Real-time Monitoring of the PDE2 Activity of Live Cells

HORMONE-STIMULATED cAMP HYDROLYSIS IS FASTER THAN HORMONE-STIMULATED cAMP SYNTHESIS

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Cyclic nucleotide phosphodiesterases (PDEs) are the enzymes that catalyze the hydrolysis of cAMP and cGMP, thereby restricting the activity of these second messengers in cells. A unique ability to shape gradients of cyclic nucleotides and compartmentalize their signaling implies a high potency and a rapid action of PDEs. However, it has not been demonstrated how fast PDEs can hydrolyze cAMP in a living system. Here we perform a real-time monitoring of PDE2 activity in aldosterone-producing adrenal cells using a recently developed genetically encoded, fluorescent cAMP sensor, which reveals enormously rapid kinetics of cAMP degradation. Activation of PDE2 results in a rapid decrease of intracellular cAMP from high micromolar to the sub-micromolar range within a few seconds. Moreover, the kinetics of atrial natriuretic peptide-stimulated PDE2 activity (measured as decline of cAMP) are much faster than the speed of ACTH and isoprenaline-induced cAMP-synthesis (measured as cAMP accumulation) in the cells, revealing high catalytic activity and fast action of PDEs in regulating cAMP signaling in a physiological system.

Cyclic nucleotide phosphodiesterases (PDEs)1 are the enzymes responsible for hydrolysis of cAMP and cGMP to terminate intracellular signaling of these second messengers (1, 2). For several of the eleven PDE families an intriguing ability has been discovered to shape cAMP gradients in cells, which leads to compartmentalization of the signaling, effectively orchestrating multiple physiological responses (3–6). This function of PDEs implies a high catalytic activity and a rapid action of these enzymes. PDE activity has been routinely measured in biochemical studies, based on in vitro hydrolysis of radioactively labeled cyclic nucleotides (7) or with the recently developed enzyme-cleavable fluorescent sensors (8). However, these techniques give little information about the behavior of PDEs in living cells, leaving unclear how fast PDEs work in vivo. The studies using modified cyclic-nucleotide-gated channels to measure cAMP at the plasma membrane have demonstrated the role of PDEs in regulation of cAMP in localized microdomains by using different PDE inhibitors (9, 10).

Here we sought to directly monitor PDE activity in real-time using a physiological system. It seemed important to analyze such a PDE that could be selectively activated by a specific stimulus and would represent a predominant isoform in the cells of interest to avoid a possible interplay with other PDEs. Among numerous PDEs only PDE2 has been characterized as a cGMP-stimulated isoform hydrolyzing cAMP (11). In PDE2, cGMP binding to an allosteric site in the regulatory GAF-B domain stimulates catalytic activity (12), which has numerous physiological consequences in vivo. For example, atrial natriuretic peptide (ANP) stimulation of cGMP production and subsequent activation of PDE2 in the adrenal cortex decreases aldosterone secretion, thereby mediating the effect of the hormone on blood volume (7). ANP stimulates its NPR-A type receptors containing an intracellular guanylyl cyclase domain, thereby generating cGMP, which diffuses in the cytosol and specifically activates PDE2 (13). The activity of this enzyme results in the degradation of cAMP, a messenger that induces aldosterone production in response to adrenocorticotropic hormone (ACTH) or β-adrenergic receptor stimulation (Ref. 14; Fig. 1A). Moreover, in bovine adrenal zona glomerulosa (ZG) cells PDE2 is not only a predominant isozyme providing almost 100% of PDE activity (7) but also a unique cGMP-binding protein, because in contrast to rat ZG cells cGMP-dependent protein kinase (PKG; Ref. 15) and cGMP-gated ion channels are not expressed in this cell type (data not shown).

For real-time monitoring of PDE2 activity we decided to use our very recently developed, genetically encoded, fluorescent CAMP-indicator Epac2-camps, which is capable of detecting cAMP in living cells with high temporal resolution within a physiologically relevant concentration range of 100 nM to 20 μM (16). Using this approach we monitor the real-time PDE2 activity in adrenal ZG cells and demonstrate unexpectedly rapid kinetics of ANP-induced PDE2 action, which dynamically overcomes ATCH and isoprenaline-induced cAMP production.

