Immunoochemical Visualization and Quantitation of Cyclic AMP in Single Cells*

Anthony P. Wiemelt‡‡, Mark J. Engleka‡‡, Annette F. Skorupai‡, and F. Arthur McMorris‡‡‡**

From ‡‡The Wistar Institute, Philadelphia, Pennsylvania 19104 and the ‡‡David Mahoney Institute of Neuroscience, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Adenosine 3′:5′-cyclic monophosphate (cAMP) is a key second messenger in signaling pathways governing many cellular processes. To define the subcellular localization and relative abundance of cAMP, we developed a novel immunoochemical approach based on acrolein fixation to visualize cAMP within cells. We describe here the fixation and immobilization of cAMP within cells and the production of specific, high titer polyclonal antibodies that recognize cAMP. Relative levels of cAMP immunofluorescence were quantitated in glial cells (oligodendrocytes, astrocytes, Schwann cells, and glioma cells) that were either untreated or treated with activators of endogenous adenylyl cyclase to raise cAMP levels. In treated cells, cAMP immunofluorescence is strongly localized in the perinuclear cytoplasm.

Many signals that regulate the growth, development, and metabolism in cells use cAMP as a second messenger (1, 2). Neurotransmitters, growth factors, and hormones signal to appropriate receptors, causing either the stimulation or inhibition of adenylyl cyclase through intermediate guanyl nucleotide regulatory proteins. Adenylyl cyclase catalyzes the conversion of ATP to cAMP, which acts by binding to protein kinase A (PKA) and causing the release of activated catalytic subunits, or by binding and activating cAMP-gated ion channels (3–6). Activated PKA regulates the function of pre-existing cellular proteins by selective phosphorylation and also regulates the synthesis of new cellular proteins by phosphorylating and altering the activity of transcription factors (7–9).

In the oligodendrocyte, the myelinating cell of the central nervous system, cAMP is thought to play independent roles in oligodendrocyte development (10, 11). Whereas experimentally induced cAMP can have potent regulatory effects in these processes, the role of cAMP in normal oligodendrocyte development is not well understood. Cyclic AMP has similar effects in the peripheral myelinating glia, Schwann cells (13), and it is thought that axonal contact causes an elevation in cAMP, inducing proliferation and differentiation (14).

Many questions remain about how cAMP relays signals, due in part to technical limitations that have prevented determination of the subcellular distribution of cAMP or comparison of cAMP levels between single cells. Homogeneous populations of cells may vary in their cAMP content from cell to cell, and conventional assays of cAMP extracted from homogenates of such a population generate a simple average level for the set (15). These assay procedures also mask any useful information about cell-specific changes in cAMP levels in the heterogeneous environments of tissue or cell culture. Finally, there have been no methods for accurately measuring free cAMP levels within the local environment of a single cell. It is quite possible that differential cAMP elevation occurs in different regions of the cell, e.g. the soma versus the processes of neuronal cells. Such information may be invaluable in deciphering the role of cAMP in cellular regulation.

Although antibodies against cAMP have been available for over two decades, they have not proven useful for cell staining, because conventional fixation techniques using formaldehyde or glutaraldehyde form unstable cross-linked products between cAMP and intracellular proteins so that cAMP is lost from the cell (16–18). We have developed methodology based on previous findings (19) that enables immobilization of cAMP by cross-linking it to intracellular proteins while fixing the cell or tissue with acrolein, an unsaturated aldehyde that forms a stable covalent bond between cAMP and proteins when used in the presence of acidic buffers (19, 20). Cyclic AMP can be linked to a carrier protein with acrolein, and this conjugate can be used as an immunogen for antibody production. We generated polyclonal antibodies that recognize acrolein-derivatized cAMP at a high titer and specificity on enzyme-linked immunosorbent assay screens. Use of this antisera to stain a variety of cell types (oligodendrocytes, astrocytes, Schwann cells, and glioma cells) revealed significant differences between untreated cultures and cells treated with forskolin (FSK), cholina toxin, and isobutylmethylxanthine (IBMX) to elevate intracellular cAMP levels. Our results demonstrate that cAMP can be reliably visualized and quantitated by this methodology.

