

Imaging of cAMP-dependent protein kinase activity in living neural cells using a novel fluorescent substrate

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Abstract In order to visualize the activity of the cAMP-dependent protein kinase (PKA) in living cells, we have constructed a new fluorescence PKA substrate by conjugating a fluorescence probe to a partial amino acid sequence of PKA regulatory domain II which contains a specific autophosphorylation site. The fluorescent peptide was cell-permeable and became phosphorylated when the intracellular cAMP concentration was increased, resulting in a decrease in its fluorescence intensity. In NG108-15 cells, PKA activity was localized to the cytosol around the nucleus. In cultured hippocampal neurons, addition of L-glutamate caused PKA activation associated with increase of the cellular cAMP.

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1. Introduction

Cyclic AMP is one of the main second messengers and plays an important role as a signal transducer in many cellular activities, the signal being transmitted by activation of cAMP-dependent protein kinase (PKA). PKA regulates specific gene expression by phosphorylating specific nuclear proteins, such as the cAMP-responsive element (CRE) binding protein (CREB), which, in turn, stimulates transcription of the gene corresponding CRE [1]. PKA is thought either to initiate long term potentiation (LTP), a model for memory formation [2], or to modulate its late phase [3]. Using fluorescent conjugates of exogenous PKA, it has been shown that an increase in intracellular cAMP levels results in dissociation of the catalytic domain of PKA from the regulatory domain, with the catalytic domain translocating into the nucleus [4–7]. Moreover, compartmentalization of PKA may be important in the phosphorylation of specific target proteins [8]. However, the dynamics of endogenous PKA are currently unknown. A suitable means for investigating this would be an imaging system using a fluorescent substrate. We recently developed such an imaging system to measure the intracellular activity of Ca²⁺/calmodulin-dependent protein kinase II (CaMKII), using a cell-permeable fluorescent CaMKII substrate [9].

In the present study, in order to visualize the dynamics of endogenous PKA activity in living cells, we constructed a new cell-permeable fluorescent PKA substrate, consisting of a fluorescent probe and an amino acid sequence comprising part

of regulatory domain II (RII) of PKA, that contains a specific serine autophosphorylation site. Using this fluorescence conjugate and fluorescence microscopy image processing, we have successfully observed dynamic features of endogenous PKA activation in neural cells and glutamate-induced PKA activation in cultured hippocampal neurons.

2. Materials and methods

2.1. Materials

6-Acryloyl-2-dimethylaminonaphthalene (acrylodan) was obtained from Molecular Probes, OR, USA, *N*-(7-dimethylamino-4-methylcoumarinyl)maleimide (DACM) from Teika Seiyaku, Toyama, Japan, calpeptin from Funakoshi, Tokyo, Japan, H-89 from Seikagaku, Tokyo, Japan, and Rp-cAMPS from Research Biochemicals International, MA, USA. FK506 was a gift from the Fujisawa Pharmaceutical Company, Osaka, Japan.

2.2. Preparation of fluorescent substrates

Peptide and phosphopeptide were synthesized using the Fmoc solid-phase method [10,11]. Fluorescent substrates were constructed by conjugating the synthetic peptide, via a Cys residue, to either acrylodan or DACM (Table 1), as follows: 1 ml of a 2.5 mM aqueous peptide solution was mixed with 0.525 ml of 4.8 mM fluoroprobe solution in CH₃CN and 0.1 ml of 100 mM NaHCO₃ and left at room temperature for 24 h in the dark, then the labelled peptide was purified by HPLC on a C8 column, as described below. The structures of the fluorescent peptides were confirmed by FAB-mass spectrometry, which showed a 1:1 molar coupling of fluoroprobe and peptide.

2.3. HPLC of fluorescent peptides

The fluorescent peptide (up to 0.5 mg) was applied to a C8 column (Aquapore RP-300, 7 µm pore size, 30×4.6 mm, Applied Biosystems, CA, USA) and eluted using a 20 ml linear gradient consisting of 10–50% CH₃CN in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The fluorescent peptide was detected by absorption at 215 nm and by fluorescence (524 nm emission, 366 nm excitation).

2.4. Cell lysate

Cell lysates were prepared by homogenization of neuroblastoma or neuroblastoma×glioma cells in 10 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 0.5 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride, 5 mg/l of leupeptin, and 20 mg/l of trypsin inhibitor, followed by centrifugation at 10 000×g for 15 min, as described by Hashimoto et al. [12].

