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Rapid and reversible translocation of the catalytic subunit of cAMP-dependent protein kinase type II from the Golgi complex to the nucleus

E.A.Nigg, H.Hilz[1], H.M.Eppenberger and F.Dutly

Institut für Zellbiologie, ETH-Hönggerberg, CH-8093 Zürich, Switzerland, and [1]Institut für Physiologische Chemie, Universität Hamburg, Martinistrasse 52, D-2000 Hamburg 20, FRG

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In unstimulated interphase bovine epithelial (MDBK) cells, both regulatory (R II) and catalytic (C) subunits of the type II enzyme of cAMP-dependent protein kinase (cAMP-dPK II) are associated with the Golgi complex. However, as demonstrated by indirect immunofluorescence microscopy, within 5 min after stimulation of adenylate cyclase by forskolin, the C subunit dissociates from the Golgi-associated R II and becomes diffusely distributed. With increasing time of forskolin treatment, C subunits accumulate in the nucleus, while R II subunits remain associated with the Golgi complex. The effect of forskolin is rapidly reversible in that C subunits begin to reassociate with the Golgi complex within a few minutes after drug removal. C subunit translocations similar to those produced by forskolin also occur after treatment of MDBK cells with dibutyryl-cAMP, confirming that the observed effects are most likely mediated by elevation of intracellular cAMP levels. These results suggest that nuclear translocation of activated protein kinase subunits may represent an important link between hormonal stimuli and physiological responses.

Key words: cAMP-dependent protein kinase/catalytic subunit/forskolin/nuclear translocation

Introduction

Most if not all physiological effects produced by elevations of cellular cAMP levels in vertebrate cells are mediated by the activation of cAMP-dependent protein kinases (cAMP-dPKs) (for reviews, see Rubin and Rosen, 1975; Nimmo and Cohen, 1977; Greengard, 1978; Krebs and Beavo, 1979; Cohen, 1982). In most species and tissues two major forms of cAMP-dPKs have been distinguished; these are commonly referred to as type I and type II enzymes, respectively. Both of these cAMP-dPKs are composed of two regulatory and two catalytic subunits that together constitute an inactive holoenzyme of the form R_2C_2. While the catalytic subunits of type I and type II enzymes appear to be very similar if not identical, the regulatory subunits R I and R II are readily distinguished. Activation of cAMP-dPKs occurs via elevation of intracellular cAMP according to the formula (Corbin *et al.*, 1978; Weber and Hilz, 1979).

$$(R_2C_2)_{inactive} + 4cAMP \rightleftharpoons (R\text{-}2\text{-}cAMP)_2 + 2\ C_{active}$$

While the structures and modes of activation of cAMP-dPKs have been elucidated in considerable detail (for review, see Flockhart and Corbin, 1982), comparatively little is known about the subsequent steps along the pathways leading to physiological responses. It is widely believed, however, that the subcellular locations of protein kinases and their potential target proteins may represent important factors in determining the range of physiologically relevant phosphorylation reactions (e.g., Lohmann and Walter, 1984). In this context it has been proposed that interactions of kinases with appropriate substrates might be achieved after translocation of activated kinase subunits among different cellular compartments (for review, see Jungmann and Kranias, 1977). Accordingly, a considerable amount of effort has been invested to determine the subcellular location of protein kinases and to investigate whether or not subcellular redistributions of activated subunits occur under physiological conditions. Unfortunately, the significance of many earlier biochemical studies appears uncertain because cAMP-dPKs have shown a pronounced tendency to redistribute artefactually in the course of subcellular fractionation experiments (Keely *et al.*, 1975; Zick *et al.*, 1979). The results of many immunocytochemical studies are equally difficult to interpret because of lacking documentation of antibody specificity (for critical discussion, see Lohmann and Walter, 1984).

In an immunofluorescence study we recently showed that in unstimulated MDBK cells the type II enzyme of cAMP-dPK is associated with the Golgi complex and with centrosomes (Nigg *et al.*, 1985). To investigate the possibility that cAMP-dPK subunits might redistribute upon elevation of intracellular cAMP, here we have studied the effect of adenylate cyclase activation on the subcellular location of these proteins. Adenylate cyclase was stimulated by treatment of MDBK cells with the diterpene forskolin (Seamon and Daly, 1981; Seamon *et al.*, 1981) and elevation of intracellular cAMP was determined by a radioimmunoassay. Using characterised antibodies (Schwoch *et al.*, 1980; Weber *et al.*, 1981; Nigg *et al.*, 1985), we show that elevation of intracellular cAMP produces a rapid and reversible nuclear translocation of the catalytic subunit of cAMP-dPK; by contrast, R II, the regulatory subunit of the type II enzyme, remains associated with the Golgi complex.