** EXPERIMENTAL PROCEDURES

Production of Anti-cAMP Antibodies—Anti-cAMP polyclonal antibodies were raised in rabbit against cAMP (sodium salt, Sigma) conjugated to keyhole limpet hemocyanin (KLH) (Sigma). Briefly, 25 mM cAMP was incubated with KLH (30 mg) in 30 ml of 1% acrolein (v/v) in 0.1 M sodium acetate buffer, pH 4.75 (acetate buffer) for 4 h at room temperature. Glycine (600 mg) was added for 30 min to quench the
reaction and react with free acrolein, followed by 400 mg of sodium cyanoborohydride (Sigma) for 30 min to reduce double-bonded oxygen and nitrogen, completing cross-linking reactions (21). This solution was dialyzed overnight against three changes of distilled water at 4 °C, frozen and lyophilized. The lyophilized product was white, with a light, spongy consistency. Coupling efficiency of cAMP to KLH by this reaction was 2.5% as determined by quantitating the incorporation of a trace amount (5 μCi) of [3H]cAMP (Amersham) added to the reaction. To optimize immunization with the cAMP-KLH conjugate, two series of rabbits were pretreated to identify an appropriate adjuvant and to determine an optimal immunization schedule that avoids nonspecific cross-reactivity to cAMP. In both series, tyrosylarginine (Kyotorphin, Sigma), which is similar to cAMP in molecular weight and in structure, containing an unsaturated ring structure and both primary and secondary amine groups, was conjugated to KLH and used as antigen. Antigens were presented in Freund’s complete, TiterMax (Cytex), or aluminum hydroxide adjuvants. TiterMax produced the most robust response, and immunization could be carried out to five boosts before cAMP nonspecific reactivity was detected.

To generate anti-cAMP antibodies, rabbits were immunized subcutaneously with 500 mg of cAMP hapten-carrier conjugate emulsified in 0.5 ml of a 1:1 mixture of TiterMax adjuvant and phosphate-buffered saline (PBS). Boosts were given with 200 mg conjugate in 0.5 ml TiterMax/PBS at 21 days, and then every 14 days for a total of four boosts. Blood samples were analyzed at day 14, and then 10 days after each boost. Comparison to control immunizations with a non-cAMP acrolein conjugate and several different adjuvants showed that this regimen produced a robust immune response without raising nonspecific cAMP reactivity.

**Immunofinity Purification**—Antiserum (5 ml) was mixed with cAMP (5 ml) linked to cyanoagel-activated agaro (C-8 attachment, Sigma) for 48 h at 4 °C with rotation. The agarose was transferred to a 2.5-cm column (Bio-Rad) and washed with 100 ml of 10 mM Tris-HCl (pH 7.5), followed by 100 ml of 500 mM NaCl, 10 mM Tris-HCl (pH 7.5). The anti-cAMP antibodies bound to the column by base-sensitive interactions were eluted with two 45-ml washes of 100 mM glycine (pH 2.5). Samples were collected in separate 50-ml polypropylene tubes containing 5 ml of 1 mM Tris-HCl (pH 8.0) to neutralize the pH, and washed with 10 ml Tris-HCl (pH 8.5) until the eluate pH was 8.8. Antibodies bound to the column by base-sensitive interactions were eluted by passing two 45-ml washes of 100 mM triethanolamine (pH 11.5) over the column, and collecting the eluate in separate 50-ml polypropylene tubes containing 5 ml of 1 mM Tris-HCl (pH 7.5). The eluant was concentrated by centrifugal microconcentration (Amicon) and brought to the original starting volume with PBS. Antibody was stored at −80 °C in aliquots after addition of 1% bovine serum albumin and 0.02% sodium azide.

