Intracardiac cAMP levels are modulated by hormones and neuromediators with specific effects on contractility and metabolism. To understand how the same second messenger conveys different information, mutants of the rat olfactory cyclic nucleotide-gated (CNG) channel α-subunit CNGA2, encoded into adenoviruses, were used to monitor cAMP in adult rat ventricular myocytes. CNGA2 was not found in native myocytes but was strongly expressed in infected cells. In whole cell patch-clamp experiments, the forskolin analogue L-858051 (L-85) elicited a non-selective, Mg²⁺-sensitive current observed only in infected cells, which was thus identified as the CNG current (I_{CNG}). The β-adrenergic agonist isoprenaline (ISO) also activated I_{CNG}, although the maximal efficiency was ~5 times lower than with L-85. However, ISO and L-85 exerted a similar maximal increase of the L-type Ca²⁺ current. The use of a CNGA2 mutant with a higher sensitivity for cAMP indicated that this difference is caused by the activation of a localized fraction of CNG channels by ISO. cAMP-dependent protein kinase (PKA) blockade with H89 or PKI, or phosphodiesterase (PDE) inhibition with IBMX, dramatically potentiated ISO- and L-85-stimulated I_{CNG}. A similar potentiation of β-adrenergic stimulation occurred when PDE4 was blocked, whereas PDE3 inhibition had a smaller effect (by 2-fold). ISO and L-85 increased total PDE3 and PDE4 activities in cardiomyocytes, although this effect was insensitive to IBMX. However, in the presence of IBMX, H89 had no effect on ISO stimulation of I_{CNG}. This study demonstrates that subsarcolemmal cAMP levels are dynamically regulated by a negative feedback involving PKA stimulation of subsarcolemmal cAMP-PDE.

Recent evidence indicates that multimolecular signaling complexes between cell surface receptors and intracellular targets is essential for the speed and specificity of signal transduction events (1, 2, 3). However, how such modules maintain specificity when small diffusible molecules are generated during the signaling cascade is difficult to investigate. This question is particularly relevant for cAMP in the heart, where this cyclic nucleotide second messenger exerts diverse effects in response to a number of different neuromediators and hormones. For instance, the β-adrenergic agonist isoprenaline (ISO),¹³ prostaglandin E₁ (PGE₁), and glucagon-like peptide 1 (GLP-1) elevate intracardiac cAMP levels with different effects on contractility; ISO augments the force of contraction, PGE₁ does not, and GLP-1 exerts a negative inotropic effect (4, 5). In order to explain these results, subcellular compartmentation of cAMP was proposed more than 20 years ago (6).

Localized cAMP signals may be generated by the interplay between discrete production sites and restricted diffusion within the cytoplasm. In addition to specialized membrane structures that may circumvent cAMP spreading (6, 7), degradation of cAMP into 5'-AMP by cyclic nucleotide phosphodiesterases (PDEs) appears critical for the formation of dynamic microdomains (8–14). Cardiac PDEs fall into four families: PDE1, which is activated by Ca²⁺+calmodulin; PDE2, which is stimulated by cGMP; PDE3, which is inhibited by cGMP; and PDE4. Whereas PDE1 and PDE2 can hydrolyze both cAMP and cGMP, PDE3 preferentially hydrolyzes cAMP, and PDE4 is specific for cAMP. There is abundant biochemical evidence that PDE3 and PDE4 are activated by cAMP-dependent protein kinase (PKA) phosphorylation in several tissues, providing a putative negative feedback mechanism by which cAMP may regulate its own levels (15, 16, 17).

A deeper understanding of the mechanisms involved in cAMP homeostasis requires appropriate methods for the direct and continuous measurement of the second messenger in intact cells. The approaches developed so far in cardiac myocytes are based on the use of fluorescent PKA as a biosensor of cAMP (18, 14). However, careful evaluation of PKA-based indicators re-
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veals a number of drawbacks, which complicate the interpretation of the results. Among these, the kinase activity inherent to the probe and the poor dynamic range may be regarded as the most limiting (18, 19). An alternative approach, initially developed by Rich et al. (7), uses genetically modified α-subunits of rat olfactory cyclic nucleotide-gated channel (CNG) as CAMP sensors. The wild-type α-subunit (CNGA2), on its own, forms a cationic channel directly opened by cyclic nucleotides with fast kinetics, wide dynamic range, and lack of desensitization. Mutants of CNGA2 with increased cAMP sensitivity and selectivity have proven to be valuable tools for monitoring subsarcomemal CAMP in model cells (19).