Results

Figure 1 shows the effects of forskolin treatment on the subcellular distribution of cAMP-dPK II subunits in MDBK cells. In unstimulated control cells, both R II (Figure 1A) and C (Figure 1B) subunits were concentrated within a conspicuous perinuclear area. As shown previously, this location reflects the association of cAMP-dPK II with the Golgi complex (Nigg *et al.*, 1985). Treatment of MDBK cells with 10^{-4} M forskolin for 1 h produced little effect on the distribution of the R II subunit (Figure 1C). However, under the same conditions a drastic redistribution of the C subunit to the cell cytoplasm, and, most prominently, to the cell nucleus occurs (Figure 1D).

Figure 2 illustrates the dose and time dependence as well as the reversibility of the forskolin effect. Compared with control MDBK cells (Figure 2A), dissociation of C subunits from the Golgi complex was clearly discernible after treatment with forskolin at concentrations as low as 10^{-5} M for as little as 5 min (Figure 2B). With increasing time of treatment (e.g., 30 min, Figure 2C), accumulation of C subunits within the cell nucleus

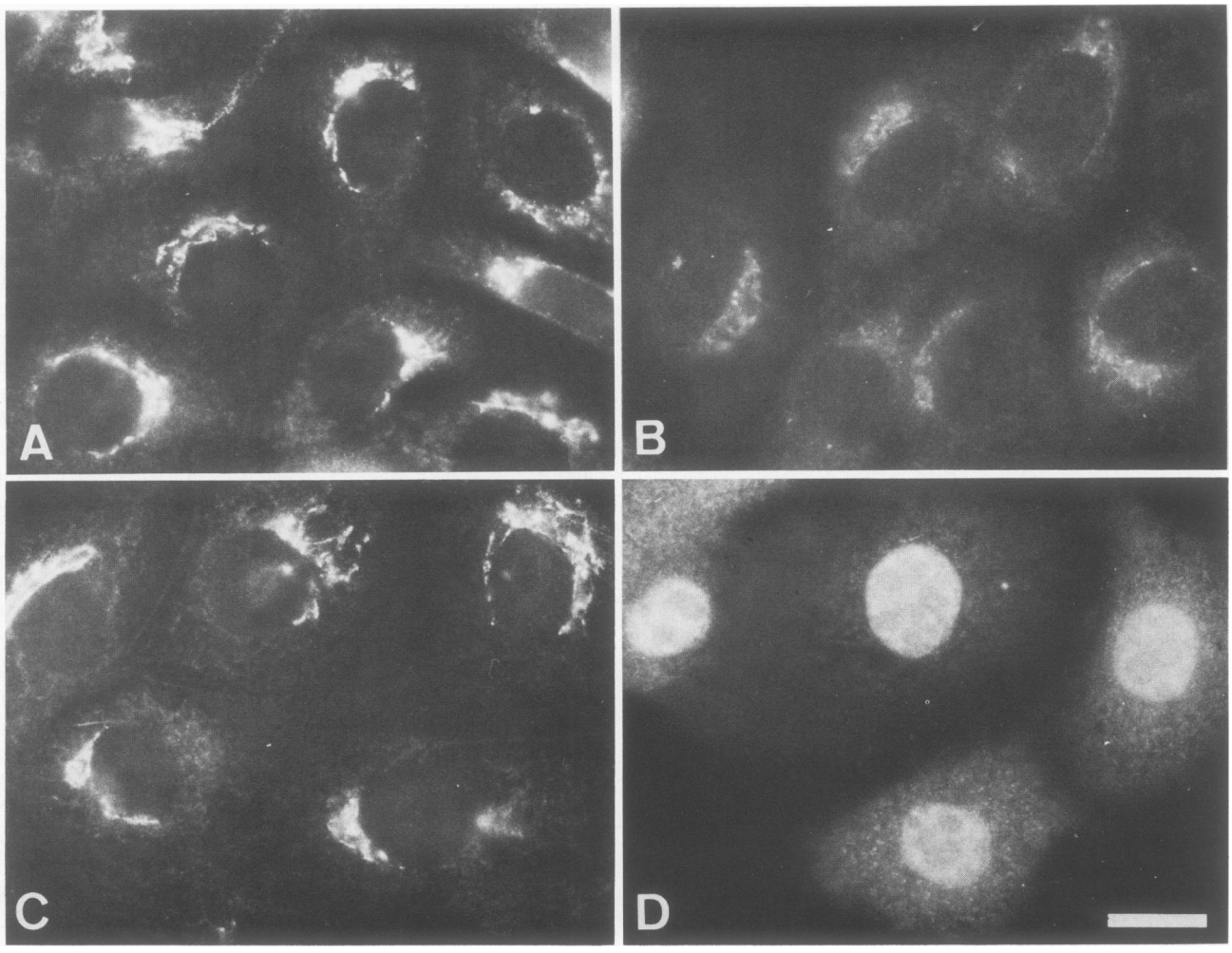

Fig. 1. Effect of forskolin on the subcellular distribution of cAMP-dPK II subunits. Untreated or forskolin-treated MDBK cells were fixed with formaldehyde and permeabilized with Triton X-100. They were then incubated with anti-cAMP-dPK II sera (diluted 1:300 in the case of anti-R II, 1:200 in the case of anti-C), followed by rhodamine-conjugated goat anti-rabbit IgG. **Panels A** and **B:** untreated MDBK cells stained with anti-R II **(A)** or anti-C **(B)** antibodies; **panels C** and **D:** MDBK cells treated for 1 h with 10^{-4} M forskolin and stained with anti-R II **(C)** or anti-C **(D)** antibodies. Bar in **D** represent 20 μm.

became very prominent. These results were not dependent on protein synthesis, as C subunit translocations also occurred in the presence of cycloheximide (not shown). While little further qualitative changes were observed upon forskolin treatments up to 16 h, the total amount of C subunits appeared progressively reduced as judged from the intensity of the fluorescent staining (not shown). Upon removal of forskolin, C subunit translocations were rapidly reversible; the beginning of reassociation of C subunits with the Golgi complex was visible as early as 5 min after removal of the drug from cells which had been treated for 30 min (Figure 2D). In all likelihood this result reflects re-binding of C subunits to the Golgi-associated R II.