2.5. Cell cultures for single cell analysis

NG108-15 (neuroblastoma×glioma cell line) cells (5×10⁴) in 2 ml of DMEM containing 5% FCS, 100 µM hypoxanthine and 16 µM thymidine were cultured at 37°C for 24 h in 35 mm glass-bottom microwell dishes, coated with poly-D-lysine (MatTek, MA, USA) and used after 2–10 days of serum depletion. Primary cultures of rat hippocampal neurons were prepared from 18-day embryonic rat brain, and cultured on glass coverslips for 7 days, as described previously [13].

2.6. Kinase assay in living cells

The cells were loaded with the fluorescent substrate conjugate by

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incubation with 100 µg/ml of ARII or 25 µg/ml of DRII (Table 1) in balanced salt solution, pH 7.3, consisting of 130 mM NaCl, 5.4 mM KCl, 20 mM HEPES, 5.5 mM glucose, 1.8 mM CaCl₂, 0.8 mM MgSO₄ and 1 µM calpeptin, a protease inhibitor, (solution A) for 30–60 min at 23°C. The substrate-loaded cells were conditioned by passage of solution A (1.4 ml/min) at 30°C, then exposed to the stimulating drug; the fluorescence of the stained cells was then measured using 360 nm or 400 nm excitation for ARII or DRII, respectively, and subjected to image analysis, using an image processor (Argus 100 or Argus 50, Hamamatsu Photonics, Shizuoka, Japan). Pseudo color ratio images were obtained by dividing each fluorescent image, recorded at a given time-point, by a reference image recorded before stimulant administration.

2.7. Quantitation of cellular cAMP

Cellular cAMP, extracting using 65% ethanol, was measured using the Biotrak cAMP EIA System (Amarsham). The values were analyzed by *F*-test to compare variances.

3. Results and discussion

3.1. Fluorescent PKA substrate conjugate

We have developed a cell-permeable fluorescent substrate which can be used to measure the real-time activity of PKA. A 19-residue peptide (RII19, Table 1), residues 81–99 of regulatory domain II of bovine cardiac muscle PKA, produced by digestion with *S. aureus* protease and which contains an autophosphorylatable Ser residue, is known to be readily phosphorylated by PKA [14]. Enz et al. replaced Cys-97 with Ala and found that the resultant peptide was still readily phosphorylated by PKA [15]. In the present work, we started

Table 1

Structure of fluorescent PKA peptide substrate conjugates and related peptides

	81	91
RII19 ^a		
	DLDPVPIPGRFDRRVSVCAL	
ARII	DLDPVPIPGRFDRRVSVAAC-Acrylodan	
DRII	DLDPVLPKADRRVSVAAC-DACM	
Rat RII19 ^b	DLEVPKPAKFTRRVSVCAL	

^aRII19: sequence from bovine cardiac muscle [20].

^bRII19: sequence from rat skeletal muscle [21].

with this latter peptide and replaced the C-terminal residue, Glu-99, with Cys, then used this residue to conjugate the peptide to the fluorescent probe, acrylodan. The resultant fluorescent peptide, ARII (Table 1) was also readily phosphorylated by PKA, the 524 nm fluorescence emission peak (excitation at 366 nm) of acrylodan being decreased on phosphorylation; when 15 µg/ml of ARII was incubated with 5–10 µg/ml of purified PKA in 10 mM MgAc₂, 1 mM EGTA and 0.5 mM ATP, pH 7.5, little, or no, phosphorylation occurred until 0.5 mM cAMP was added (Fig. 1A). Under the conditions used, the ARII fluorescence decreased by 3.3% on phosphorylation. To determine whether our system using changes in ARII fluorescence could be applied not

