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Immunocytology on microwave-fixed cells reveals rapid and agonist-specific changes in subcellular accumulation patterns for cAMP or cGMP

(signal transduction/catecholamine/calcitonin/prostaglandin/atrial natriuretic peptide)

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ABSTRACT **We developed a method for cAMP and cGMP immunocytology based upon fixation by microwave irradiation. Fixation by microwave irradiation prevented three problems found with other fixation methods: nucleotide loss from cells, nucleotide diffusion within cells, and chemical modification of immunologic epitopes. Six agonists (four that stimulate adenylate cyclase and two that stimulate guanylate cyclase) produced cAMP or cGMP accumulation patterns that were agonist-specific, dose-dependent, detectable at physiologic concentrations of hormone, and time-dependent within 15 sec to 30 min. cAMP accumulation after 1 mM forskolin was greatest in the nucleus. Isoproterenol, prostaglandin E_2, or calcitonin caused initial accumulation of cAMP along the plasma membrane, but later accumulation was greater in the cytoplasm. With calcitonin the later accumulation of cAMP was selectively perinuclear and along the nuclear membrane. Sodium nitroprusside stimulated cGMP accumulation diffusely throughout the cytoplasm. Atrial natriuretic peptide initiated cGMP accumulation near the plasma membrane, and cGMP accumulation moved from there into the cytoplasm. In conclusion, microwave irradiation preserved cell structure and allowed visualization of expected as well as unsuspected changes in intracellular accumulation patterns of cAMP and cGMP.**

Biochemical and cytologic studies have identified adenylate cyclase and guanylate cyclase activity in many cell compartments (including plasma membrane, cytoplasm, and nucleus) and have shown that cAMP and cGMP couple to protein kinases, to ion channels, and perhaps to other effectors (1–8). Signal transduction is believed often to involve intracellular movement and redistribution of cAMP or cGMP after it is synthesized in response to agonists (1, 3). Reliable analysis of intracellular distribution of cAMP or cGMP could enhance our understanding of hormone action (5).

cAMP and cGMP have been visualized after fixation by freezing (9–11), but retention of cyclic nucleotides has been low, presumably due to elution during processing. Formaldehyde fixation improves the retention of cyclic nucleotides but is too slow to prevent cyclic nucleotide degradation by enzymes or movement during fixation (12). Moreover, it reduces immunoreactivity by altering the chemical structure of these small molecules (12). Some improvement in sensitivity was achieved by using antiserum raised against formaldehyde-conjugated protein–cyclic nucleotide complexes (13, 14), but this technique still did not permit visualization of subcellular compartmentalization (especially intranuclear localization). Specificity of these antibodies was dependent on nucleotide microenvironment (15), and antibody penetration (12, 16) was a persistent problem with this method.

Because cAMP and cGMP are heat-stable, we evaluated fixation with microwave irradiation. Microwave fixation of tissues was introduced in the early 1970s. As a result of recent technical developments, it became suitable for fixation and histologic analysis of intra- and extracellular proteins (17–21). Microwave radiation has also been used to prepare tissues for homogenization and subsequent radioimmunoassay of cyclic nucleotides (22, 23). Here we report its use for visualizing rapid subcellular changes of cAMP and cGMP.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium was from Biofluids (Rockville, MD); Eagle's medium without phenol red but with glutamine and gentamicin was from GIBCO; defined bovine serum was from HyClone; insulin (beef/pork) was from Eli Lilly; ITS-Premix culture medium additive (ITS = insulin, transferrin, selenium, linoleic acid, bovine serum albumin), human fibronectin, and mouse epidermal growth factor were from Collaborative Research. Lab-Tek tissue culture chamber slides were from Nunc. CytoPrep spray fixative was from Fisher; mounting solution was from Zymed Laboratories. Sodium nitroprusside, cGMP, cAMP, prostaglandin E_2 (PGE_2), forskolin, 3-isobutyl-1-methylxanthine (IBMX), and aprotinin were from Sigma; (−)-isoproterenol was from Sterling–Winthrop Research Institute; rat atrial natriuretic peptide (ANP)-(8–33) was from Peninsula Laboratories. ^{125}I-labeled succinyl-cGMP-tyrosine methyl ester (specific activity, >2000 μCi/μM; 1 Ci = 37 GBq) was from Biomedical Technologies. 8-(Fluoresceinyl)thioguanosine 3′,5′-cyclic monophosphate and 8-(fluoresceinyl)thioadenosine 3′,5′-cyclic monophosphate were from Molecular Probes. Gelatin was from Bio-Rad. Immunocytology reagents were from Vector Laboratories or from Sigma. Polyclonal antiserum against cAMP (raised in goat 122G) was provided by Gary Brooker (24), polyclonal rabbit antibody against cGMP (raised in rabbit AB33/5-6-81) was provided by Kevin J. Catt (National Institutes of Health), and monoclonal mouse antibody against cGMP (4B6) and cAMP (10C2) were from Michael A. Kaliner (25).