In all vertebrate cells tested to date, forskolin was found to activate adenylate cyclase (for review, see Seamon and Daly, 1981). This is the case also in the MDBK cells (Table I). cAMP-dPK II is known to dissociate only at cAMP concentrations significantly higher than those required for the activation of cAMP-dPK I both *in vitro* and *in vivo* (Corbin *et al.*, 1975; Schwoch, 1978; Byus *et al.*, 1979; Schwartz and Rubin, 1983); however, the forskolin effects produced in our experiments were sufficient to effect complete activation of both protein kinases. As shown in Table I, cAMP rose to values nearly 1000-fold above controls after 15 min of forskolin treatment, and still surpassed unstimulated levels by a factor of >200 after 60 min. Removal of forskolin resulted in rapid degradation of the second messenger, which returned to essentially basal values in <60 min.

Additional evidence for the involvement of cAMP in mediating the forskolin-induced C subunit translocations is provided by the observation that dibutyryl-cAMP could mimic the action of forskolin (Figure 3). Incubation of MDBK cells with dibutyryl-cAMP resulted in C subunit translocations similar to those produced by forskolin (compare Figure 3B,C with Figure 2B,C). Again, C subunit translocations were not accompanied by any major redistributions of R II subunits (not shown). Butyrate, used as a control, did not significantly affect the distribution of C subunits (Figure 3D). Substantial C subunit translocations were produced by dibutyryl-cAMP at a concentration of 5 mM, while partial effects could be observed down to a concentration of 1 mM. Compared with the response of MDBK cells to forskolin, the response of these same cells to dibutyryl-cAMP was considerably slower; incubation times of the order of $1-3$ h were required before major effects became visible. It is likely that these rather high concentrations and relatively long incubation times reflect poor penetration of dibutyryl-cAMP into MDBK cells and slow formation of N^6-monobutyryl-cAMP as the true kinase ac-

