Direct Cytochemical Localization of Catalytic Subunits Dissociated from cAMP-dependent Protein Kinase in Reuber H-35 Hepatoma Cells. I. Development and Validation of Fluorescinated Inhibitor

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Abstract A specific and sensitive procedure has been developed that reliably localizes intracellular sites of free catalytic unit (C) dissociated from cAMP-dependent protein kinase. The method is based on a FITC conjugate (F:PKI) of affinity column-purified heat-stable protein inhibitor (PKI) of free C. The fidelity of this cytochemical probe was determined using cultures of Reuber H-35 hepatoma cells that had been stimulated for 2 h with 0.1 mM DBcAMP, or with diluent, then fixed with anhydrous acetone at −30°C. In these preparations the F:PKI probe complexed with free C in cytoplasm, nucleolus, and, to a minor extent, in nucleoplasm. Binding of the F:PKI molecule to free C was competitively diminished by arginine analogues, guanidinium HCl and polyarginine, each used over a 2-log dose range. When the inhibitor’s arginine residues were blocked by reaction with cyclohexanedione it no longer inhibited phosphotransferase activity of free C, and when fluorescinated it failed to localize C in stimulated cells. Similarly, when F:PKI was preabsorbed with excess pure C it no longer functioned as a cytochemical stain. Affinity column-purified antibody to free C also reduced significantly the ability of F:PKI to complex with C in cell cultures stimulated with 0.1 mM DBcAMP. 1 μg of antibody reduced by ~10% the binding of F:PKI to all cell compartments while 5 μg of antibody diminished binding by >50%. Together, these results indicate that the F:PKI binds specifically, perhaps exclusively, to the catalytic units of cAMP-dependent protein kinase. The cytochemical procedure, unlike its biochemical counterparts, is able to locate the dissociation of cAMP-dependent protein kinase in individual cells of functionally or histologically complex cultures. Also, it reveals variations in the time- and dose-dependent activation of the kinase amongst clonal cells stimulated with cyclic nucleotide analogues or hormones.

Numerous cellular responses are regulated by the binding of a hormone or other agent to a cell surface receptor. This event leads to the activation of adenylate cyclase (1) and a consequent increase in cyclic 3′:5′ adenosine monophosphate (cAMP). The influence of cAMP on subsequently activated processes appears to be further mediated and amplified through the activation of a cAMP-dependent protein kinase (1–6). The holoenzyme form of the kinase whether type I or type II consists of a dimeric regulatory subunit (R) and two catalytic subunits (C). The binding of cAMP to R causes the reversible dissociation of the cAMP-independent C which is then able to catalyze the phosphorylation of a variety of protein substrates (2, 3, 7–9).

Although the dissociation of cAMP-dependent protein kinase is thought to mediate a diversity of cellular functions, each of these is initiated by a similar event, the generation of cAMP (1–6). How these responses are thereafter specifically brought about is poorly understood but they may result from the different affinities of the type I and type II isozymes for cAMP (2, 3), from the ratio of isozymes (2, 3, 10), or from the temporal and spatial kinetics of the kinase subunits relative to their specific substrates (2, 9, 11, 12). In this regard, little is known about the subcellular distribution of R-cAMP and C during the minutes and hours after their separation, i.e., upon activation of the holoenzyme.
Studies using differential centrifugation methods have furnished tentative evidence that the holoenzymes dissociate in cytoplasm where freed regulatory and catalytic units may remain or can, with time, translocate to the nucleus (2, 11, 12). The presence of free C in the nucleus is thought to be important to the regulation of a variety of transcriptional events (2, 3, 9–12) and may be relevant to cAMP-dependent protein kinase-mediated control of cellular growth and differentiation (10).

Immunocytochemical methods with heterospecific antisera to the holoenzymes or their RI and RII subunits have confirmed the concept of nuclear translocation (13). Unfortunately, all of the available antibodies to the catalytic unit crossreact extensively with both forms of the holoenzyme and are thus unusable for locating only the free C (13). This and other problems inherent to indirect immunocytochemical procedures make them unsuitable for localizing C at unit cell resolution. Even so, a morphological probe for the sites of phosphotransferase activity is desirable, if not necessary, in order to gain insight into how the cAMP-dependent kinases though activated by a single event can engender a wide variety of effects.