Cell Treatment. Normal human skin fibroblasts (26) and LLC-PK_1 porcine kidney epithelial cells (American Type Culture Collection) were grown as described (26). Cells at 10,000 cells per chamber were subcultured in two-chamber tissue culture slides coated with human fibronectin at 10 μg per well. When cells reached 70% confluency, the culture medium was changed to phenol red-free and serum-free medium supplemented by 1% ITS, and cells were maintained in this medium for 48 hr before agonist exposure. Agonists (forskolin, PGE_2, (−)-isoproterenol, salmon calcitonin, so-

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Abbreviations: FITC, fluorescein isothiocyanate; PGE_2, prostaglandin E_2; ANP, atrial natriuretic peptide; IBMX, 3-isobutyl-1-methylxanthine.

dium nitroprusside, or ANP) were added at various concentrations and times in the following assay buffer: Eagle's medium without phenol red, without sodium bicarbonate, but containing 25 mM Hepes, 0.5 mM Ca^{2+}, 0.5 mM Mg^{2+}, bovine serum albumin (0.3 g/liter), and aprotinin (800 units/liter) at pH 7.4 and 37°C.

Microwave Fixation. We used a commercial microwave oven (Amana model RCS 700) with a maximum power of 650 W and an operating frequency of 2.45 GHz, with continuous mode (no on/off cycles). We fixed only a single slide at a time, placing it at a defined locus. Because of its ceramic shelf, we did not have to put into the oven an additional water load. The latter would have resulted in uneven fixation and burning artefacts (26). Irradiation of such a small sample was possible because of magnetron protection against mismatch (27) in this model. Uniformity of heating was further improved by a mode stirrer (28). Before cells were placed into the chamber, microwave power was turned on for 1 min (preheating the oven floor to approximately 42°C). After removal of assay buffer, the total volume of cells and residual buffer was 200 μl per well (calculated from microscope-assisted measurement of sample thickness). During irradiation, slide wells were covered by the lid to prevent dehydration of cells. Optimal exposure time at maximal power output was 10 sec for fibroblasts and 9 sec for LLC-PK_1 cells, yielding final temperature (monitored with a needle microprobe thermometer) of 50 ± 6°C. Immediately after irradiation, 1 ml of ice-cold isotonic phosphate-buffered saline (PBS) was added to each slide well. Optimal irradiation conditions were established by monitoring integrity of histologic structure and cGMP retention with various heating and cooling times and by monitoring sample temperature and temperature homogeneity at different locations in the oven.

Other Fixation Methods. Some control samples were fixed by one of the following procedures: (*i*) freezing, (*ii*) freezing followed by an ethanol-based spray fixative (CytoPrep) (29), (*iii* and *iv*) covering cell monolayers in each chamber with 500 μl of 3.5% (vol/vol) formaldehyde for 30 min with or without being followed by 0.1% Triton-X 100 for 10 min, respectively, and (*v* and *vi*) microwave irradiation of samples covered with 3.5% formaldehyde as above, with or without Triton-X 100.