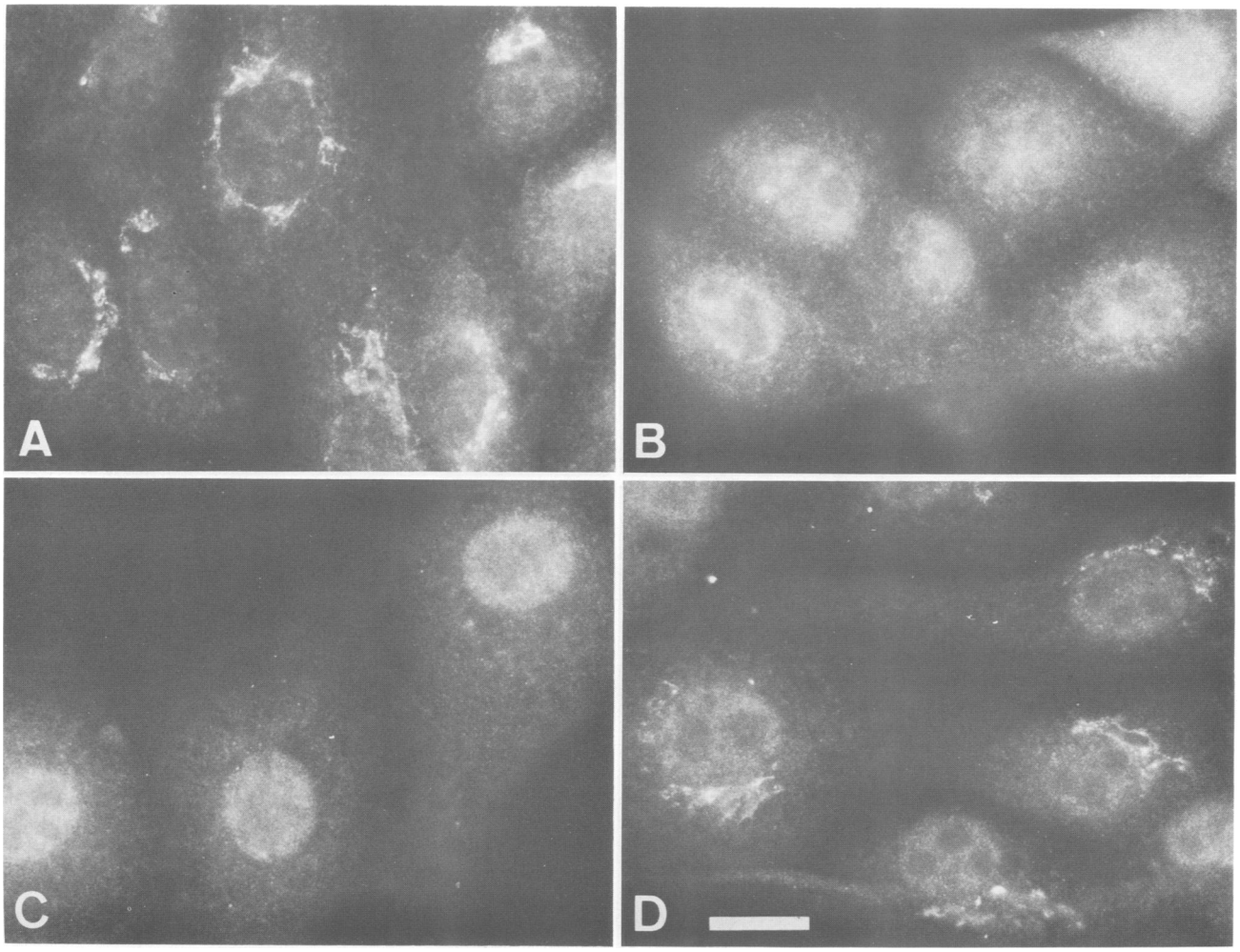

Fig. 2. Time dependence and reversibility of forskolin effect on C subunit distribution. Immunofluorescent labeling of untreated or forskolin-treated MDBK cells with anti-C subunit antibodies was carried out as described in the legend to Figure 1. **Panel A:** untreated MDBK cells; **panel B:** 5 min forskolin 10^{-5} M; **panel C:** 30 min forskolin 10^{-5}M; **panel D:** 30 min forskolin 10^{-5} M, followed by a 5 min incubation of cells in drug-free culture medium at 37°C. Bar in **D** represents 20 μm.

tivating derivative of dibutyryl-cAMP (Kaukel *et al.*, 1972). In any event, the similarity of the effects produced by forskolin and dibutyryl-cAMP strongly suggests that C subunit translocations occur after cAMP-induced dissociation of intracellular protein kinases.