**Antibody Titration**—Antiserum titer and specificity were evaluated using standard enzyme-linked immunosorbent assay (ELISA). Ovalbumin (Sigma) in 0.1 mM carbonate buffer (pH 9.5) was used to coat 96-well micro-ELISA plates (Nunc) at a concentration of 10 μg/ml (50 μl/well) at 4 °C overnight. After rinsing three times with 100 μl/well of 0.05% Tween-20 (Sigma) in PBS (T-PBS), wells were incubated with 25 μl of acetate buffer and 25 μl of 2% acrolein (control), or with 25 μl of 20 mM cAMP in acetate buffer and 25 μl of 2% acrolein for 1 h at room temperature to conjugate cAMP to the adherent ovalbumin. The contents of the wells were then discarded, and 50 μl of 1% glycine (w/v) in acetate buffer was added to the wells for 30 min. This solution was removed, and 50 μl of 1% NaCNBH₃ (w/v) in acetate buffer was added for 30 min. Wells were rinsed three times with T-PBS (100 μl/well) and incubated with anti-cAMP antibody diluted in 0.5% gelatin (w/v), 0.5% Tween-20 (Sigma) in PBS. After 1 h at 37 °C, the plate was rinsed with T-PBS three times, and incubated with peroxidase-conjugated goat anti-rabbit IgG antibody (1:400 dilution, Boehringer Mannheim) for 1 h at 37 °C. These antibodies were removed, the plates washed with T-PBS, and color was developed by incubating wells with 50 μl of perox-

**Fig. 1. Purification of Anti-cAMP Antiserum.** ELISA plates were coated with ovalbumin and fixed with acrolein alone (OA-Acrolein), or with acrolein and 20 mM cAMP (OA-Ac-cAMP). Wells were incubated with unpurified anti-cAMP antiserum or following one round of immunoaffinity purification with cAMP-agarose. Antibody was used at a 1000-fold dilution. Unpurified antiserum shows 3.8-fold greater absorbance in wells containing cAMP than in control wells, whereas purified antiserum shows 121.3-fold greater absorbance. Error bars indicate S.E.

**Fig. 2. Anti-cAMP Antibody Titer.** A, ELISA plates were coated with ovalbumin and fixed as in Fig. 1. Anti-cAMP antiserum was added to ELISA wells at the indicated dilutions. Significant differences between control and cAMP-containing wells are seen to 50,000-fold dilutions (p < 0.01). B, primary astrocyte cultures were treated with 100 μM FSK and 1 mM IBMX for 30 min to elevate intracellular cAMP levels. Cells were fixed with 5% acrolein as described under “Experimental Procedures” and incubated with serial dilutions of anti-cAMP antibody. Confocal imaging and subsequent image analysis was used to quantitate immunofluorescence in perinuclear regions (within 5 μm of the nucleus). All antibody dilutions showed significant differences between treated and untreated cells (p < 0.0001). Error bars indicate S.E.
idase substrate solution (1 vol of 0.182 mM 2,2′-azino-di(3-ethylbenzothiazoline sulfonate), 1 vol of 0.09% hydrogen peroxide, in 0.1 mM citric acid, 0.2 mM sodium phosphate, pH 5.2) for 5 min. The reaction was quenched by the addition of 50 μl of 1 M sulfuric acid, and absorbance was read at 490 nm. For competition assays, diluted antiserum was incubated in the absence of cAMP or cGMP prior to addition to ELISA wells, and antibody to cAMP or cGMP reduced immunoreactivity against cAMP fixed to ovalbumin. Antibody specificity.

Cell Culture—Cloned C6 rat glioma cells (22) obtained from the American Type Culture Collection were used for the characterization of antibody staining. Cells were grown in oligodendroglial cell medium-5 (OM-5) (11) that contains 10% fetal bovine serum, Schwann cells were isolated from 3-day-old rat pups as described (23), explanted onto glass coverslips, and grown for 1 week. To elevate cAMP levels, cells were treated for 30 min at 37 °C with forskolin (Sigma) to directly activate endogenous adenylyl cyclase activity and with isobutylmethylxanthine (Sigma) to inhibit endogenous cAMP-phosphodiesterase activity. Concentrations are indicated in the individual experiments.

Primary glial cell cultures were isolated from dissociated postnatal day 1 rat cerebra as described (24). Such cultures contain mixed glial cell populations, including oligodendrocytes, astrocytes, and microglial cells. Mixed glial cultures were grown in OM-5 medium at 37 °C for 5 days, and enriched for oligodendrocytes by differential shakeoff (25). These cells were inoculated onto 16-well polylysine-coated culture slides and grown an additional day at 37 °C. Astrocytes were treated with FSK and IBMX to elevate cAMP.