Cell-Free System. We prepared a gelatin matrix model system [modified from version of deVente *et al.* (15)] to analyze lower detection limits and recovery for imaging of cyclic nucleotides with graded concentrations of cAMP and cGMP (2×10^{-14} M to 2×10^{-3} M) or with ^{125}I-labeled succinyl-cGMP-tyrosine methyl ester (0.25 μCi/100 μl). Aliquots (200 μl per well) of these solutions were spread on the surface of two-chamber slides and chilled at 4°C. After gelation, slides were fixed with microwave or with the other methods (see above). After microwave irradiation, or other fixation, the gelatin-containing slides underwent the same immunostaining procedure as the cells. To calculate retention we measured ^{125}I in treated and untreated gelatin samples.

Cell Loading with Fluorescent Cyclic Nucleotides. Fibroblasts were incubated with 1 mM fluorescein isothiocyanate (FITC)-cAMP or 1 mM FITC-cGMP in assay buffer under four conditions, resulting in distinct spatial distributions of fluorescence.

Stabilization of fluorescence patterns was monitored under the microscope, and, at the end of incubation, cells were fixed with various methods. Slides were then subjected to immunocytology procedures to image exogenously loaded cyclic nucleotide. Images were recorded before fixation, after fixation (by direct fluorescence microscopy), and after immunostaining (by transmitted light microscopy).

Immunocytology Procedures. After we removed the upper segment of the slide unit, we incubated the lower glass slide for 30 min with blocking serum (normal serum from species of second antibody), 15 min with avidin, and 15 min with biotin (blocking kit). Primary antibodies were incubated with specimens for 1 hr at the following final dilutions [in 4% (vol/vol) human serum]: mouse monoclonal against cAMP diluted 1:1000, goat polyclonal against cAMP diluted 1:1200, mouse monoclonal against cGMP diluted 1:800, or rabbit polyclonal against cGMP diluted 1:500. Affinity-purified biotinylated second antibody (in the dilution recommended by the manufacturers) was applied for 30 min and then an avidin–biotinylated detector reagent (alkaline phosphatase, horseradish peroxidase, or phycoerythrin) was applied for 30 min. Alkaline phosphatase substrates and levamisol were from Vectra kits. Peroxidase substrates (Sigma) were 3-amino-9-ethylcarbazole or diaminobenzidine tetrahydrochloride. Alternatively, in some experiments (with and without agonists), FITC-labeled second antibodies were also used. All incubation steps were followed by three 5-min rinses with PBS. All staining procedures were at room temperature.

Photomicroscopy and Image Processing. A Zeiss photomicroscope model 3 equipped for epifluorescence was used unless indicated otherwise. Pictures were taken with automatic exposure setting, with transmitted light on Kodachrome KPA-135/40 ASA color reversal film and with fluorescence on Ecktachrome 800/1600 film. The shutter was opened for the same duration for paired experimental-control samples.

RESULTS

Comparisons Among Fixation Methods. *Morphology.* The cell morphology after rapid microwave fixation was indistinguishable from that after other conventional fixation methods, except in small regions close to the field edges. After microwave fixation the penetration of first and second antibodies was uniform for all cell compartments.

Nucleotide recovery after fixation. Recovery of ^{125}I-labeled succinyl-cGMP-tyrosine methyl ester in a gelatin matrix was the following: 4 ± 3% without fixation, 5 ± 3% after freezing and CytoPrep spray treatment, 25 ± 1% after formaldehyde, 48 ± 2% after microwave irradiation, and 68 ± 5% after microwave irradiation of formaldehyde-covered cells (mean ± SD, $n = 5$).

Sensitivity and specificity. The lower limits for detection of cAMP and cGMP in the gelatin matrix after microwave fixation were the following: 10 pM cAMP with 122G goat polyclonal antibody, 1 pM cGMP with 4B6 monoclonal antibody, and 0.1 nM cGMP with AB33/5-6-81 rabbit polyclonal antibody. Cross-reaction from rabbit polyclonal anti-cGMP antibody with cAMP was less by a factor of about 10 (0.1 mM) than from the monoclonal anti-cGMP antibody (1 mM).