EXPERIMENTAL PROCEDURES

Preparation of Adenovirus Expressing Epac2-camps—A E3-deleted adenoviral vector encoding Epac2-camps was generated following standard procedures. After tittering adenoviral concentrations by plaque assays conducted on monolayer 293 cultures, a multiplicity of infection (m.o.i.) between 10 and 50 was used to infect primary adrenal ZG cells 48–72 h prior to fluorescence resonance energy transfer (FRET) measurements.

Preparation of Zona Glomerulosa Cells—Bovine (adrenal glands were obtained from a local slaughterhouse) adrenal ZG cells attached to
the adrenal capsule were digested with collagenase and mechanically disaggregated as described (15, 17). Isolated cells were suspended in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. For FRET measurements, cells were plated on glass coverslips (20 mm diameter) at a density of 10,000 cells/ml and after 2 days were infected with Epac2-camps adenovirus. For experiments on aldosterone production and Western blotting, the cells were plated into 6-well plates at a density of 10–12 × 10^4 cells/dish. After 3 days in culture the medium was replaced by serum-free medium for 12 h and cells were incubated for 1 h with forskolin, ANP, and ACTH-1–24 peptide (Sigma, Deisenhofen, Germany), then the medium was collected for aldosterone radioimmunoassay (RIA), and cells were harvested in SDS gel loading buffer. Protein kinase A (PKA) activity in samples was analyzed by Western blot using the phosphorylation of the well known PKA and PKG substrate vasodilator-stimulated phosphoprotein (VASP) detected by monoclonal anti-phospho-Ser239 mouse antibody (18). Equal loading of protein was determined by RIA using a commercially available aldosterone kit (DPC Biemann, Bad Nauheim, Germany).

Aldosterone RIA—Aldosterone concentration in the culture medium was determined by RIA using a commercially available aldosterone kit (DPC Biemann, Bad Nauheim, Germany).

Statistical Analysis—The data shown are from at least triplicate experiments. Values are expressed as the mean ± S.E. Differences between groups were analyzed by Student’s t test, and p < 0.05 was considered statistically significant.

FRET Measurements—FRET measurements to monitor PDE2 activity in live glomerulosa cells were done 48–72 h after infection with Epac2-camps adenovirus. Microscopic experiments and data analysis were performed as described previously (16). Cells were continuously superfused with a buffer (144 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES, pH = 7.3) and challenged with ACTH, isoprenaline, forskolin, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), and rat 1–28 ANP (Sigma) in different combinations.

Fluorescence Measurements—Fluorescence measurements in vitro were performed as described (16). Epac2-camps purified from SF9 cells was diluted in 5 mM Tris buffer, pH = 7.4, to a final concentration of 40 nM (in 600 μl) and saturated with 20 μM cAMP. 0.12 unit (10 μM final concentration) of PDE (EC 3.1.4.17 from bovine heart, Sigma) were added to degrade unbound cAMP and monitor ligand dissociation from the sensor.

RESULTS AND DISCUSSION

Epac2-camps Rapidly Responds to Binding and Dissociation of cAMP—Epac2-camps is a cAMP sensor, which is based on a single binding domain of exchange protein directly activated by cAMP (Epac2) fused between cyan and yellow fluorescent proteins (Fig. 1C). The binding of cAMP leads to a conformational change of the sensor (black arrow, Fig. 1C), which is detected by a decrease in FRET between CFP and YFP. To test whether our fluorescent indicator could be applied to monitoring of PDE activity in live cells, we investigated how fast bound cAMP dissociates from the sensor. In case of slow dissociation this step might become time-limiting for our system, making correct measurements of PDE2 action impossible. After saturating purified Epac2-camps with 20 μM cAMP (with a fast on-rate of 2.0 ± 0.2 s), a high amount of purified catalytically active PDE (10 μM final concentration) was added to the cuvette to rapidly degrade unbound ligand. cAMP depletion led to a fast dissociation of the sensor-bound ligand fraction with an apparent half-life of 2.9 ± 0.3 s (Fig. 1B). At a catalytic activity of the PDE-preparation used of 0.3 mol of cAMP/s/mol of PDE, degradation of cAMP in the cuvette can be estimated to occur with a half-life of ~3 s. Since cAMP depletion in this experiment is limited by this speed of PDE-mediated cAMP hydrolysis, the real kinetics of cAMP dissociation from the sensor are difficult to estimate but should be even faster than measured, indicating that we can use Epac2-camps to monitor PDE2 activity provided that the latter process occurs with a half-life significantly above 2 s.