Marker Antibodies—Monoclonal antibodies recognizing antigens expressed during specific oligodendrocyte developmental stages were prepared as tissue culture supernatants from hybridoma cells. Antibody to the O4 antigen, which recognizes oligodendrocytes and intermediate-stage oligodendrocyte precursors (27), was used to identify oligodendroglial cells in mixed glial cell cultures. Schwann cell identity was confirmed by the expression of P0 protein (28).

Immunocytochemistry—Cells were fixed in a 5.5% (v/v) acrolein, 0.1 mM acetate buffer solution for 30 min at room temperature. Slides were removed from the fixation solution and quenched in 1% glycine in acetate buffer for 30 min and then placed in 1% NaCNBH3 in acetate buffer for a final 30 min. Anti-cAMP antibody was serially diluted 5–1000-fold in 50 mM Tris-HCl (pH 7.5), 0.4 M NaCl, with 0.5% goat serum (v/v), and 0.05% Triton X-100 (v/v). Slides were incubated with the antiserum overnight at 4 °C, followed by 1 h incubations with biotinylated goat anti-rabbit IgG (1:200, Vector Laboratories, Inc.) and fluorescein isothiocyanate-coupled avidin (1:100, Sigma) both at 37 °C.

Because acrolein fixation disrupted the antigenicity of lipid epitopes recognized by diagnostic marker antibodies for oligodendrocyte development (e.g., O4), live cells were preincubated with antibody for 30 min at 37 °C before fixation to stain primary oligodendrocyte or Schwann cell cultures with antibodies to lipid antigens. Such treatment did not alter cAMP content in cells (data not shown). Fixation and cAMP staining were carried out as described for C6 glioma cells. Primary oligodendrocytes and Schwann cell markers were subsequently stained with rhodamine-labeled goat anti-mouse IgM (μ-chain specific). In experiments where cells were treated to elevate cAMP, stimuli were mixed with the marker antibody solution for presentation to the cells.

Cyclic AMP and guanine 3'-5'-cyclic mono-phosphate (cGMP) in the extract were quantitated by an acetylcholinesterase-based ELISA according to manufacturer’s procedures (Amersham Life Science, Inc.). Protein abundance was determined using a modified Lowry assay, according to the manufacturer’s procedures (Bio-Rad).

The cGMP content of C6 cells was elevated by activating endogenous guanylate cyclase with the addition of 100 μM sodium nitroprusside (Sigma) and 5 mM N-acetylcysteine (Sigma) in OM-5 medium for 30 min at 37 °C. Sodium nitroprusside spontaneously releases nitric oxide (30), which activates intracellular soluble guanylate cyclase (31). N-acetylcysteine forms an S-nitrosothiol adduct with nitric oxide increasing its bioavailability (32).
Visualization and Quantitation of cAMP

RESULTS AND DISCUSSION

A large effort has been devoted to developing methods to visualize, localize, and quantitate the intermediates involved in signal transduction. For protein intermediates, antibody generation has been straightforward and successful. In calcium biology, efforts have also been quite fruitful, and it is now possible to dynamically image small rapid changes in concentrations over time using calcium-sensitive fluorescent dyes (33–35). Using a novel fixation procedure with the aldehyde acrolein, we have developed an antiserum that successfully detects cAMP immunoreactivity.

Antibody Purification—Unpurified antiserum showed high reactivity toward cAMP cross-linked to protein but also showed significant reactivity toward acrolein-modified protein alone. However, following a single affinity purification step using cAMP bound to an agarose support, 98% of all nonspecific immunoreactivity to acrolein-derivatized protein was lost, whereas most of the reactivity toward cAMP was recovered (Fig. 1).