Pattern retention. The subcellular distribution patterns of exogenously added fluorescent cAMP or cGMP that we imaged by direct fluorescence were dependent upon composition of incubation medium, incubation temperature, and incubation time during cell loading (Fig. 1).

Fixation by different methods resulted in major differences in signal intensity and pattern retention of exogenously added cyclic nucleotide. Fixation by freezing resulted in substantial loss in the amount of retained fluorescent cyclic nucleotide; however, the speckled cytoplasmic and the nuclear fluorescence patterns were retained at lower intensity and could also be recognized by immunocytology. When an ethanol-based fixative (CytoPrep) was used, more fluorescence was retained than after cryofixation alone, but the subcellular distribution pattern of fluorescence was never retained. After formaldehyde fixation or after combined formaldehyde-microwave fixation, although fluorescence was better retained than after freezing, all four primary antibodies failed to give any cAMP or cGMP signal. After Triton X-100 treatment, the fluorescent cyclic nucleotides redistributed ran-

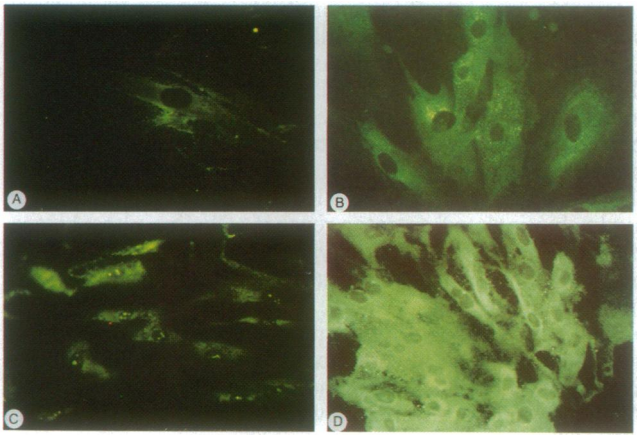

FIG. 1. Spatial distribution of FITC-labeled cGMP after microwave fixation. Fibroblasts were incubated with 1 mM FITC-cGMP as follows. (*A*) At 4°C for 15 min (signal along the cell surface). (*B*) At 37°C for 3 min (speckled cytoplasmic signal). (*C*) In the presence of 1 mM calcium at 37°C for 15 min (signal in the cytoplasm but most prominent in the nucleus). (*D*) At 37°C for 5 min with albumin, saponin, and 0.5 mM calcium (diffuse cytoplasmic signal). (×85.)

domly. After microwave fixation, the intensity of incorporated fluorescent cyclic nucleotide and its spatial distribution were almost completely retained (Fig. 1). Furthermore, the exogenously loaded fluorescent cAMP and cGMP, after microwave fixation, showed the same localization by direct fluorescence microscopy as by immunocytology with any of the four primary antibodies.

Controls for validation. We confirmed the specificity of immunodetection in experiments with fibroblasts fixed by microwave irradiation after exposure to 10 μM forskolin (for cAMP) or 1 mM sodium nitroprusside (for cGMP). Controls included (*i*) substitution of primary antibody with nonimmune serum or nonimmune ascites fluid; (*ii*) preincubation of primary antibody with its target nucleotide or with the other nucleotide (1 mM, overnight at 4°C) in the presence of 0.5 mM IBMX (immuno-inhibition test); (*iii*) comparison between two primary antisera to the same cyclic nucleotide; and (*iv*) comparison among at least two second antibody detection systems. There was no detectable staining when primary antibody was omitted, replaced by nonimmune serum or ascites fluid, or preincubated with the homologous nucleotide in the immuno-inhibition test. Changes in detection methods affected to a modest degree sensitivity and background but not subcellular distribution pattern.