The fact that free C appears to be the effector component of the holoenzyme suggested an alternative method for localizing its subcellular distribution and movements, if any, with time once dissociated from the intact isozymes. A number of laboratories have reported the presence of a heat-stable protein that inhibits free C phosphotransferase activity (4). This inhibitor (PKI), found in a variety of tissues and characterized (14–20) has a high affinity and specificity only for the free catalytic unit of CAMP-dependent protein kinase (4, 14, 16). It does not bind to either form of the holoenzyme nor to the cyclic 3':5' guanosine monophosphate (cGMP)-dependent protein kinase (15, 16). It seemed reasonable therefore that the inhibitor protein could be useful as probe for the direct cytochemical localization of free C.

Here we describe the development of a cytochemical method employing a fluorescein isothiocyanate-conjugated inhibitor (F:PKI) for the localization of the catalytic subunit and the procedures used to assure its fidelity and sensitivity. Our companion article which follows will describe the kinetic of free C in a hormone-responsive tumor cell, as determined with the localization procedure.

MATERIALS AND METHODS

To examine the effects of various stimulating agents on intact, living cells, we used monolayer cultures of Reuber H-35 hepatoma kindly provided by Dr. Wesley Wicks (University of Tennessee). Cells were seeded onto a sterile 12-mm square glass coverslip in 10 x 50 mm plastic petri dishes. The cells were grown in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY) containing 5% fetal calf + 5% calf serum (Gibco Laboratories). Studies using differential centrifugation methods have furnished tentative evidence that the holoenzymes dissociate in cytoplasm where freed regulatory and catalytic units may remain or can, with time, translocate to the nucleus (2, 11, 12). The presence of free C in the nucleus is thought to be important to the regulation of a variety of transcriptional events (2, 3, 9–12) and may be relevant to cAMP-dependent protein kinase-mediated control of cellular growth and differentiation (10).

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**Purification of Inhibitor**

The protein inhibitor of adenine 3':5'-monophosphate-dependent protein kinase was purified to homogeneity from both rabbit skeletal muscle and bovine brain according to Demaille et al. (16) with a protein kinase catalytic subunit affinity column used to obtain homogenous inhibitor (16, 17). Using this procedure essentially as described (16), we routinely obtained inhibitor which was 400,000-500,000-fold pure and migrated as a single band on 12.5% polyacrylamide gels. While the inhibitor protein is apparently quite stable during most of the purification procedure with total yields approaching 30%, in solution it is susceptible to denaturation in terms of its ability to inhibit the free catalytic subunit. For this reason, the eluate from the phosphocellulose column (16), the homogeneous inhibitor and the fluoresceinated inhibitor were each stored at -20°C as a lyophilized powder.

**Conjugation of Inhibitor Protein with Fluorescein**

Affinity chromatography-purified inhibitor (100 µg) was dissolved in 2 ml of 50 mM sodium carbonate, pH 8.5. Two mg of fluorescein isothiocyanate (FITC) bound to Celite (Sigma Chemical Co., St. Louis, MO) was then added to the inhibitor solution and allowed to react at room temperature for 2 min with intermittent mixing. After this, the inhibitor was rapidly cooled to 2–4°C and centrifuged at 20,000 g for 5 min to remove the majority of the unreacted FITC. The FITC-inhibitor conjugate (F:PKI) could then be separated from the remainder of the FITC by extensive dialysis in 10 mM Tris-HCl, pH 7.5, or by chromatography on a Bio Gel P4 column (0.5 x 5 cm) equipped with a Gilson fluorescent column monitor. Each preparation of F:PKI was then assayed for its ability to inhibit the free catalytic subunit under standard assay conditions (see below), placed in 100-µl aliquots, lyophilized, and stored desiccated at -20°C.