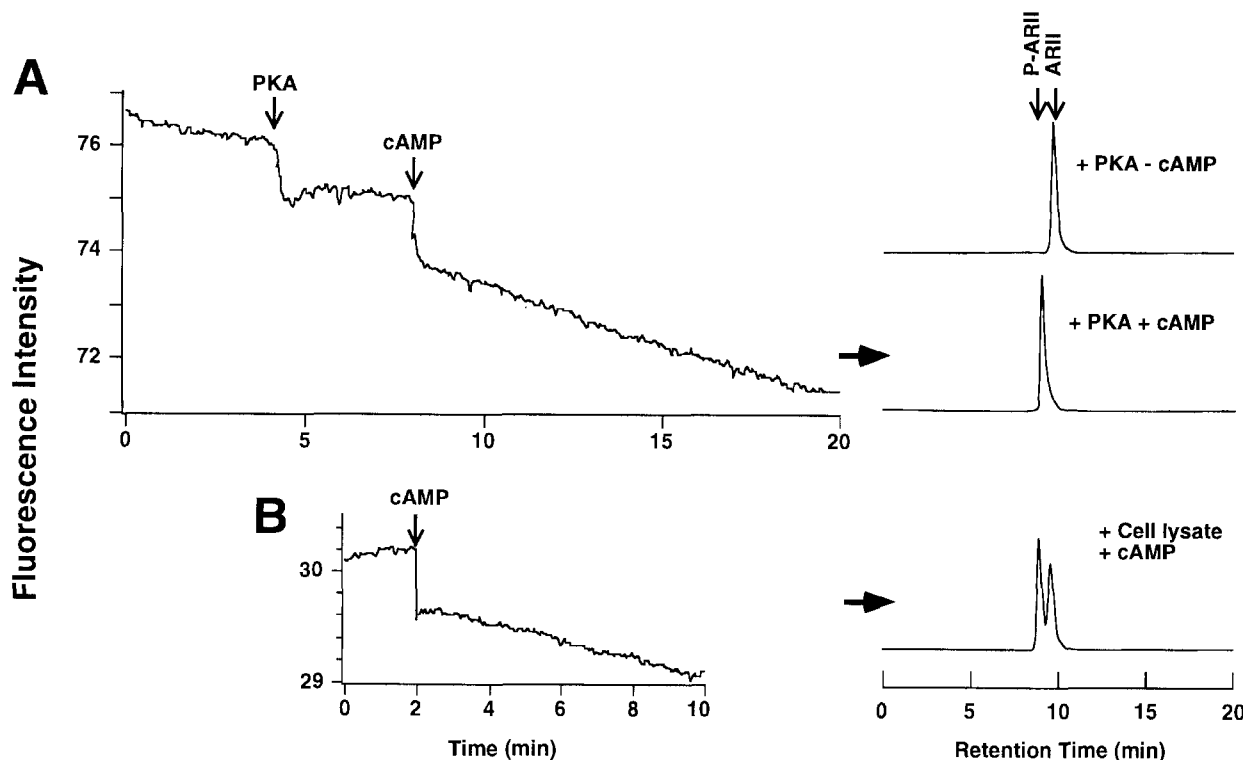


Fig. 1. Cyclic AMP-mediated ARII phosphorylation by purified PKA (A) or neuroblastoma cell lysate (B). Fifteen µg/ml of ARII was pre-incubated with 1 mM EGTA, 10 mM MgAc₂, 0.5 mM ATP, 50 mM HEPES, pH 7.5, at 25°C for 2 min and 10 µg/ml of purified PKA (holoenzyme from bovine heart, Sigma, P-5511) or neuro2A neuroblastoma cell lysate (final concentration of 40 µg protein/ml) was added. Two minutes later, phosphorylation was initiated by addition of 0.5 mM cAMP. The fluorescence intensity decreased gradually by phosphorylation following the straight drop by reagent dilution. The left trace shows the time course of the fluorescence intensity and the right trace the HPLC profile of the experimental material compared with standard phosphorylated peptide conjugate. The phosphorylated substrate conjugate eluted at the same position as standard phosphorylated ARII on HPLC analysis. The presence of the phosphoserine group in the isolated product was confirmed by amino acid sequencing.

