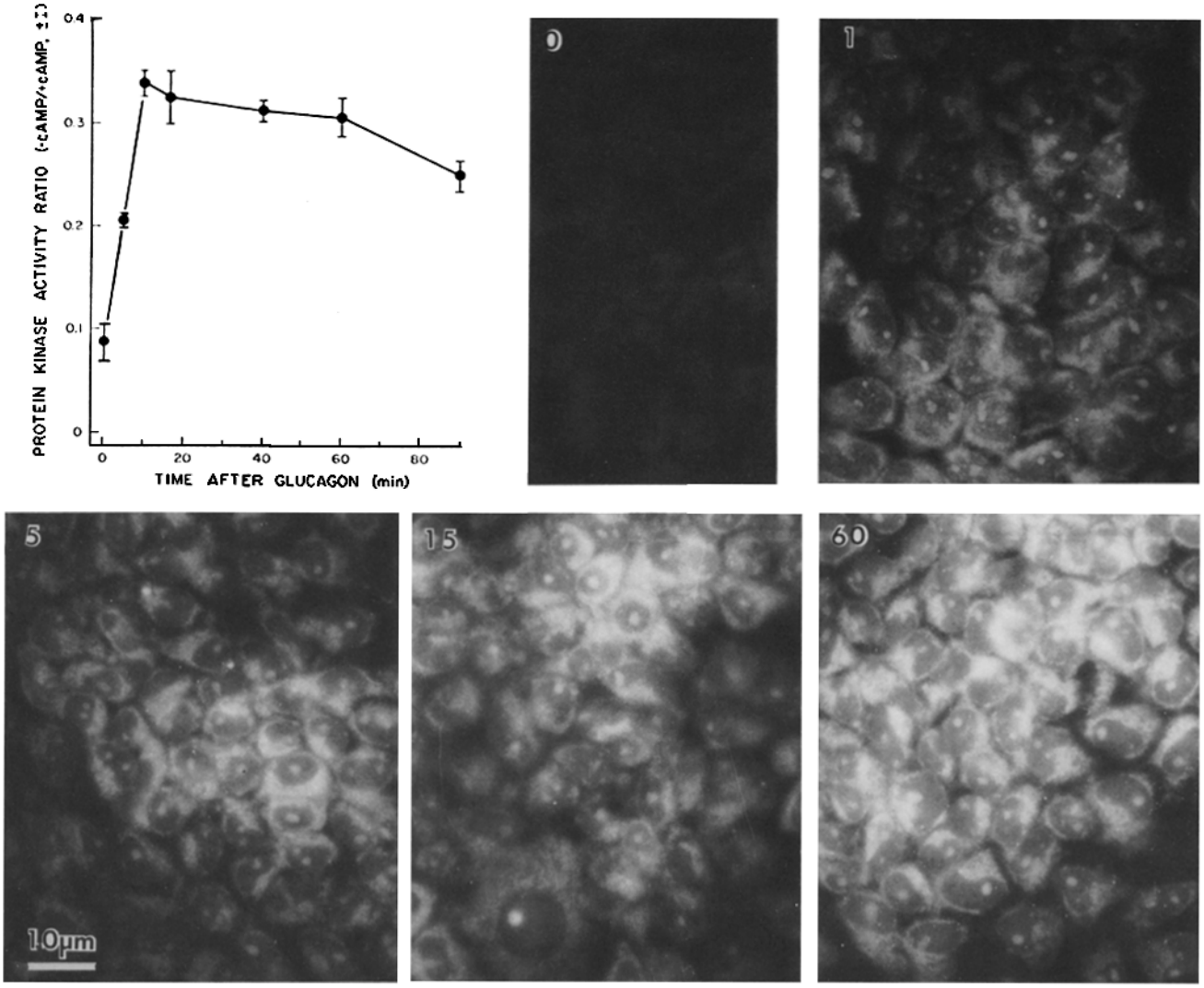

FIGURE 3 Fluorescent localization of free catalytic subunits in Reuber H35 hepatoma cells incubated with glucagon. Reuber H35 hepatoma cells were cultured on glass cover slips to ~50% confluence and glucagon was added in PBS to a final concentration of 10^{-8} M. At the times indicated, the cover slips were removed and prepared for cytochemistry as described in Materials and Methods. Photographs were obtained using the 60-min cells for the standard exposure time as described previously. The unstimulated culture (0) was not incubated with glucagon whereas the remaining cultures were treated with glucagon (10^{-8} M) for periods of 1, 5, 15, and 60 min. Graphically shown is the effect of glucagon upon the activation of cAMP-dependent protein kinase in Reuber H35 hepatoma cells. Glucagon dissolved in PBS was added to a final concentration of 10^{-8} M. The cells were scraped from the culture dishes at the time indicated and the protein kinase activity ratio was determined in cellular supernatants as described in Materials and Methods. The data are represented as the mean ± SE of the activity ratio determined from five replicate culture dishes at each time following the addition of glucagon.

the appearance of free catalytic unit in different compartments of the cell than do cyclic nucleotide analogues that activate protein kinase directly.