**Assay for Inhibitor Activity**

The protein kinase inhibitor was assayed according to a modification of the method of DeLange et al. (21). The assay mixture (0.10-0.15 ml) contained 0.01M potassium phosphate buffer, pH 6.8, 0.025 M Mg acetate, 1 mM diethylthiourea, 100 lµM mixed calf thymus histone, and -32 P ATP (1 mci/mmol) plus sufficient unlabeled ATP for a fixed ATP concentration of 0.15 mM. Purified catalytic subunit prepared according to Beavo et al. (22) as modified by Demaille et al. (16) was preincubated for 5 min at 30°C with varying amounts of native inhibitor or its fluoresceinated derivative. The reaction was initiated by the addition of the ATP and allowed to proceed for 10 min at 30°C. The assay was terminated by pipetting 150 µl of the reaction mixture onto Whatman 3 M filters (2.3 cm) which were then washed extensively in 10% trichloroacetic acid and 95% ethanol, dried, and counted in an omnifluor/toluene liquid scintillation cocktail.

**Cyclohexanedione**

Affinity column-purified inhibitor was reacted with cyclohexanedione as described by Demaille et al. (16). Briefly, this consisted of treating inhibitor for 3 h with 150 mM cyclohexanedione in 250 mM sodium borate buffer at pH 9.0.

**Catalytic Subunit Antibody**

Antibody to purified bovine heart catalytic subunit was generously provided by Drs. Michael Murtaugh, Alton Steiner, and Peter Davies (University of Texas). Free C was derivatized to keyhole limpet hemocyanin and injected into recipient goats. The antibody obtained was prepared and characterized as described elsewhere (23). The heterospecific antiserum was purified by chromatography on a catalytic subunit affinity column (16) and shipped to us packed in dry ice. All of the affinity-purified antibody used in this study was from the same bleed (Affi #3).

**Preparation of Cultures for Cytochemistry**

During preliminary protocols a number of agents (cyclic nucleotide analogues or hormones) were incubated with HT3 cells at various doses and periods of time. Using the kinase activation assay and cytochemical methods in parallel it was found that 2-h exposure to 0.1 mM DBcAMP was optimal for dissociation of the holoenzyme. These parameters were then held constant in order to establish the remainder of the preparative methods. The time- and dose-related effectiveness of other cyclic nucleotide analogues and of hormones is described in the accompanying article.

Cells grown on coverslips from control and DBcAMP-stimulated cultures were washed for 3 min at 4°C with pH 7.4 phosphate-buffered saline (PBS). Fixation was achieved by a variety of methods at either 4°C or -30°C for 15-20 min. The criteria by which the various fixation procedures were compared were that: (a) the intracellular free catalytic subunit could not be structurally altered to the point of impairing its binding to the F:PKI probe; (b) prefraction cellular morphology should be conserved; and (c) fixation alone should not induce an appreciable "background" fluorescence.

Paraformaldehyde (Polysciences Inc., Warrington, PA) was diluted with PBS from a 10% stock solution in distilled water and adjusted to pH 7.4. Glutaraldehyde from a variety of sources was tried. The best results were obtained by diluting freshly opened ampoules of an 8% solution (Polysciences Inc.) with PBS, then readjusting the pH to 7.4. The didehydroxy mixture was made with equal volumes of the 0.25% solutions of paraformaldehyde and glutaraldehyde. Dime-thylsuberimidate (DMS, Sigma Chemical Co.) was prepared and used as described by Hand and Hassell (24).

The paraformaldehyde-lysine-periodate (PLP) (25) fixative consisted of 50
mm l-lysine, 10 mM sodium metaperiodate, and 0.25% paraformaldehyde in 50 mM PBS which was made isometric by the addition of sucrose. Methanol and methanol: acetic acid (1:3 vol:vol) were tried only twice, as they greatly disrupted cellular morphology. Ethanol or acetone (Mallinkrodt Inc., Science Products Div., St. Louis, MO) were each made essentially anhydrous with 4A molecular sieves (Linde PO Box 372, South Plainfield NJ) and used to treat the cells at 4°C or −30°C. The 4°C preparations were unsuitable and no results are presented here. An attempt was made to quench the background fluorescence induced by the fixatives containing aldehydes or DMSO. L-lysine monohydrochloride (Sigma Chemical Co.) was added to PBS to achieve the desired concentration (20–100 mM) and stirred continuously until solubilized. Sodium borohydride (Sigma Chemical Co.) solutions (10–100 mM) were similarly made and their pH was adjusted to 8.2 at which point they most effectively react with free aldehyde groups (26). After fixation, cover slips were immersed in one of these reagents and allowed to react at 4°C for 15 min. Triton X-100 (scintillation grade, Research Products International Corp., Mt. Prospect, IL) was diluted to 0.0125 or 0.10% with PBS. Filipin (Upjohn Co., Kalamazoo, MI) was solubilized with dimethylsulfoxide (DMSO) (Mallinkrodt Inc.) and brought to the final concentration (10 or 100 μg/ml) with PBS (0.5% DMSO in working solution). Fixed cultures were exposed to either Triton or Filipin for 15 min at 4°C. Dimethylsulfoxide (DMSO) was diluted with PBS and adjusted to pH 7.4. Prefixed cultures were placed in DMSO for 15 min at 4°C.