Discussion

Using monospecific antibodies directed against bovine cAMP-dPK II subunits, we have previously shown that within epithelial and fibroblastic tissue culture cells this enzyme is predominantly concentrated in the area of the Golgi complex (Nigg *et al.*, 1985). In addition, throughout the cell cycle cAMP-dPK II subunits were found to be associated with centrosomes (Nigg *et al.*, 1985). Although R II and C subunits were found to co-distribute to a large extent in unstimulated cells, some additional amount of C subunit appeared to be diffusely distributed throughout the entire cells, presumably reflecting the association of C subunits with a relatively small amount of RI subunits (Nigg *et al.*, 1985). Here, by immunofluorescence microscopy, we show that treatment of MDBK cells with the adenylate cyclase activator forskolin induces a rapid and reversible dissociation of C subunits from the Golgi-associated R II. Most prominently, C subunits were translocated to the cell nucleus. Similarly, C subunits also appeared to dissociate from centrosomes (unpublished results), but for technical limitations this latter result is difficult to prove rigorously. The subcellular distributions of R II subunits were not substantially affected by forskolin treatment. As shown by radioimmunoassay, the effects of forskolin were accompanied by elevations of cellular cAMP levels; moreover, they could be mimicked by dibutyryl-cAMP, confirming that C subunit translocations were mediated by elevations of intracellular cAMP.

The anti-C subunit antibodies used here do not discriminate between C subunits forming part of either the type I or type II holoenzyme or free subunits. In our experiments we therefore monitor the distribution of the total amount of C subunits. While

Table I. Effect of forskolin on cellular cAMP content[a]

Untreated MDBK cells	47 ± 3	pmol/10^7 cells
15 min forskolin 10^{-5} M	39.670 ± 8960	pmol/10^7 cells
60 min forskolin 10^{-5} M	12.625 ± 840	pmol/10^7 cells
60 min forskolin, 60 min reversal	55 ± 5	pmol/10^7 cells

[a]After treatment of cells with forskolin, they were briefly washed and heated to 100°C for 5 min. Following centrifugations, the content of cAMP in the supernatants was determined by a radioimmunoassay.