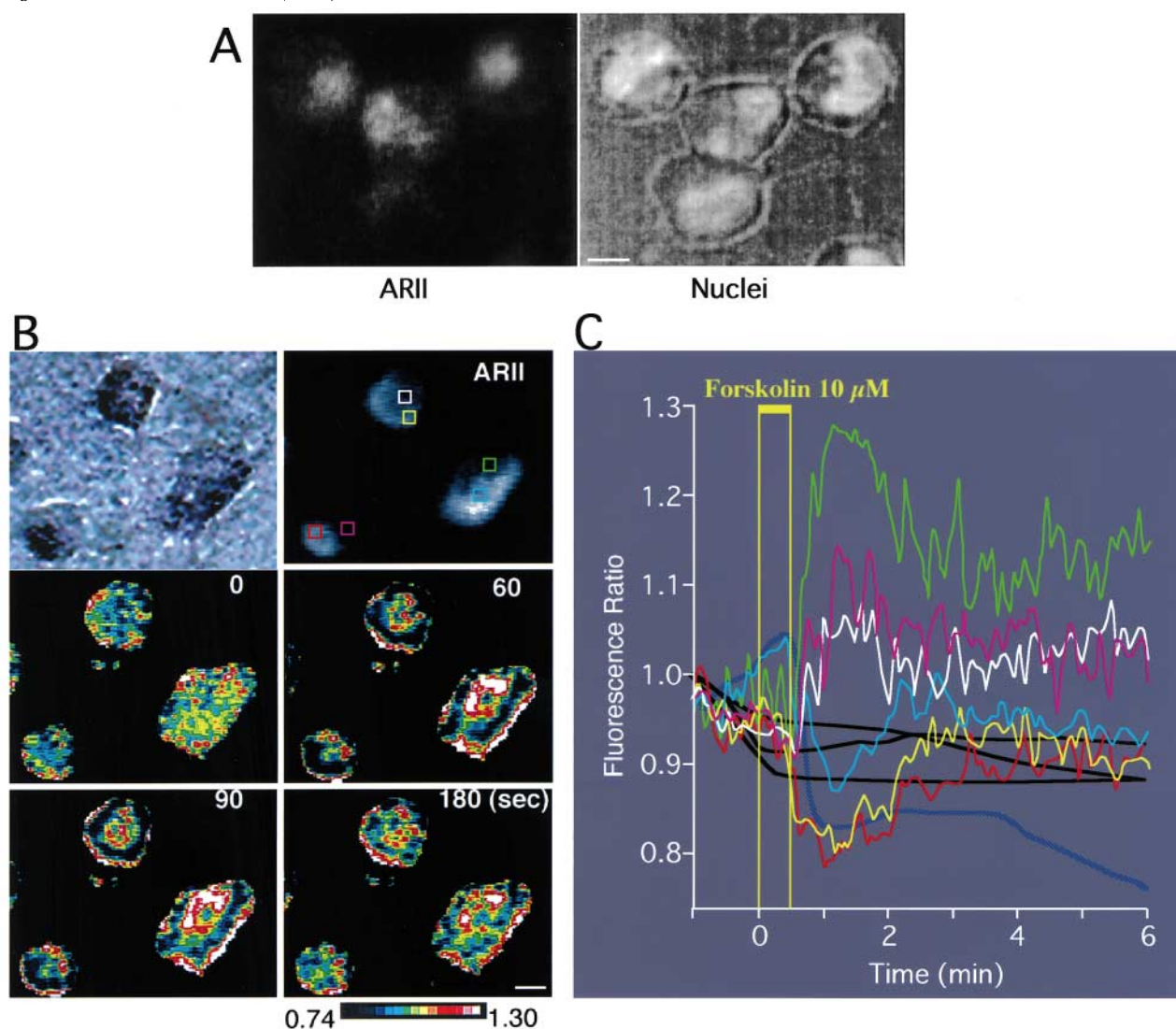


Fig. 2. (A): ARII loading of living neural cell lines. The ARII fluorescence image is shown on the left, while the right panel shows the fluorescence image of the nuclei of the same cells, overlapped by a transmitted light image of the cells. Cell nuclei were stained using 5 $\mu\text{g}/\text{ml}$ of Hoechst 33342 (Molecular Probes, OR, USA) in solution A for 10 min. The white bar corresponds to 10 μm . (B and C): Activation of PKA in ARII-loaded NG108-15 cells by stimulation with forskolin. The upper left and right panels in B show transmitted light and fluorescence images of the cells, respectively. At time 0, the pre-loaded cells were stimulated by exposure to 10 μM forskolin for 30 s. The time course of the fluorescence ratios in the colored squares in the upper right panel in B was plotted every 5 s to give the respective colored lines in C. The fluorescence of the cytosolic areas (yellow, red and blue lines) decreased, and that of the nuclear areas (green, magenta and white lines) increased, after addition of forskolin. The fluorescence decrease began to reverse 1 min after removal of forskolin. The reversal was prevented when the cells were exposed to 1 μM FK506 10 min before and during the experiment; typical fluorescence ratio in the presence of FK506 are shown in C by a slashed line which shows the averaged fluorescence ratios for 7 cytosolic areas. The effect of forskolin was completely blocked in both areas when the cells were exposed to 1 μM H-89 10 min before, and during, the experiment; typical fluorescence ratios in the presence of H-89 are shown in C by black lines which are smoothed to differentiate individual curves. The lower 4 panels in B show pseudo color fluorescence ratio images at the indicated times. The scale on the colored bar indicates the fluorescence ratio. The white bar corresponds to 10 μm .