Intracellular cAMP and cGMP Without Agonists. Cells incubated in assay buffer for up to 30 min showed very little immunoreactivity representing cAMP or cGMP. Cells incubated in buffer with 0.5 mM IBMX showed staining for cAMP and cGMP, and the subcellular accumulation patterns were dependent on incubation time (data not shown). In the experiments reported below, all agonists were tested without IBMX on human dermal fibroblasts. Some of the agonists were also tested on LLC-PK_1 kidney epithelial cells.

cAMP Accumulation Pattern After Agonists. *Forskolin.* Forskolin for 3 min caused cAMP accumulation in the cytoplasm close to the plasma membrane and diffusely in the nucleus at 0.1–10 nM. Forskolin at 0.1 μM or greater caused cAMP accumulation as well in the cytoplasm and along the nuclear membrane. At 1 mM the cAMP accumulation was maximal and was most concentrated in the nucleus (Fig. 2).

The time course with forskolin (1 mM) showed nuclear and perinuclear accumulation of cAMP within 15 sec (the earliest time evaluated); after 30 sec both cytoplasmic and nuclear staining were accentuated. Intranuclear accumulation was the most prominent at all time points, reaching maximum

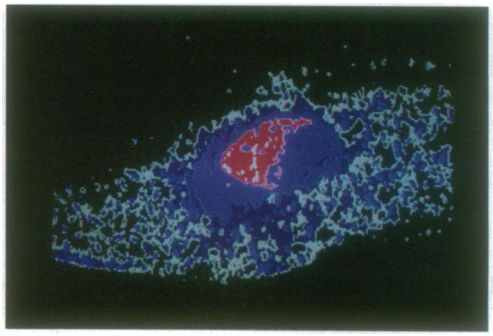

FIG. 2. Intracellular cAMP accumulation pattern after stimulation with forskolin (10 μM for 3 min). Samples were immunostained with goat polyclonal antibody against cAMP and with FITC-labeled second antibody. The image was taken from a Zeiss epifluorescent microscope, digitally processed, and color coded for fluorescent intensity (image processor from Baltimore Instrument). Red is highest, blue is intermediate, and green is moderate intensity. (×150.)

after 5 min. cAMP accumulation was maximal in cytoplasm at 1 min and by 3 min was also along the plasma membrane and along the nuclear membrane. cAMP distribution after forskolin was similar in human skin fibroblasts and in LLC-PK_1 cells. Forskolin at 1 mM caused no accumulation of cGMP for up to 20 min.

Isoproterenol. We tested graded doses of (−)-isoproterenol for 3 min. Isoproterenol at 1 nM caused detectable cAMP accumulation in the plasma membrane and in the cytoplasm. cAMP accumulation was maximal at 10 μM, at which concentration cAMP accumulated also, though to a lesser degree, in the nucleus.

The time course during (−)-isoproterenol (0.1 μM) showed cAMP accumulation in the plasma membrane and cytoplasm at 30 sec with maximum accumulation at these locations at 5 min; small amounts accumulated in the nucleus only beyond 15 min.

PGE_2. We found a similar dose-dependent distribution of cAMP after 3 min with PGE_2 as with (−)-isoproterenol. The threshold dose was 1 nM, and maximal effect was at 1 μM.

The time course of cAMP accumulation pattern with 0.1 μM PGE_2 also was similar to that with (−)-isoproterenol. Time-dependent changes in patterns of cAMP accumulation after isoproterenol and after PGE_2 were similar in LLC-PK_1 cells and in fibroblasts.

Calcitonin. We tested graded doses of salmon calcitonin for 3 min. Calcitonin at 0.1 nM caused detectable cAMP accumulation in the cytoplasm. Higher concentration (maximum at 0.1 μM) resulted in increasing immunoreactivity in cytoplasm close to the nuclear membrane.