After fixation or a postfixation treatment, cover slips were rinsed for 3 min in PBS at 4°C, placed in a humidity chamber, and covered with 100 μl of the F:PKI diluted with PBS or with PBS alone. Cover slips were then incubated at 4°C for 3–96 h. rinsed with PBS, then either covered with glycerol/PBS or dehydrated with anhydrous acetone at −30°C, and mounted on glass slides with Entellan (EM Laboratories, Inc., Elmsford NY).

**Fluorescence Microscopy**

All microscopy was performed with an Olympus Vanox microscope equipped with a 200-W HBO ultraviolet source, a BG excitation filter, and a 515-nm barrier filter or with a Leitz Ortholux microscope fitted with a specific excitation barrier filter (k2:LEITZ), and varirotochromat camera. Kodak Tri-X film was exposed at ASA 800 and developed for 12 min with Kodak D-76. To display the relative fluorescence intensity of each preparation, all images were exposed for the same amount of time. This time was determined by making 10 exposures of the most fluorescent preparation with the automatic photometer. The average of these times, which varied by <10%, was then used to photograph all other preparations within the same experiment. For each experiment all rolls of film were processed together and prints were made with the grade of paper and exposure and development times held constant.

**RESULTS**

In order for the F:PKI to be a specific probe for localization of the free catalytic subunit the inhibitor protein must retain its high affinity for C following treatment with fluorescein isothiocyanate. Fig. 1 shows the relative affinity of equivalent concentrations of PKI and F:PKI to inhibit the activity of the free catalytic subunit in vitro under standard assay conditions. Comparison of the effects of PKI and F:PKI upon the activity of protein kinase indicated that if an appropriate technique for the fluorescination of PKI was employed (see Materials and Methods) no loss in inhibitory activity could be observed. More rigorous conditions for fluorescination (greater concentration of FITC and/or longer reaction times) did result in a significant reduction in the ability of F:PKI to inhibit C (data not shown).

A major difficulty in establishing any cytochemical or immunochemical procedure is the method of fixation chosen. Early trials using unfixed or critical-point-dried cells were not successful. Of the various fixation methods tried (see Materials and Methods), anhydrous acetone at −30°C best satisfied all of our requirements.

**Specificity of Fluorescinated Inhibitor Localization**

Once the method of fixation was established it became possible to test the specificity of the F:PKI interaction with free C. The inhibitor protein binds via its arginine residues to the substrate site of free C (3, 16). Arginine analogues, therefore, act as competitors toward PKI for the substrate site as judged by biochemical assays (16). To determine whether they do so in acetone-preserved cells, cultures were stimulated for 2 h with 0.1 mM DBcAMP or with diluent only, then fixed and prepared for cytochemistry as described above. Preparations were then stained with 100 μl of the F:PKI diluted 1:20 (i.e. 1.25 μg/ml) with PBS alone or with PBS containing 10–200 mM guanidinium hydrochloride or 10–200 μM polyarginine.

Fig. 2a depicts a culture stained with PBS diluted F:PKI. About 75% of the cells exhibit intense fluorescence in cytoplasm and nucleolus. Companion cultures that were incubated with an identical amount of the probe containing in addition 100 mM guanidinium HCl (Fig. 2b) or 100 μM polyarginine (Fig. 2c) show significantly less fluorescence. Approximately the same percentage of cells contain dissociated C in cytoplasm and nucleolus but the amount of F:PKI bound is reduced appreciably by each of the analogues.