Antibody Titer and Specificity—ELISA screen of serially diluted antiserum indicated that anti-cAMP antiserum was high titer recognizing cAMP bound to ovalbumin at dilutions of 50,000 and greater (Fig. 2A). Antibody titer was also tested in cell staining where dilutions as high as 1:1000 gave significant staining in astrocytes treated for 30 min with FSK and IBMX to elevate cAMP (Fig. 2B). All dilutions between 10- and 1000-fold yielded significantly more staining in treated cultures than in untreated controls (Fig. 2B).

To test the specificity of the antiserum for cAMP, antibody was incubated for 30 min at 37 °C with 1 mM concentrations of various competitors prior to addition of the antibody to ELISA wells. ATP, ADP, AMP, 2′-3′-cAMP, adenosine, and CMP did not compete with the anti-cAMP antibody (Fig. 3A), suggesting that the antigenic determinant recognized by the antiserum does not include the adenosine group of the molecule but that the 3′-5′-cyclic moiety is necessary for antibody binding. Cross-reactivity toward cGMP was detected. When the anti-cAMP antibody was incubated with serial dilutions of cAMP and cGMP, cAMP was found to compete more effectively (Fig. 3B), reducing absorbance by 50% at 16 μM, whereas 36 μM cGMP was required for a similar reduction in absorbance. In the cAMP and cGMP molecules, the most probable reactive regions in the acrolein cross-linkage are the 1-nitrogen group and the amino group attached to the 6-carbon on the purine structure. These regions would be bound to protein during the fixation process, leaving the ribose group of both molecules facing away from the protein. It is likely that antibodies recognize this ribose 3′-5′-cyclic monophosphate portion of both molecules, consistent with the inability of the antiserum to recognize adenosine or any other adenosine nucleotide, including adenosine 2′,3′-cyclic monophosphate, which differs only in the phosphate moiety.

Normal endogenous cellular cAMP levels vary from 5–20 pmol/mg of protein under basal conditions to 100–1000 pmol/mg of protein under treated conditions, whereas cGMP is present at much lower concentrations from basal levels of 0.1–0.3 pmol/mg of protein stimulated levels of up to 10 pmol/mg of protein (36). To determine whether cGMP cross-reactivity interferes with cell staining, C6 cells were treated with 100 μM sodium nitroprusside and 5 mM N-acetylcysteine to activate endogenous intracellular soluble guanylate cyclase and thus elevate cGMP levels (31). ELISA of cGMP in treated cultures showed a 6.6-fold elevation over basal cGMP levels (Fig. 4A), which corresponds well to previously reported maximal values for cGMP in C6 cells (37). When these treated cells were immunostained with anti-cAMP antibody, there was no significant difference in immunofluorescence over control untreated cells (Fig. 4B, p = 0.66). By contrast, treatment with 10 μM FSK and 1 mM IBMX to elevate cAMP resulted in a 3.2-fold elevation of cAMP determined biochemically (Fig. 4A) and a 2.5-fold increase in immunofluorescence staining intensity (Fig. 4B). Maximally stimulated cGMP levels are significantly lower than even basal cAMP levels in the cell. Thus, cross-reactivity with cGMP interferes negligibly with the determination of cellular cAMP content by this method.

Cell Staining—Cell fixation and staining procedures were optimized using astrocytes, and cAMP-specific immunofluorescence was imaged and quantitated by confocal microscopy.
Cells were treated with 10 μM FSK and 1 mM IBMX for 30 min and fixed with acrolein concentrations between 1 and 8% (v/v); 5.5% acrolein yielded optimal immunofluorescence staining intensity (data not shown). Analysis of fixation times between 15 min and 1 h, indicated similar immunofluorescence staining intensity at all intervals (data not shown). We used 5.5% acrolein and 30 min as our standard fixation conditions.

Astrocytes were stained for cAMP immunofluorescence in both basal and treated conditions (Fig. 5). Basal cAMP staining intensity was weak but significantly greater (p < 0.001) than controls in which primary antibody to cAMP had been omitted (Figs. 5, B and F, and 6). The cAMP staining of cytoplasm in untreated cells was slightly more apparent than the nuclei (Figs. 5F and 6), and the staining appeared to be uniformly dispersed (Fig. 5F).