The cAMP accumulation patterns with calcitonin (0.1 μM) had the following time course. The first detectable cAMP accumulation was near the plasma membrane after 30 sec. Accumulation near plasma membrane was no longer recognizable after 1 min. Cytoplasmic accumulation increased from 45 sec up to 2 min. After 3 min with calcitonin cAMP accumulated most intensely in the perinuclear region and along the nuclear membrane (Fig. 3). After 10 min a small amount of diffuse nuclear accumulation also occurred. Calcitonin caused similar time-dependent changes in cAMP accumulation pattern in LLC-PK_1 cells and in skin fibroblasts (Fig. 3).

cGMP Accumulation Pattern After Agonists. *Sodium nitroprusside.* A 3-min incubation with graded doses of sodium nitroprusside caused an increase of cGMP in the cytoplasm near the plasma membrane at 1 nM and distributed diffusely in the cytoplasm with higher concentrations (Fig. 4). At a high dose (1 mM), there was also some diffuse intranuclear accumulation of cGMP.

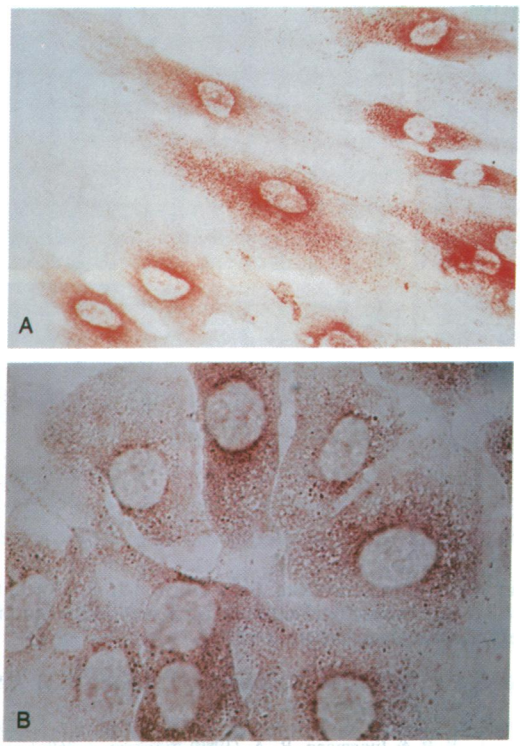

FIG. 3. Intracellular cAMP accumulation pattern after salmon calcitonin (0.1 μM for 3 min). (*A*) Human skin fibroblasts. (*B*) LLC-PK_1 porcine kidney epithelial cells. Samples were immunostained with goat polyclonal antibody against cAMP, a biotinylated second antibody, and an avidin–biotinylated alkaline phosphatase and levamisol/red substrate solutions. (×120.)

Analysis of the time course with sodium nitroprusside (1 mM) showed peripheral cytoplasmic cGMP accumulation at 30 sec; accumulation was diffuse throughout the cytoplasm at 1 min; at 3 min a small amount of diffuse nuclear accumulation was also seen. The amount of cGMP accumulation reached maximum at 5 min at which time it was most intense in the cytoplasm.

ANP. Graded doses of ANP for 3 min resulted in cGMP accumulation in the plasma membrane and in the cytoplasm at 0.1 nM, maximal accumulation at 0.1 μM, and less cGMP at higher doses.

The time course during ANP (0.1 μM) showed cGMP accumulation along the plasma membrane at 15 sec (the earliest time tested); by 30 sec the accumulation was greater and was also further into the cytoplasm. Cytoplasmic cGMP became evenly distributed by 45 sec and became maximal at 3 min. There was no nuclear accumulation of cGMP throughout 30 min with ANP (Fig. 5).

DISCUSSION

We confirmed that cell structure was satisfactory for light microscopy after fixation by microwave irradiation (17–21). Fixation by microwave or formaldehyde allowed good retention of exogenously added fluorescent cyclic nucleotides whereas cryofixation did not; the poor recovery of cyclic nucleotide accounted for poor sensitivity in immunocytology after cryofixation. Microwave fixation also allowed immunodetection of cAMP and cGMP in unique subcellular distribution patterns; these patterns were not detected by immunolabeling after formalin fixation because of loss of cyclic nucleotide reactivity with the primary antibody. Furthermore, we found that ethanol or detergent, often used to improve access for staining reagents, caused disruption of cyclic nucleotide patterns. Unlike other fixation methods, fixation with microwave directly increased permeability of cells sufficiently to allow proper access of reagents without need for any further permeabilization (16, 19).