only to simple reaction mixtures but also to complex mixtures, we then tested cell lysates from a neuroblastoma cell line, neuro2A (Fig. 1B), and from a neuroblastoma $\times$ glioma hybrid cell line, NG108-15 (data not shown), and found that these cell lysates were also able to phosphorylate ARII under the same conditions. Phosphorylation was confirmed both by HPLC analysis, using the phosphorylated fluorescent peptide as a standard (Fig. 1), and by sequencing of the isolated peptide, and was inhibited by a specific PKA inhibitor, H-89 (0.2–1 μM , data not shown). No phosphorylation was detected under CaMKII activating conditions, despite the fact that ARII contains a consensus sequence for a CaMKII phosphor-

ylation site. These results indicate that phosphorylation is PKA-specific.

3.2. Single cell analysis

ARII was readily incorporated into NG108-15 cells when added to the extracellular medium and we therefore were able to apply our system to single cell analysis using NG108-15 cells cultured on a poly-lysine-coated glass surface. The cells, pre-loaded with ARII, were exposed to forskolin for 30 s (Fig. 2). The fluorescence started to decrease 15–30 s after forskolin application, reached a minimum at 60–90 s, then returned gradually to normal within 3–4 min. Exposure to 0.1–1 mM

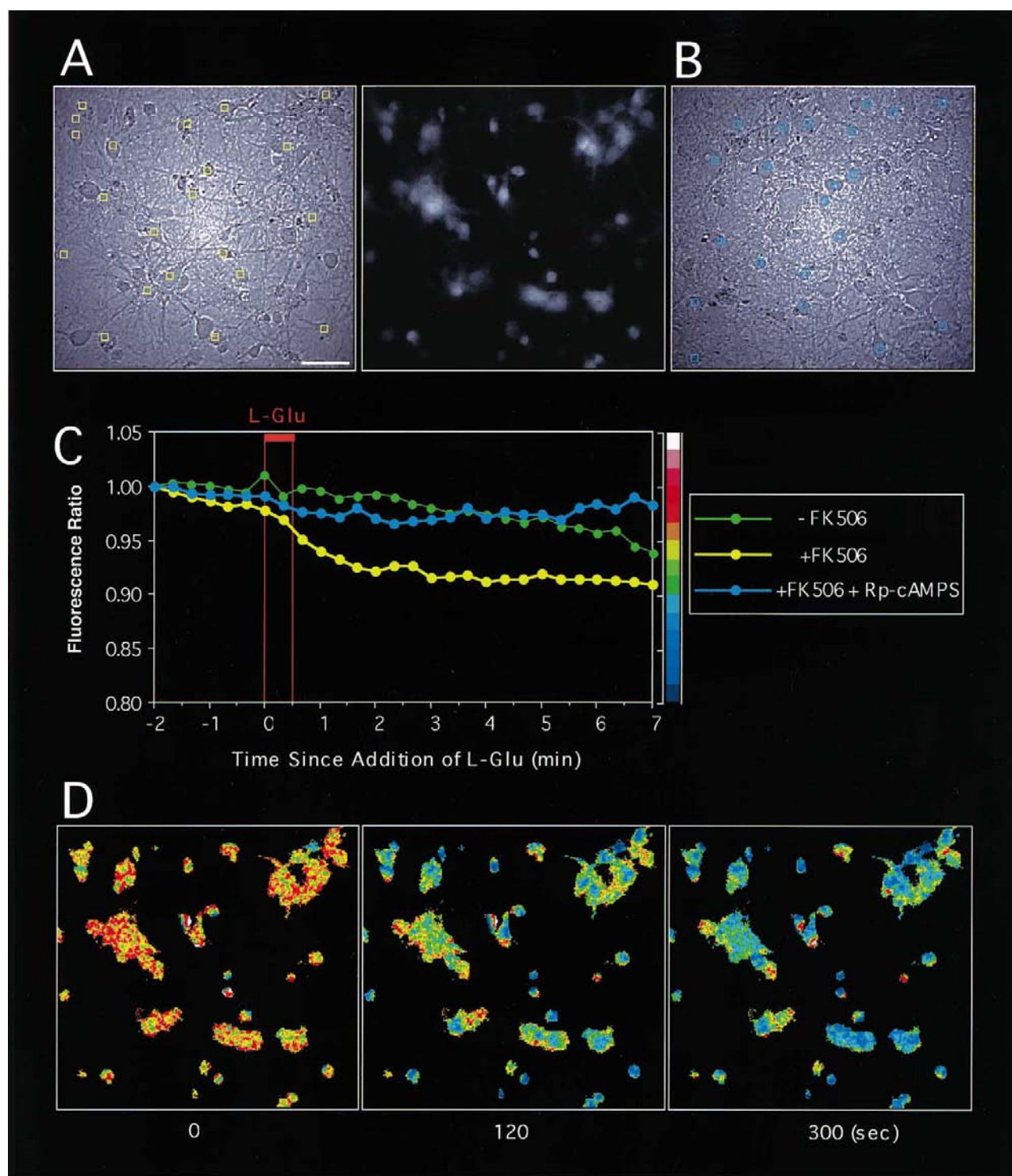


Fig. 3. L-Glutamate activation of PKA in primary cultures of hippocampal neurons. A (left panel) and B show the transmitted light images. The cells were loaded with DR11 (shown in the fluorescence image in the right panel in A), then the cells in A were treated with 1 μ M FK506 from time -5 min to the end of the experiment; the cells in B were treated identically, but 100 μ M of Rp-cAMPS, a PKA inhibitor was also included. At time 0, the cells were stimulated by a 30 s exposure to 100 μ M L-glutamate (C). The averaged fluorescence ratios for the areas in 21 yellow and blue squares in A and B, respectively, are shown in C, using the same colors. In the absence of the PKA inhibitor, cell fluorescence clearly decreased; this change was not seen in the presence of the inhibitor. The green circles in C show the averaged fluorescence ratios for the areas in 21 in the same experiment as A but without FK506. (D): pseudo color fluorescence ratio images, at the indicated times, of the cells shown in A. The colored bar in C indicates the fluorescence ratio. The white bar in A (left panel) corresponds to 50 μ m.

dibutyl cAMP also caused a decrease in fluorescence intensity. Nuclear staining showed the ARII incorporation and its

forskolin-induced fluorescence decrease to be marked in the cytosol around the nucleus (Fig. 2A). The fluorescence de-