Astrocytes treated with 8 μM FSK, 1 mM IBMX show a marked increase in cytoplasmic cAMP immunofluorescence over basal cAMP levels (Figs. 5L and 6). The majority of the immunofluorescence staining in treated cells was perinuclear (Fig. 5L), although in cells that were most intensely immunoreactive, the staining was uniform throughout the cytoplasm (not shown). Quantitative measurements taken in areas of the perinuclear region showed a 8.0-fold increase in treated cells in staining over basal unstained staining of the same region (Fig. 6). This immunoreactivity was completely blocked by preincubating the antiserum with 1 mM cAMP for 30 min at 37 °C prior to staining (Fig. 5J). In treated cells, nuclear cAMP staining was 4.3-fold more intense than in untreated cells (Fig. 6).

Conventional fluorescence microscopy of Schwann cell cultures treated with FSK and IBMX revealed strong cAMP immunofluorescence in treated cells (Fig. 7D), whereas untreated cells stained weakly (Fig. 7B).

Untreated primary oligodendrocyte-enriched cultures were fixed and double-stained with antibodies to cAMP and to the O4 antigen, which marks intermediate oligodendrocyte precursor cells. Like untreated astrocytes, the untreated O4-positive cells exhibited low intensity nuclear staining with faint cytoplasmic staining (Fig. 8F) localized primarily in the cell body and was less intense in the processes (Figs. 8F and 9). Upon stimulation with 2 μM FSK, 250 ng/ml cholera toxin, and 1 mM IBMX, O4-positive cells dramatically increased cAMP immu-
noreactivity (Figs. 8L and 9). Again, the greatest increase in staining occurred in the perinuclear region and spread outward toward the processes. Perinuclear staining was increased 8.3-fold on average compared with untreated O4-positive cell staining (Fig. 9). Nuclear staining and an unusual punctate pattern of cytoplasmic staining was seen in a subpopulation of treated cells from oligodendrocyte and astrocyte cultures. These patterns may reflect the presence of type II PKA, which contains the cAMP-binding RII regulatory subunit, and is associated with plasma membrane, cytoskeleton, and nuclear membrane (38, 39), in contrast with type I PKA, which is primarily cytoplasmic (7, 8).

The development of an immunological approach to the detection of cAMP has been hindered by the small size of the molecule and its ubiquitous presence in all living systems. Successful presentation of cAMP to an immune system has involved conjugation to a large antigenic protein which elicits a host immune response (16). In prior studies, cAMP was cross-linked to protein by the addition of an acetyl- or succinyl-reactive group to the 2′ hydroxyl on the ribose (40, 41). In a second step, this reactive group was covalently linked to protein carboxyl groups with ethylene dicarbamide. Cyclic AMP protein conjugates were generated with these reactions and used to produce antiserum toward the derivatized cAMP molecule. Indeed, numerous biochemical ELISA kits for cAMP have been developed based on this technique and the antibodies used recognize acetyl- or succinyl-derivatized cAMP with high sensitivity. However, antibody recognition of unmodified cAMP such as naturally occurring cAMP in cells is dramatically lower (42).

Previous approaches to cAMP immunohistochemistry have also suffered from the ineffective immobilization of cAMP within the cell, resulting in its loss during processing (17, 43–45). Formaldehyde and glutaraldehyde fixatives produce unstable adducts between cAMP and cellular proteins and thus do not effectively immobilize cAMP. Microwave irradiation has also been used for fixation of cAMP within the cell (46), but this procedure has proven difficult to replicate. With all of these methods, free cAMP in the cell is washed away during fixation 

FIG. 7. Immunofluorescence staining of Schwann cells. Schwann cells were treated with 2 μM FSK and 1 mM IBMX for 30 min at 37 °C, and fixed with acrolein. Cyclic AMP is stained with fluorescein isothiocyanate (green, panels B and D), whereas Schwann cells are identified by positive staining of the P0 antigen indirectly conjugated to rhodamine (red, panels A and C). Cells were untreated (panels A and B) or treated (panels C and D). Cyclic AMP immunofluorescence is greatly increased in treated cells.