The unique patterns of intracellular accumulation of cAMP and cGMP in response to agonists were a further confirmation of the utility of the microwave fixation method. The agonist dose and time dependency as well as specific accumulation patterns of cyclic nucleotides correlate well with biochemical results (1, 5, 10).

Our methods differ in detail from prior reports on microwave fixation methods. We used higher power and reduced exposure time (10 sec versus 30 sec–4 min) (16–20). We omitted additional water load from the oven chamber, and we irradiated one small single sample at a time. These changes and a wave guide and mode stirrer on our apparatus facilitated better homogeneity of fixation and integrity of structure without need for aldehyde fixation. In some methods (30), cells were allowed to dry during fixation; this not only compromises preservation of cell structure but also may increase nonspecific binding of antibodies. We prevented drying by shortening irradiation time, covering the samples, and rapidly replacing medium. We also omitted aldehyde

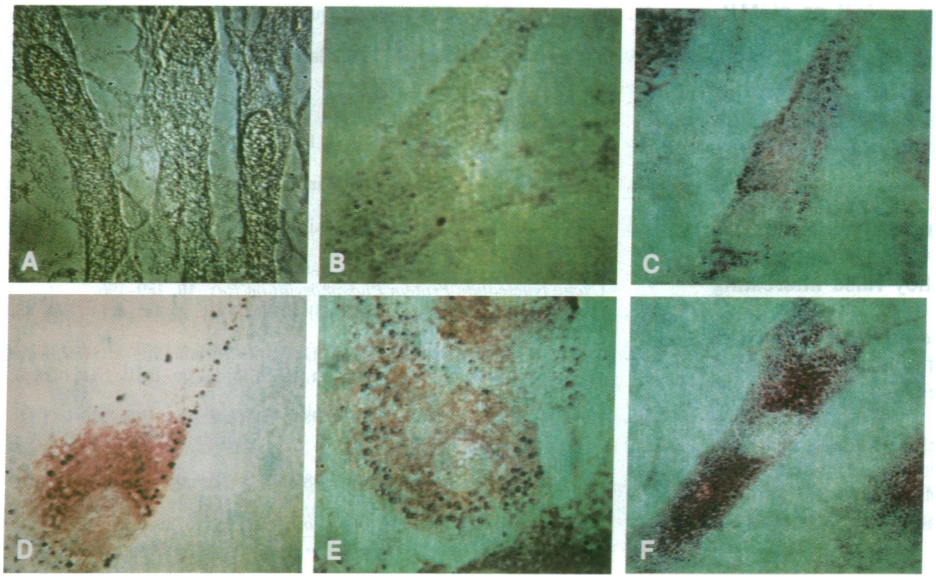

FIG. 4. Dose–response observations of sodium nitroprusside (for 3 min) on accumulation pattern of cGMP in human skin fibroblasts. (*A*) Control. (*B*) cGMP at 1 nM. (*C*) cGMP at 10 nM. (*D*) cGMP at 0.1 μM. (*E*) cGMP at 10 μM. (*F*) cGMP at 1 mM. Samples were immunostained with a mouse monoclonal antibody against cGMP, a biotinylated second antibody, an avidin–biotinylated alkaline phosphatase, and levamisol/red substrate solutions. Image processing was by G. W. Hannaway and Assoc. (Boulder, CO). (×150.)