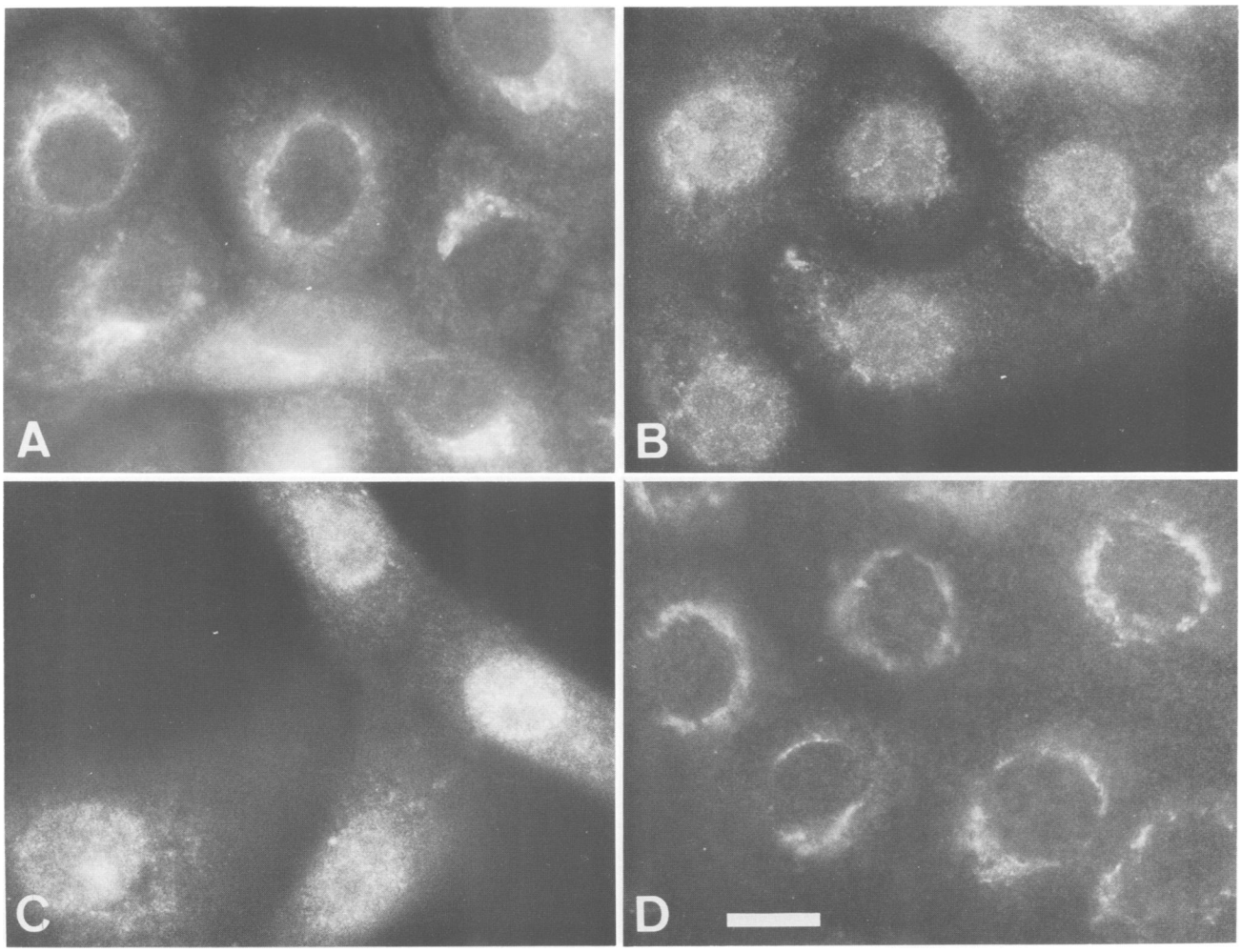

Fig. 3. Effect of dibutyryl-cAMP on the subcellular distribution of C subunits. Immunofluorescent labeling of MDBK cells with anti-C subunit antibodies was carried out as described in the legend to Figure 1. **Panel A:** untreated MDBK cells; **panel B:** 1 h dibutyryl-cAMP at 5 mM; **panel C:** 16 h dibutyryl-cAMP at 5 mM; **panel D:** 16 h butyrate at 5 mM. Bar in **D** represents 20 μm.

we know that R II subunits do not undergo any major redistributions in response to cAMP, we have no information about the fate of R I; however, the relative amount of the type I enzyme is rather low in MDBK cells (Nigg *et al.*, 1985; Hilz, unpublished results). Because cAMP-dPK I is known to dissociate at cAMP levels below those required to activate the type II enzyme (Corbin *et al.*, 1975; Schwoch, 1978; Byus *et al.*, 1979; Schwartz and Rubin, 1983), it is likely that C subunits dissociating from the type I enzyme may also undergo nuclear translocation. In any event, most of the C subunits which are translocated to the nucleus almost certainly exist in a free and hence enzymatically active form.

The occurrence of cAMP-dPK subunit translocations has long been considered an attractive possibility to account for some of the physiological effects mediated by cAMP (for reviews, see Jungmann and Kranias, 1977; Johnson, 1982; Lohmann and Walter, 1984). However, biochemical studies aimed at demonstrating cAMP-dPK subunit translocations have produced contradictory results. While several groups have presented evidence suggesting the occurrence of C subunit translocations (e.g., Castagna *et al.*, 1975; Spielvogel *et al.*, 1977; Kurosawa *et al.*, 1979; Schwartz and Costa, 1980; Laks *et al.*, 1981), other workers have pointed out the danger of artifacts inherent in cell fractionation experiments (Keely *et al.*, 1975; Zick *et al.*, 1979). Immunocytochemical studies have also produced conflicting results (for discussion, see Lohmann and Walter, 1984; Nigg *et al.*, 1985). For example, Murthaugh *et al.* (1982) used conventional immunofluorescence microscopy for the localization of C subunits; these authors did not detect any C subunit redistributions after stimulation of cells by 8-bromo-cAMP. In contrast, Byus and Fletcher (1982, 1985) used a fluorescent derivative of the heat-stable protein kinase inhibitor (Walsh *et al.*, 1971); this reagent was expected to recognize only free catalytic subunits but not cAMP-dPK holoenzymes. While Byus and Fletcher did report accumulation of free C subunits within nuclei of appropriately stimulated cells, they consistently observed strong labeling of nucleoli by their fluorescent probe (Byus and Fletcher, 1982, 1985; Murray *et al.*, 1985). This latter observation is in contrast to our results; we cannot definitively account for this discrepancy, but the nucleolar labeling produced by the fluorescent kinase inhibitor might conceivably represent a feature of this particular technique. Finally, our results agree well with recent data from Jungmann's laboratory. Studying the distribution of cAMP-dPK subunits in regenerating rat liver and in glucagon-treated rat hepatocytes, these authors also reported increases in the amounts of nuclear C subunits with no concomitant increases in nuclear R II subunits (Laks *et al.*, 1981; Kuettel *et al.*, 1984; Squinto *et al.*, 1985).