Through the use of the microspectrophotometer total cellular fluorescence indicative of the catalytic subunit was quantified in several of our protocols (Fig. 2). Measurements made in this manner (see Materials and Methods) of the F:PKI-stained H35 cells also serve to illustrate a similar time course for appearance of free catalytic subunit following 8-BrcAMP and glucagon as does the measurement of the protein kinase activity ratio. In addition through the use of the F:PKI-procedure the distinct dose-dependent activation of kinase holoenzyme (i.e., appearance of the catalytic subunit) can be measured fluorometrically and provides quantitative information concerning protein kinase activation at unit cell resolution.

It should be emphasized that little can be concluded from our data concerning the movement of catalytic subunit. Since F:PKI does not bind to the holoenzyme the appearance of catalytic subunit in a given cellular compartment which is not fluorescent in unstimulated control preparations might indicate that (*a*) the catalytic subunit translocated into this part of the cell from another location or that (*b*) activation of the holoenzyme by cAMP in this compartment led to the release of the catalytic subunit which can then bind F:PKI.

The translocation or movement of the catalytic subunit has been studied for many years using biochemical procedures and various cell fractionation techniques. After activation of cAMP-dependent protein kinase by hormones or cyclic nucleotide analogues, the catalytic subunit has been shown to appear in the nucleus in the rat liver (16, 17), porcine ovary (18), adrenal medulla (19, 20), C_6glioma cells (21), and in neuroblastoma cells (22). It has, however, been suggested that in such studies the positively charged catalytic subunit may become artifactually associated with various subcellular organ-

elles during tissue homogenization (23). Although this nonspecific binding has often times been minimized by the appropriate choice of nonaqueous or high-ionic-strength buffer it has proven difficult to show conclusively that the protein kinase in fact translocated into the nucleus. Specific nuclear and some nucleolar localization of the catalytic subunit by immunocytochemical techniques has been demonstrated previously (23, 24) and adds further support for a physiological role for the catalytic subunit in the nucleus.

A marked and rapid increase in nucleolar free catalytic subunit was consistently observed in the H35 cells which were incubated with cyclic nucleotide analogues or glucagon (Figs. 1, 2, and 3). The kinetics of diffusion of macromolecules in cytoplasm does not preclude the possibility that the catalytic subunit could translocate within 1 min from the cytoplasm to the nucleus upon activation of the holoenzyme. However, it is equally possible that the catalytic subunit may be in the nucleus or nucleolus of unstimulated cells in a condition in which the enzyme is not able to bind F:PKI. In this regard, the catalytic subunit could be present in the nucleus in the holoenzyme form or tightly bound to chromosomal proteins (potential substrates) and only becomes capable of binding the inhibitor when cAMP levels increase. We have observed a large increase in nucleolar and nuclear catalytic subunit employing the F:PKI procedure in isolated nuclei purified from rat liver incubated with 8-BrcAMP (data not shown). For this reason we suggest that the rapid appearance of free catalytic subunit in the nucleus and nucleolus after incubation of the cells with cyclic nucleotide analogues or glucagon may be due to activation of protein kinase holoenzyme already present in these subcellular compartments. Translocation or movement of free catalytic subunit may also occur from cytoplasm to the nucleus or from the nucleolus to the nucleoplasm and could be indicative of the nucleoplasmic fluorescence which occurs following 1-h incubation with DBcAMP (Fig. 1).

The fluorescence observed predominantly in the nucleus after 240-min incubation with DBcAMP is a very consistent yet surprising observation (Fig. 1). It can be shown in Fig. 1 that as the DBcAMP is metabolized by the cells the total soluble protein kinase reassociates and the activity ratio begins to return to control values. A possible explanation of the continued presence of free catalytic subunit in the nucleus at 240 min is that nucleoplasmic free catalytic subunit is not able to reassociate with regulatory subunits as rapidly as the cytoplasmic kinase and thus remains capable of binding the F:PKI.

A more intriguing observation was the rapid and consistent appearance of free catalytic subunit in the nucleolus of H35 cells upon activation of cAMP-dependent protein kinase. The physiological role of the catalytic subunit in the nucleolus remains unknown. However, there is a large variety of nucleolar proteins which are postulated to have specific functions, some of which have been shown to be phosphorylated (33). It is conceivable that the catalytic subunit could be phosphorylating any of these proteins and altering their function. The nucleolus itself is the location of ribosomal RNA synthesis and as such is closely involved in the trophic response of a cell or tissue to hormones or drugs and may also be involved in a number of cell-cycle specific events. Cyclic AMP has been known for many years to affect growth processes in both a positive and negative manner, presumably through the activation of cAMP-dependent protein kinase (6, 26–28). In this regard even the total amount of cellular cAMP-dependent protein kinase has been reported to change during the cell cycle (29), cardiac hypertrophy (30), tumor promotion (31), and steroid hormone treatment (32). The poorly understood effects of cAMP upon cell growth might be explained through further studies of the nucleolar accumulation of the catalytic subunit and the subsequent phosphorylation of specific nucleolar proteins.

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