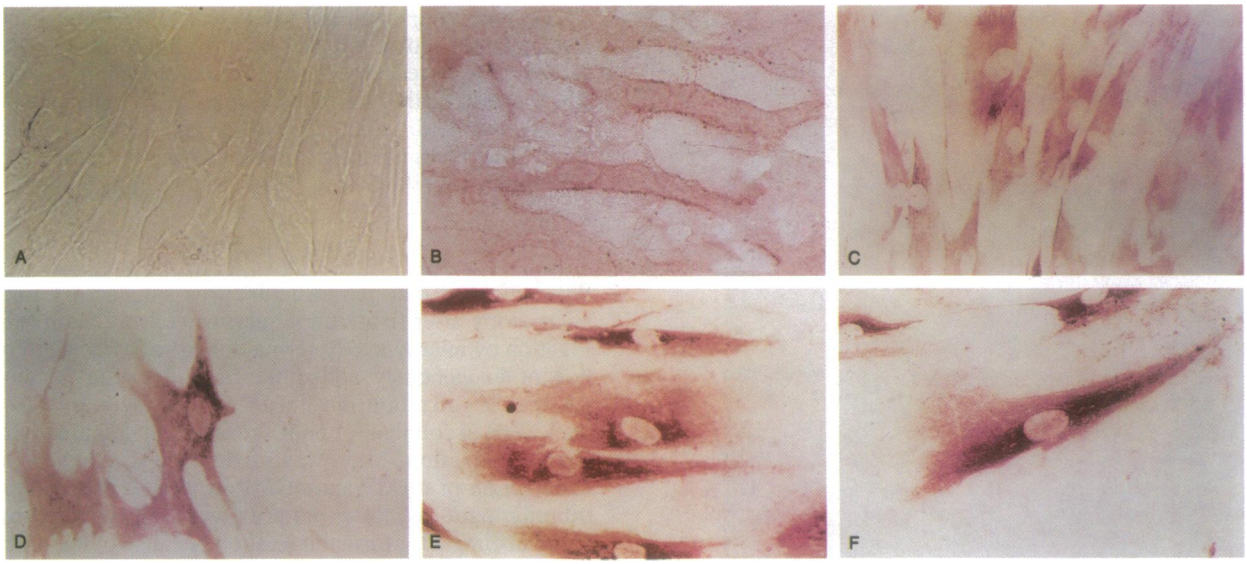

FIG. 5. Time course of ANP (0.1 μM) effect on cGMP accumulation pattern in human skin fibroblasts. (*A*) Control. (*B*) At 15 sec. (*C*) At 30 sec. (*D*) At 45 sec. (*E*) At 1 min. (*F*) At 3 min. Samples were immunostained with a mouse monoclonal antibody against cGMP, a biotinylated second antibody, an avidin–biotinylated horseradish peroxidase, and 3-amino-9-ethylcarbazole substrate. (×170.)

fixatives during microwave irradiation to preserve immunoreactivity of cAMP and cGMP (21, 28, 31).

The mechanisms whereby microwave radiation causes fixation of cAMP and cGMP are unknown as are the mechanisms for microwave fixation of proteins. Local heat generated by oscillation of polar molecules cannot be the only factor, since rapidly raising the temperature to the same extent by other methods did not produce similar fixation in our hands (data not shown) or in other studies (32). Some possible mechanisms include alteration of molecular hydrogen bonds, proton tunneling, and the formation of new disulfide bonds between proteins and small polar molecules (17).

The intracellular patterns of cyclic nucleotide accumulation might result from combinations of differences in local production rates, local degradation rates, local binding to proteins, and intracellular flow. Previous biochemical studies suggested that cAMP and cGMP can be produced in the immediate vicinity of their agonist receptors (1, 5). Thus the cyclic nucleotide accumulation patterns we observed at early time points probably represent accumulation near sites of synthesis.

Cells loaded with fluorescent-labeled cAMP or cGMP, showed spatial distributions of fluorescence dependent on calcium, buffer, and temperature. Similar factors may also influence redistribution of endogenously generated free or protein-bound cAMP or cGMP (3, 9). The cyclic nucleotide accumulation patterns we observed at later time points may represent redistribution.

The perinuclear accumulation of cAMP beyond a 3-min incubation with calcitonin and the principally intranuclear accumulation of cAMP after forskolin were not anticipated from prior biochemical findings, and they raise interesting issues for further study.

The current method for fixation should lead to further understanding of the direct and indirect actions regulated in cell compartments by cyclic nucleotides.

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