When affinity column-purified inhibitor was treated with cyclohexanedione its ability to inhibit the phosphotransferase capacity of C was virtually abolished, as reported by Demallie et al. (16). The reaction mixture alone, in the absence of cyclohexanedione, reduced by ~10% the inhibitor's biologic activity, but this effect was insignificant compared to that caused by the complete reaction mixture. When the chemically altered inhibitor was fluorescinated it conjugated with an equivalent amount of FITC as did the untreated PKI (determined with a fluorometer). However, the cyclohexanedione-modified F:PKI no longer functioned as a cytochemical probe (Fig. 2d). In fact, fluorescence of these preparations was nearly equivalent to that of unstimulated-unstained cultures (blank controls).

If the F:PKI was binding only to the free catalytic unit it was reasonable to expect that excess exogenon C could reduce the amount of the probe available to complex with endogenous C. Fluorescininated inhibitor (1.25 μg/ml) was prereacted with homogeneously pure bovine heart catalytic subunit (10 μg/ml) for 3 h at 4°C. 100-μl aliquots of this material was then used to stain fixed cultures that had been stimulated for 2 h with 0.1 mM DBcAMP. As shown in Fig. 2e, when preabsorbed with C the F:PKI no longer is usable for the localization of endogenously dissociated catalytic unit.

To establish that the FITC-conjugated inhibitor interacts with free C of acetone-fixed cells in a manner equivalent to that of the unlabelled inhibitor, cultures were stained with F:PKI and PKI mixed in varying molar ratios. Fig. 2f shows the results of one of three such competitive inhibition experiments. In all cases the H35 cells were stimulated for 2 h with 0.1 mM DBcAMP.

When the PKI:F:PKI ratio is 10:1 (Fig. 2f), the fluorescence intensity of cells declines markedly from that observed when diluted F:PKI (1.25 μg/ml) is used alone (Fig. 2e). Fluorescence intensity is reduced further, to an unworkably low level, at a 20 molar excess of PKI (Fig. 2g) and it is abolished at a 40-fold molar excess (Fig. 2h). Although the amount of F:PKI bound declines in proportion to the increasing amount of unlabeled inhibitor protein, the subcellular distribution of the probe is unaltered.

It appears from these results that the F:PKI probe and PKI bind specifically to free C in acetone-fixed cells in a manner which parallels their ability to inhibit catalytic subunit activity measured biochemically. In an attempt to determine whether
or not the F:PKI bound to cellular proteins in addition to C, the cytochemical procedure was performed in the presence of antibody to catalytic subunit.

Cultures were stimulated for 2 h with 0.1 mM DBCAMP, then fixed and prepared for cytochemistry. Two procedures were used to treat the fixed cells with F:PKI and antibody to the catalytic subunit. In the first fixed cultures were preincubated for 3 h at 4°C with 1 or 5 μg of affinity chromatography purified antibody to C or with 5 μg of preimmune serum protein. They were then stained with the probe at one of two dilutions (1.25 μg/ml or 0.63 μg/ml) in the continued presence of antibody. Alternatively, cultures that had been similarly stimulated with DBCAMP were stained with a mixed solution containing 1 or 5 μg of antibody or 5 μg of preimmune serum and one of the two concentrations of the F:PKI probe. The first procedure (preincubation) was expected to provide the antibody ample opportunity to complex with C such that when the F:PKI was added afterwards it would not be able to quickly displace C. The coincubation preparations, where antibody and F:PKI were added simultaneously, should, on the other hand, furnish the antibody and the F:PKI equal access to dissociated C. Figs. 3a–h shows the results of one of two such studies. Unstimulated cultures stained with the high concentration of F:PKI (1.25 μg/ml) in the presence of 5 μg of preimmune serum exhibited the normal number (~5%) of H-35 cells with spontaneously active cAMP-dependent protein kinase (Fig. 3d). In parallel, control cultures exposed to the F:PKI and 5 μg of antibody, the endogenously dissociated free C was rendered unavailable to the probe by 5 μg of the catalytic unit antibody (Fig. 3h).