FIG. 8. Immunofluorescence staining of primary oligodendrocytes. Live primary glial cell cultures enriched for oligodendrocytes were incubated for 30 min at 37 °C with O4 antibody to detect oligodendrocytes and precursors in the absence (A–F) or presence (G–L) of 2 μM FSK, 250 ng/ml cholera toxin, and 1 mM IBMX to elevate intracellular cAMP. Cells were then fixed with acrolein and stained with anti-cAMP antibody (1:50). O4 immunoreactivity is shown in red (rhodamine), whereas cAMP immunoreactivity is shown in green (fluorescein isothiocyanate). As in Fig. 5, cells in the top row were treated in the absence of the primary anti-cAMP antibody, cells in the middle row were stained with anti-cAMP antibody preincubated with 1 mM cAMP for 30 min, and cells in the bottom row were stained with anti-cAMP antibody.
Visualization and Quantitation of cAMP

**Figure 9. Quantitation of immunofluorescence staining in oligodendrocytes.** The mean fluorescence intensities from each cellular region (nuclear, perinuclear cytoplasmic, or peripheral cytoplasmic) from 20 cells at each condition (untreated and treated) were quantified using image analysis as described under “Experimental Procedures.” Error bars indicate S.E.

(17, 43–45) and any detectable immunoreactivity is likely due to cAMP that was bound to protein (e.g. PKA) at the time of fixation (44, 45). Thus, accurate measurement of total cellular cAMP requires that it be locked into the cell before or during the fixation procedure as achieved by acrolein fixation.

Another approach to the imaging of cAMP within cells involves microinjection of PKA conjugated with fluorescein on the catalytic subunit and rhoadmine on the regulatory subunit (47). Because of the different fluorescence energy transfer characteristics of the derivatized PKA in the cAMP-bound and free forms, it acts essentially as a cAMP-sensitive dye (47). The method allows analysis of changes in cAMP content with time in the same cell, but because the signal depends on the amount of PKA microinjected into the cell as well as the amount of endogenous PKA, it is not as useful for the comparison of cAMP content in different cells. This method also relies on the labile microinjection of individual cells and the use of specialized equipment for spectral analysis of emitted light. Our method is much less laborious and allows comparison of different cells.

The ability to monitor total cAMP levels in single cells will help to elucidate many longstanding questions in the biology of signal transduction. The immunochemical approach presented here provides a rapid and convenient method to address these questions.