crease was inhibited when the cells were exposed to 1 μ M H-89 before and during the experiment, indicating that it was due to PKA activation. In some cases, the fluorescence decrease in the cytosolic area was followed by increased fluorescence in the nucleus (Fig. 2B, C), the increase starting 30 s after forskolin application, peaking at 90–120 s, then gradually declining, with the fluorescence at 6 min still being higher than in the untreated cell. As the catalytic domain of PKA translocates to the nucleus [4–7], a possible explanation for the fluorescence increment in the nucleus is the nuclear translocation of the fluorescence substrate in association with the catalytic domain although the association remains to be proved. The nuclear fluorescence increase was also inhibited by pretreatment with 1 μ M H-89, again indicating that the reaction is PKA-specific (Fig. 2C). It should be noted that if the large fluorescence increase is caused by the substrate translocation, the amount of the translocated substrate is likely to be small because of the following reason. As we measure the ratio of the fluorescence at individual time point to that at initial time, even a small change in nuclear content would cause a large change in the ratio, since only a small amount of substrate is initially present in the nucleus. Since the fluorescence decrease in the cytosol (>10%) was larger than the difference in fluorescence intensity seen with the cell-free system (3.3%), possible effects of substrate outflow on fluorescence changes in the cytosol cannot be excluded and the system cannot therefore precisely measure phosphorylation of substrate. However, since the reactions in the cytosol and nucleus were both inhibited by a PKA inhibitor, they do reflect changes in the magnitude of PKA activity. We expected the return of fluorescence intensity, seen following the cytosolic decrease, to be due to dephosphorylation of the P-ARII produced, since calcineurin specifically dephosphorylates the original phosphorylated peptide [14] and when the cells were treated with a calcineurin inhibitor, FK506 (1 μ M) before, and during, forskolin exposure, this reversal was indeed prevented (blue slashed line in Fig. 2C). Increased cytosolic levels of cAMP may possibly activate calcineurin indirectly. These results suggest that calcineurin and the catalytic domain of PKA are co-localized, or closely localized, consistent with the report of Coghlan et al. [16] that both enzymes interact with the same anchor protein. We have also developed an imaging system for calcineurin activity, using a phosphorylated substrate (manuscript in preparation). It has been reported that the catalytic domain of PKA translocates to the nucleus [4–7] and phosphorylates transcription factors, such as CREB [1], leading to expression of the specific gene, and calcineurin may act by modulating such gene expression by reversing the PKA-mediated phosphorylation. Co-localization would therefore be advantageous for rapid modulation of a common substrate.