In cultures stimulated with DBCAMP the intensity of fluorescence was essentially unaffected by 5 μg of preimmune serum (Fig. 3a and e). In the presence of a constant amount of preimmune serum the fluorescence intensity was, however, directly dependent on the concentration of F:PKI used (compare Fig. 3a and e). When stimulated, fixed cultures were exposed to C antibody before being stained with F:PKI, the amount of probe bound was significantly diminished in proportion to the concentration of antibody used. 1 μg of antibody (Fig. 3b and f) reduced F:PKI binding relative to that of their respective control cultures treated with preimmune serum (Fig. 2a and e). This decline in fluorescence was similar for both concentrations of F:PKI employed (in Fig. 3, compare b–a and f–e). When the amount of antibody was increased to 5 μg/culture (Fig. 3c and g) the quantity of F:PKI bound was reduced to about one-half that of preimmune serum controls in those cases where the high concentration (1.25 μg/ml) of F:PKI was used (Fig. 3c). When a lesser amount of the F:PKI (0.63 μg/ml) was employed the 5 μg of antibody was even more effective in its ability to prevent binding of the probe to free C (Fig. 3g). Irrespective of the amount of antibody or F:PKI used, the fluorescence observed relative to the preimmune serum controls was a reduction only in intensity, not in the subcellular location of the residual F:PKI.

**DISCUSSION**

Our purpose was to develop a morphologic probe that could be used for observing the subcellular location of cAMP-dependent protein kinase after its dissociation. Others have found that antisera to the free C subunit of the kinase extensively crossreact with both forms of the holoenzyme (13, 28). To overcome this problem will most likely require generating a monospecific antibody that recognizes determinants of C that are inaccessible when it is associated with the regulatory subunit. Alternatively, we have devised a direct cytochemical method employing a fluorescinated conjugate of affinity column-purified heat-stable inhibitor protein. The inhibitor protein (PKI) binds specifically to free C but not to either of the holoenzyme forms or to the cyclic GMP-dependent kinase (2, 4, 15, 16, 29). The affinity of PKI for C (Kₚ = 2 x 10⁻⁹ M) is much greater than that of C for any of its known substrates (Kᵢ for Histone 2b 3.2 x 10⁻⁵ M) (16). The high affinity of the inhibitor and its relatively low molecular weight suggested that it would be an excellent species for direct cytochemistry.

Of the fixation procedures tried, anhydrous acetone at -30°C gave the best results. Although this mode of preservation slightly disrupted cellular morphology, the acetone adequately stabilized substructure such that it did not deteriorate appreciably during the 48-h incubation with the F:PKI. Also, acetone did not impair the affinity of C for the F:PKI probe. In this regard the catalytic subunit has been reported to retain full activity in 1.33 M urea or 1 mM SDS (16) and has been purified in active form from acetone powder of rat liver (30).

Of the other fixatives tried, only paraformaldehyde at concentrations of 0.25% or less appears to be usable. However, at those low concentrations cellular preservation was no better than that achieved with -30°C acetone. Further, the aldehyde induces a nonspecific fluorescence, mainly in cytoplasm, i.e., in the blue-green wavelength, which interferes with observing the F:PKI (emission 515 nm). This hindrance renders all of the aldehydes used in this study unsuitable fixatives for studying the early stages (~1 min) of kinase activation following stimulation of cultures with low doses of hormone or cyclic nucleotide analogues (see accompanying manuscript).

The times of incubation with the F:PKI (1.25–0.63 μg/ml) ranged from 3 to 96 h. At the earliest interval a low level of specific subcellular fluorescence was observed. After 24 h of staining at 4°C, the fluorescence intensity increased significantly although it was confined to the same cellular compartments as before. Maximal binding of the F:PKI probe was reached at 48 h of incubation, after which no increase occurred. In all cases the subcellular distribution of the F:PKI-C complexes was similar.

We found that by using higher concentrations of the F:PKI
FIGURE 2. Cultures of H-35 cells were stimulated with 0.1 mM DBcAMP then stained with 100 nl of F:PKI in PBS alone (1.25 μg/ml) (A) or with F:PKI:PBS made 100 mM with guanidinium hydrochloride (B) or 100 μM with polyarginine (C). The preparation in D was stained with inhibitor fluorescinated after reaction with cyclohexanone. E displays a culture stained with F:PKI (1.25 μg/ml) preabsorbed with free catalytic unit (10 μg/ml). F-H demonstrate the effect of competition between unlabeled inhibitor and FITC-conjugated inhibitor for the free C of H35 cells stimulated for 2 h with 0.1 mM DBcAMP. All preparations were stained with the same amount of F:PKI (1.25 μg/ml) used routinely (A) but in the presence of a 10 (F), 20 (G) or 40 (H) molar excess of unlabeled inhibitor.