ANP-activated PDE2 Rapidly Blocks Forskolin-induced cAMP Accumulation—To monitor PDE2 activity in primary adrenal ZG cells, we transfected them with Epac2-camps (Fig. 1D), an inert fluorescent cAMP indicator, without any additional manipulations of the cells. After 2–3 days in culture we performed single-cell microscopic measurements. cAMP degradation by PDE was monitored as an increase of the FRET signal (Fig. 1E). Addition of 10 μM forskolin resulted in a substantial increase of intracellular cAMP (monitored as a decrease in FRET ratio) from basal sub-micromolar levels up to a plateau (Fig. 2A), indicating a saturation of the sensor (~20 μM). Next, we activated PDE2 in the cells by stimulation with ANP and observed an unexpectedly rapid signal, demonstra-
ing a fast degradation of cAMP by PDE2. After a few seconds delay, PDE2 became apparently active and hydrolyzed the major amount of intracellular cAMP with a half-maximal time constant ($t_{0.5}$) of $15.5 \pm 0.9$ s (Fig. 2A). This is at least five times higher than the speed of cAMP dissociation from purified Epac2-camps in vitro (Fig. 1B), suggesting that we can accurately monitor PDE2 activity as a time-limiting process. Using this protocol we measured a concentration response dependence of the ANP effect on PDE2 activity with a half-maximal response at 0.43 ± 0.02 pM ANP (Fig. 2B). This curve appears steep (Hill coefficient of 2.9), presumably due to the fact that the cAMP-sensor does not cover the entire range of intracellular cAMP levels, causing truncation of the signal at higher cAMP concentrations (16). Levels of intracellular cAMP measured in real-time with our system also correlated well with the data of conventional biochemical experiments on aldosterone production and phosphorylation of VASP, a substrate of PKA and PKG (Fig. 2C). In addition to the generally accepted concept that aldosterone production in adrenal cortex cells is crit-
ically dependent on cAMP levels, which are regulated by ANP-activated PDE2 (14), we demonstrate that PDE2 activity kinetically overcomes the forskolin-induced cAMP production, providing a mechanism to hydrolyze extensive amounts of the second messenger. Being intrigued by a fast action of PDE2 on forskolin-induced cAMP in ZG cells, we sought to monitor the activity of this enzyme under different conditions including stimulation of the cells via physiologically relevant G-protein coupled receptors.

**Monitoring PDE2 Activity under Basal and Agonist-stimulated Conditions**—To further characterize PDE2 activity in ZG cells, we used its specific inhibitor EHNA, which at micromolar concentrations allows to selectively block the enzyme, inhibiting other PDEs at more than 100 μM only partially (19). When applying EHNA to the cells under basal conditions, little effect on cAMP could be observed even at 100 μM (Fig. 3A), indicating that basal PDE2 activity in unstimulated ZG cells is very low. Isobutylmethylxanthine, an unspecific PDE inhibitor, at 350 μM also had little effect comparable with EHNA (data not shown). In contrast, applying a saturating concentration of ANP activated PDE2, which led to a decrease in cAMP, even though this effect was not as prominent as after forskolin stimulation, corresponding well with the functional data on aldosterone production (Fig. 2C).