By what mechanism(s) is the rapid and reversible nuclear translocation of C subunits accomplished? In principle it is possible that active transport or facilitated uptake systems might control the distribution of C subunits. Alternatively, considering that the molecular mass of the C subunit should allow relatively unhindered passage of this protein through nuclear pore complexes (Bonner, 1978; Paine and Horowitz, 1980), an equilibrium may exist between C subunits in the nuclear and cytoplasmic compartments. The relative magnitude of the affinity constants for binding of C subunits to intranuclear sites and R subunits, respectively, might then essentially determine the subcellular distribution of C subunits. On a longer time-scale, selective turnover (Alhanaty and Shaltiel, 1979) of unbound C subunits in either the cytoplasmic or the nuclear compartment may also modulate the distribution of this protein in response to alterations of cAMP levels.

With respect to the physiological relevance of the described subcellular distributions of cAMP-dPK II subunits, we consider it intriguing that early responses to hormonally induced cAMP elevations in many tissues include stimulations of secretory processes, while later responses often comprise changes in nuclear activity. On the basis of our previous report (Nigg *et al.*, 1985) we have already discussed the possibility that phosphorylation of physiological substrates might occur within or close to the Golgi area; other substrates of cAMP-dPK II may be associated with centrosomes and hence may influence the cellular disposition and activity of microtubules and mitotic spindles (Nigg *et al.*, 1985). Our present results further strengthen the view that physiological substrates for cAMP-dPKs should also be searched for among nuclear proteins.

Two alternative hypotheses have been advanced to rationalize the effects of cAMP on nuclear events. First, changes in nuclear activity might result from phosphorylation of appropriate nuclear proteins by the activated catalytic subunit of cAMP-dPKs. Second, in analogy to the situation in prokaryotes, R subunits upon binding of cAMP might directly function as intranuclear regulatory proteins (for reviews, see Jungmann and Kranias, 1977; Kondrashin, 1985; Gancedo *et al.*, 1985). Our data provide direct evidence against a function of the cAMP-binding regulatory subunit R II analogous to the cAMP receptor protein (CRP) in *Escherichia coli* (Ullmann and Danchin, 1983). While the latter upon binding of cAMP acts as a transcriptional regulator of various genes, eukaryotic cAMP-binding R II is not translocated to the nucleus but remains at the Golgi complex (and also at the centrosome) even when cAMP has increased 1000-fold. Since, however, there is strong evidence that cAMP can directly provoke the expression of specific genes (e.g., Jungmann *et al.*, 1983; Murdoch *et al.*, 1982), the cAMP signal appears to be transmitted to the responsive genes via translocation of the catalytic subunit and subsequent phosphorylation of chromosomal proteins. The rapid kinetics of forskolin-induced C subunit translocation to the nucleus observed in the present study correlates well with the rapid cAMP-dependent stimulation of prolactin gene transcription and concomitant phosphorylation of a chromatin-associated protein reported for rat pituitary cells (Murdoch *et al.*, 1982).

Materials and methods

Bovine epithelial kidney (MDBK) cells were cultured as described previously (Nigg *et al.*, 1985). Forskolin was obtained from Calbiochem and was added to cell cultures from 10 mM stock solutions prepared in absolute ethanol; control cultures were treated with the same amounts of ethanol (final concentration 0.1%). For protein synthesis inhibition, cells were incubated for 15 min in the presence of cycloheximide at 1 μg/ml or 20 μg/ml prior to further addition of forskolin. Dibutyryl-cAMP was purchased from Sigma. The rabbit antisera used for immunofluorescent localizations of cAMP-dPK subunits have previously been characterized in considerable detail (Schwoch *et al.*, 1980; Weber *et al.*, 1981; Nigg *et al.*, 1985). Rhodamine-conjugated goat anti-rabbit IgG were from Cappel. Indirect immunofluorescence microscopy was carried out exactly as reported previously (Nigg *et al.*, 1985). The determination of cAMP by radioimmunoassay has also been described (Schumacher and Hilz, 1978).

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