(6–12.5 μg/ml) it was possible to reduce the time of incubation with the probe to 6–12 h at 4°C or 3 h at room temperature. While this yielded a fluorescence intensity equivalent to that of preparations stained for a longer time with a lesser amount of F:PKI, it occasionally caused an increase in background fluorescence. Even though this could be eliminated by washing stained cultures with 250 mM KCl (16), it interfered with observing the modest fluorescence in unstimulated-stained (control) cultures (e.g., Fig. 2D). Although it is acceptable to stain maximally stimulated preparations for short periods with higher concentrations of F:PKI, this procedure can mask the minimal fluorescence (i.e., minimal dissociation of cAMP-de-
The influence of catalytic subunit antibody on the ability of F:PKI to complex with free C. Cultures A–C and E–G were stimulated with DBCAMP. A–C were stained with 100 µl of F:PKI (1.25 µg/ml) in the presence of: 5 µg of preimmune serum (A); 1 µg of antibody (B); 5 µg of antibody (C). E–G were stained with 100 µl of F:PKI (0.63 µg/ml) and: 5 µg of preimmune serum (E); 1 µg of antibody (F); 5 µg of antibody (G). D and H are unstimulated cultures stained with F:PKI (1.25 µg/ml), with either 5 µg of preimmune serum (D) or 5 µg of antibody (H).
kinase inhibitors, based on their Ki values, that are competitive or with low doses of nucleotide analogues or hormones. In the inhibition with unfluorescinated PKI (Fig. 2F-H), only the with arginine analogues (Figs. 2 B and C) or competitive with the fact that native PKI effectively competed with F:PKI (compare Fig. 2 D and E). These findings in conjunction with the fact that native PKI effectively competed with F:PKI for dissociated C (Fig. 2 F–H) indicate that the fluorescinated inhibitor specifically occupies the substrate site of the free catalytic units. We interpret this to validate the concept that inhibitor specifically occupies the substrate site of the free catalytic subunit. To our knowledge no known protein other than free C from cAMP-dependent protein kinase (2-4, 15-20). Even so, these limitations made it necessary to establish that as a cytochemical probe the F:PKI binds predominantly, perhaps exclusively, to the free catalytic unit of cAMP-dependent protein kinase.

There are ample evidence that the arginylation of the kinase inhibitor protein are necessary to its binding to free C (3, 16). Demaiel et al. (16) described three classes of protein kinase inhibitors, based on their Ki values, that are competitive toward protein substrates. Using in vitro assays, guandinium hydrochloride and polyarginine were observed to be effective in the millimolar and micromolar range, respectively, in competition with the inhibitor protein for the substrate site of C. In the cytochemical procedure these arginine analogues were similarly effective in reducing the binding of F:PKI to all cellular constituents (Fig. 2 B–C). Because of the inhibitor's high affinity for the substrate site (Ks = 2 x 10^-8 M) it was not possible, using competitive inhibitors, to completely abolish the binding of F:PKI to cells that had been stimulated with cyclic nucleotide analogues. However, when the inhibitor protein itself was modified by reaction with cyclohexanedione such that it no longer diminished the phosphotransferase activity of free C, when fluorescinated it also did not stain cells in which cAMP-dependent protein kinase was maximally activated (Fig. 2 D). In a reciprocal study it was found that preabsorption of F:PKI with excess C caused a marked reduction in its binding to stimulated cells (Fig. 2 E). In fact, the negligible fluorescence of these preparations was equivalent to that of preparations incubated with cyclohexanedione-reacted F:PKI (compare Fig. 2 D and E). These findings in conjunction with the fact that native PKI effectively competed with F:PKI for dissociated C (Fig. 2 F–H) indicate that the fluorescinated inhibitor specifically occupies the substrate site of the free catalytic units. We interpret this to validate the concept that the F:PKI interacts with free C in acetone-fixed cells in a manner similar, if not identical to, its interaction with C as determined biochemically. The fact that this interaction is not only specific for C but may be confined to the dissociated kinase is further supported by the results using antibody to purified catalytic unit. The catalytic subunit affinity column-purified antibody significantly inhibited the binding of F:PKI to H-35 cells. As was the case for competition of the F:PKI with arginine analogues (Figs. 2 B and C) or competitive inhibition with unfluorescinated PKI (Fig. 2 F–H), only the amount of probe bound was diminished, not its subcellular distribution. Due to the scarcity of antibody we were unable to use a quantity capable of completely abolishing F:PKI binding to stimulated cells, although in unstimulated preparations 5 µg of antibody did eliminate F:PKI binding to endogenously dissociated free C (Fig. 3 H). In another study using porcine ovarian granulosa cells, 5 µg of the affinity column-purified antibody to free C reduced by 90% the amount of F:PKI bound to cells that had been stimulated for 30 min with 0.1 mM 8BrcAMP (manuscript by these authors in preparation).