Next, we monitored PDE2 activity in agonist-stimulated cells, using agonists for endogenously expressed G-protein coupled receptors (Fig. 1A). ACTH as a natural ligand capable of stimulating aldosterone secretion (14) induced a slow cAMP accumulation, which could be fully and rapidly blocked (t_{0.5} of 13.0 ± 3.2 s) by ANP-induced PDE2 (Fig. 3B). This PDE2 effect was inhibited by 10 μM EHNA, applied in addition to ACTH and ANP. Interestingly, the ACTH effect on cAMP production in this case was much stronger. Similar effects have been previously observed for specific PDE3 and PDE4 inhibitors and adrenergic stimulation in cardiac myocytes (5). Upon removal of the inhibitor, ANP-induced PDE2 activity was immediately restored and brought cAMP in cells down to the basal level. The t_{0.5} for this restoration of PDE2 activity was 18.3 ± 1.9 s, which is comparable with the speed of ANP effects in presence of forskolin (2A).

EHNA has been demonstrated to reverse the inhibitory effect of cGMP-dependent PDE2 on cAMP-stimulated calcium current in frog ventricular cardiomyocytes after isoproterenol treatment (20). Therefore we studied ANP and EHNA effects on β-adrenergic receptor-mediated cAMP production in ZG cells. The β-adrenergic receptor agonist isoproterenol induced a cAMP signal that was again blocked by ANP-stimulated PDE2 (Fig. 3C). EHNA, comparable with the ACTH experiments, fully reversed PDE2 activity and together with isoproterenol further increased cAMP, exhibiting a half-maximal effect at ~1 μM (Fig. 3D), which corresponds well with the previously measured constants (19, 20). Again, PDE2 recovery demonstrated rapid kinetics with a t_{0.5} of 19.8 ± 2.6 s (Fig. 3C).

Using the bovine adrenal cells as a model system, here we report the real-time monitoring of PDE activity in live cells. Taking an advantage of the high expression of PDE2, which is responsible for physiological regulation of aldosterone production, we specifically stimulate this predominant PDE isoform with ANP and analyze real-time kinetics of cAMP hydrolysis by PDE2. Extremely fast action of PDE2 could be monitored, which allows to kinetically overcome forskolin, ACTH, or β-adrenergic receptor-mediated cAMP accumulation, to fully block their effects on aldosterone production. Epac2-camps in this measuring system is capable of measuring PDE2 activity due to a much faster ligand dissociation from the sensor (Fig. 1B), as compared with the speed of PDE2-mediated, time-limiting cAMP hydrolysis (Figs. 2A and 3, B and C). In contrast to the classical biochemical studies where the activity of PDE is estimated on a scale of minutes (7), our real-time live cell experiments reveal that the endogenous PDE2 only needs 15–20 s to decrease intracellular cAMP from high micromolar (saturation of the sensor at ~20 μM) to submicromolar (the lower detection limit of Epac2-camps at ~100 nm) concentrations (Figs. 2A and 3, B and C). Such a high catalytic activity and rapid action of PDEs could be crucial for their physiological function to rapidly antagonize cAMP formation in the cells and shut down its signaling.

Extremely rapid PDE2-mediated cAMP degradation observed in our experiments could be important for compartmentalization of the cAMP signaling, which might be possible due to much higher speed of PDE-catalyzed cAMP hydrolysis compared with the speed of cAMP production in living cells. Compartmentalization of cAMP is supposed to be regulated by constitutively active PDEs as demonstrated by various experimental approaches, using cyclic nucleotide-gated channels or fluorescent PKA as sensors (5, 6, 9, 10). This can be concluded from the fact that PDE inhibitors led to the diffusion of cAMP out of the restricted microdomains. In our previous study using Epac-camps in neurons we measured a high speed of cAMP propagation at 40 μm/s without visible barriers (16). The speed of ANP-activated PDE2-mediated cAMP hydrolysis in ZG cells, however, is much lower since the cGMP-dependent PDE activity of the whole cell has been monitored. Further studies will need to define the rate-limiting elements in this signaling cross-talk between cGMP and cAMP and to determine the physiological relevance of cAMP compartmentalization achieved by this mechanism.

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