3.3. PKA activity in primary cultures of hippocampal neurons

As described above, we succeeded in observing the dynamics of PKA in neuroblastoma cells following stimulation which resulted in an increase in levels of intracellular cAMP. However, in the case of primary neuron cultures, very little ARII incorporation was seen (data not shown). To overcome this problem, we increased the hydrophobicity of the substrate by substituting 2 amino acid residues (Ala-88 and Lys-89) from the rat skeletal muscle RII19 sequence into the bovine cardiac muscle-derived ARII and also substituting

an additional 2 residues (Leu-86 and Ala-90); this modified peptide was then conjugated to DACM, yielding the conjugate DRII (Table 1). DRII had similar characteristics to ARII when tested in the cell-free PKA systems, except that its fluorescence emission peak is at 475 nm (excitation at 386 nm). The DRII fluorescence decreased by 4.0% on phosphorylation. However, in contrast to ARII, it was readily incorporated into primary cultures of hippocampal neurons, as well as neuroblastomas, and was ubiquitously distributed throughout the cell (Fig. 3A). When the cell was exposed to dibutyl cAMP, the fluorescence throughout the cell decreased (data not shown); similar results were obtained when the cell was exposed to 100 μ M L-glutamate in the presence of the calcineurin inhibitor, FK506 (Fig. 3). Both reactions were completely inhibited by a specific PKA inhibitor, Rp-cAMPS. The fluorescence decrease was markedly inhibited when FK506 was not present in the reaction mixture (green circles in Fig. 3C), and in some cases, a slight increase in fluorescence was seen. Thus, substrate phosphorylation was enhanced when the cells were exposed to FK506 before, and during, the experiment, again suggesting a close association between PKA and calcineurin and high basal activity of calcineurin in this cell. In a separated experiment, the cAMP concentration of primary hippocampal neuron cultures increased from 3.5 ± 0.80 (average $\pm$ S.E.M., $n=7$) to 7.3 ± 2.38 ($n=7$) pmol/mg cellular protein ($P<0.01$ by *F*-test) following stimulation with 100 μ M glutamate for 7 min at 37°C. Since the hippocampal neuron possesses glutamate receptors that open Ca^{2+} channels and increase the intracellular Ca^{2+} level, activation of PKA by glutamate probably occurs via activation of a Ca^{2+} /calmodulin-dependent adenylate cyclase. Like glutamate stimulated increment of intracellular Ca^{2+} level in the same cell [17], the increment of intracellular cAMP level should show spatial heterogeneity in the cell. Tsuji et al. [18] have shown a transient glutamate-induced increase in cAMP levels in cultured spinal cord neurons, while Xia et al. [19] have reported enrichment of Ca^{2+} /CaM-sensitive type I adenylate cyclase mRNA in the hippocampus and it is possible that this may be the enzyme responsible for the cAMP production.

In summary, we have shown that dynamics of PKA activity in living neural cells was successfully visualized by fluorescence microscopic image processing using novel fluorescent substrates and that PKA in hippocampal neuron was activated by L-glutamate.

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