It appears then that the F:PKI probe binds predominantly to C and not to a host of other cellular proteins in addition. Even though the antibody crossreacts with the cAMP-dependent protein kinase holoenzyme (M. Murtaugh, A. Steiner, and P. Davies, personal communication), this would not be reflected in a diminution of F:PKI binding as inhibitor does not complex with the kinase holoenzyme (2-4, 16). If the F:PKI were able to bind holoenzyme in addition to free C, then there would be no increase in cellular fluorescence in short-term (e.g., 30 min) stimulated preparations relative to unstimulated controls. Clearly, the stimulation does lead to an increased fluorescence.

The large amount of affinity-purified antibody needed to cause a 50% decline in F:PKI binding implies that only a fraction of the antiserum protein is directed toward determinants of C that are used by the fluorescinated inhibitor. While we do not know what the determinants are, the substrate binding site is at least partially involved in that 1 µg of affinity column-purified antibody was required to reduce by 50% the phosphotransferase activity of 10 ng of catalytic subunit (Murtaugh, Steiner, and Davies, personal communication). This indicates that the antibody has a Ks for C in the micromolar range and may explain its inability to eliminate fully the binding of F:PKI which has an affinity for free C three orders of magnitude greater (reference 16 and Fig. 1).

From the accumulation of results obtained it can be concluded that the F:PKI conjugate binds specifically to the substrate site of free catalytic subunit dissociated from cAMP-dependent protein kinase. This specificity along with the high affinity for C renders the F:PKI valuable for localizing at unit cell resolution the distribution of C in cells stimulated by a variety of agents. While it is certain that the probe binds specifically to C, it remains unclear that it binds exclusively to the active kinase. If the F:PKI binds to proteins other than C they must be in minute quantities or possess a number of characteristics which are identical to those of the catalytic subunit. On the basis of the data described above, this protein(s) would have to possess the following characteristics: (a) it must exhibit the ability to bind F:PKI in a time- and dose-related fashion which is proportional to the activation of cAMP-dependent protein kinase; (b) it must require for binding the presence of specific active arginine residues in the F:PKI in a manner indistinguishable from the free catalytic subunit; and (c) it must have the capacity to cross-react with affinity column-purified antibody directed against the free catalytic subunit. To our knowledge no known protein other than the free catalytic subunit exhibits all of these properties.

Perhaps as important as the specificity and sensitivity of the F:PKI probe is its adaptability. To date we have used the procedure to follow the temporal and spatial kinetics of free C in ovarian granulosa cells (31), cells from anterior pituitary (32), and in normal and neoplastic adrenocortical cells (33). In addition to its use on culture preparations, we have recently adapted the procedure for application on cryotome sections of
intact tissues. The tissue method has been used to study the kinetics of cAMP-dependent protein kinase activation in liver (34), anterior pituitary (32), ovary (31), and adrenal gland (33).

Unlike biochemical methods such as kinase activation assays, the cytochemical procedure is capable of discerning the activation of cAMP-dependent protein kinase in single cell types of a histologically or functionally complex culture or tissue. For example, cultured cells from the anterior pituitary that respond to luteinizing hormone releasing hormone can be distinguished from neighbor cells that do not respond (32). Also, populations of granulosa cells in ovarian follicles that preferentially associate kinase in response to follicle-stimulating hormone can be differentiated from granulosa cells responding only to luteinizing hormone (31). In view of this and its established validity we anticipate that the procedure for localizing subcellular sites of free C will be useful in a variety of systems.

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