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**REFERENCES**

1. Iyengar, R. (1996) Science 271, 461–463
2. McKeown, G. S. (1991) Curr. Opin. Cell Biol. 3, 213–217
3. Zhafii, F. (1993) Exp. Suppl. (Basel) 66, 135–145
4. DiFrancesco, D., and Tortora, P. (1991) Nature 351, 145–147
5. Lester, D. S., Asher, C., and Garty, H. (1988) Am. J. Physiol. 254, C802–C808
6. Klein, P. S., Sun, T. J., Saxe, C. L., Kimmel, A. R., Johnson, H. L., and Devreotes, P. N. (1988) Science 240, 1467–1472
7. Beebe, S. J. (1994) Semin. Cancer Biol. 5, 285–294
8. Francis, S. H., and Corbin, J. D. (1994) Annu. Rev. Physiol. 56, 237–272
9. Meinkoth, J. L., Alberts, A. S., Went, W., Fantozzi, D., Taylor, S. S., Hagiwara, M., Montminy, M., and Feramisco, J. R. (1988) Mol. Cell. Biochem. 127/128, 179–186
10. Rall, D. W., and McMorris, F. A. (1993) J. Neurosci. Res. 37, 287–294
11. Rall, D. W., and McMorris, F. A. (1990) J. Neurosci. Res. 37, 43–46
12. Rall, D. W., and McMorris, F. A. (1989) Dev. Biol. 133, 437–446
13. Jessen, K. R., Misky, R., and Morgan, L. (1991) Ann. N. Y. Acad. Sci. 633, 78–89
14. Ratner, N., Glaser, L., and Bunge, R. P. (1984) J. Cell Biol. 98, 1150–1155
15. Horton, J. K., and Baxendale, P. M. (1995) Methods Mol. Biol. 41, 91–105
16. Steiner, A. L., Parker, C. W., and Kipnis, D. M. (1972) J. Biol. Chem. 247, 739–744
17. De Vente, J., Steinbusch, H. W., and Schipper, J. (1987) Neurosci. 22, 361–373
18. Rall, T. W., and Lehne, R. A. (1982) J. Cyclic Nucleotide Res. 8, 243–265
19. De Vente, J., Schipper, J., and Steinbusch, H. W. (1992) Histochemistry 99, 457–462
20. King, J. C., Lechan, R. M., Kugel, G., and Anthony, E. L. (1993) J. Histochem. Cytochem. 41, 62–68
21. Peter, M. E., Hall, C., Ruhlmann, A., Sancho, J., and Terhorst, C. (1992) EMBO J. 11, 933–941
22. Benda, P., Lightbody, J., Sato, G., Levine, L., and Sweet, W. (1968) Science 161, 370–371
23. Scherer, S. S., Wang, D. Y., Kuhn, G., Lemke, G., Wrbetz, L., and Kamholz, J. (1994) J. Neurosci. 14, 539–542
24. McMorris, F. A., Smith, T. M., DeSalvo, S., and Furlanetto, R. W. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 822–826
25. McCarthy, K. D., and de Veijis, J. (1980) J. Cell Biol. 85, 890–902
26. McMorris, F. A. (1981) J. Neurochem. 36, 506–515
27. Sommer, I., and Schachman, M. (1981) Dev. Biol. 83, 311–327
28. Mirsky, R., and Jessen, K. (1993) Semin. Neurosci. 2, 425–435
29. McMorris, F. A. (1980) Biochim. Biophys. Acta 672, 560–568
30. Bates, J. N., Baker, M. T., Guerra, B. J., and Harrison, D. G. (1991) Biochem. Pharmacol. 42, (suppl. 2) S157–S165
31. Lad, P. J., Liebel, M. A., and White, A. A. (1981) Biochem. Pharmacol. Res. Commun. 103, 629–637
32. Ignarro, L. J., Lippton, H., Edwards, J. C., Baricos, W. H., Hyman, A. L., Kadowitz, P. J., and Gruetter, C. A. (1981) J. Pharmacol. Exp. Ther. 218, 54–55
33. Bologou, S. (1995) Biochem. Soc. Trans. 23, 627–629
34. Diliberto, P. A., Wang, X. F., and Herman, B. (1994) Methods Cell. Biol. 40, 243–262
35. Tsien, R. Y. (1992) Am. J. Physiol. 263, C723–C728
36. Villegas, S., and Brunton, L. L. (1996) Anal. Chem. 68, 102–103
37. Simmons, M. L., and Murphy, S. (1992) J. Neurochem. 59, 957–965
38. Zhang, Q., Carr, D. W., Lerca, K. M., Scott, J. D., and Newman, S. A. (1996) Dev. Biol. 176, 51–61
39. Stein, J. C., Farooq, M., Norton, W. T., and Rubin, C. S. (1987) J. Biol. Chem. 262, 3002–3006
40. Harper, J. F., and Brooker, G. (1975) J. Cyclic Nucleotide Res. 1, 207–218
41. Cailia, H. L., Racine-Weibusch, M. S., and Delaage, M. A. (1973) Anal. Biochem. 56, 394–407
42. Pradelles, P., Grasso, J., Chabardes, D., and Guise, N. (1989) Anal. Chem. 61, 447–453
43. Cumming, R., Dickson, S., and Arbuthnott, G. (1980) J. Histochem. Cytochem. 28, 713–719
44. Steiner, A. L., Ong, S. H., and Wedner, H. J. (1976) Adv. Cyclic Nucleotide Res. 7, 115–155
45. Kapoor, C. L., and Steiner, A. L. (1982) in Handbook of Experimental Pharmacology (Kebabian, J. W., and Nathanson, J. A., eds) pp. 333–354, Springer-Verlag, Berlin
46. Barsony, J., and Marx, S. J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1198–1192
47. Adams, S. R., Haroutunian, A. T., Buechler, Y. J., Taylor, S. S., and Tsien, R. Y. (1991) Nature 349, 694–697