In this article, we report the first real-time measurement of CAMP with CNG channels in cardiac myocytes. This method was used to directly examine some of the regulatory mechanisms involved in shaping physiological subsarcomemal CAMP signals.

EXPERIMENTAL PROCEDURES

Isolation, Culture, and Infection of Adult Rat Ventricular Myocytes—The protocols described in the Methods for the isolation and culture of adult rat ventricular myocytes were used (17). For each experiment, the cells were plated on 60-mm dishes and infected with Ad-CNG as described above. After 24 h of culture, the cells were preincubated in a medium containing H89 (1 μM) to block nonspecific cation current measurement, nifedipine was omitted for Ca,L measurement, the cells were maintained at 0 mV holding potential and routinely perfused with external solution. Control and drug-containing solutions were applied to the exterior of the cell by placing the cell at the opening of a 250-μm inner diameter capillary tubing. Patch electrodes (0.8–1.2 MΩ) were filled with control internal solution containing (in mM): CsCl 118, EGTA 5, MgCl2 4, sodium phosphate 5, Na2ATP 3.1, NaGTP 0.42, CaCl2 0.062 (pCa 8.5), HEPES 10, adjusted to pH 7.3. ISO and IBMX were purchased from Sigma. L-858051 (L-85, a hydrolysable analogue of forskolin), H89 and cilostamide were purchased from France Biochem (Meudon, France), and RO 20-1724 was gently provided by Hoffman-La Roche (Basel, Switzerland).

Data Analysis—The maximal amplitude of \( I_{\text{CNG}} \) was measured as the difference between the peak inward current and the current at the end of the 400-ms duration pulse (22). \( I_{\text{CNG}} \) amplitude was time-independent and was measured at the end of the 200-ms pulse. Currents were not compensated for capacitance and leak currents. In a total of 154 rat ventricular myocytes, mean capacitance was 157.3 ± 5.1 pF. \( I_{\text{CNG}} \) was calculated for each experiment as the ratio of current amplitude to cell capacitance. All the data are expressed as mean ± S.E. When appropriate, the Student’s t test was used for statistical evaluation. The concentration-response curves (CRC) for the effects of ISO and L-85 on \( I_{\text{CNG}} \) were fitted to the Hill equation:

\[
\text{d}I_{\text{CNG}} = \text{E}_{\max} \left(1 + \left(\text{EC}_{50}/\text{[drug]}\right)^n\right)
\]

where \( \text{EC}_{50} \) is the drug concentration ([drug]) required to produce half-maximal stimulation, \( E_{\max} \) is the maximum response to a given concentration of [drug], and \( n \) is the Hill coefficient. Data were analyzed by a Fisher test. For both Student and Fisher tests, a p value of <0.05 was considered statistically significant.

Immunocytochemistry—Cells attached onto coverslips were rinsed once in phosphate-buffered saline solution (PBS) for 5 min, fixed in paraformaldehyde 4% (5 min) and washed in PBS (3 × 5 min). The cells were then permeabilized (30 min in PBS-BSA 1% and twice in PBS) and blocked (2 × 5 min) and once in PBS-BSA 1% (5 min). Next, they were incubated with a mouse monoclonal antibody against CNGA2 (dilution 1:800, 1 h, 37 °C). This antibody was a generous gift from Drs. F. Mueller and B. Kaupp (Juelich, Germany). After three washes in PBS and one in PBS-BSA 1%, the cells were revealed with the Alexa fluor 488 goat anti-mouse antibody (30 min, 37 °C). After three additional washes in PBS, the cells were stained with a mouse monoclonal CNGA2 antibody (dilution 1:800, 1 h, 37 °C, warm-up medium (France Biochem) and examined under a Carl Zeiss (Oberkochen, Germany) LSM 510 confocal scanning laser microscope. Optical sections series were obtained with a Plan Apochromat ×63 objective (NA 1.4, oil immersion). The fluorescence was observed with a LP 505-nm emission filter under 488-nm laser illumination.

PDE Assays—For each assay, 5 × 10^5 freshly isolated rat ventricular myocytes were plated on 60-mm dishes and infected with Ad-CNG as described above. After 24 h of culture, the cells were preincubated in control external Ringer (similar to that used in patch-clamp experiments, see composition above) supplemented or not with H89 (1 μM). After 30 min, cells were stimulated or not with 100 nM ISO or 10 μM L-85 during 5 min. Cells were homogenized at 4 °C in extraction buffer (0.5 M Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM EGTA, pH 7.4 and 1 mM dithiothreitol). The homogenates were sonicated for 5 s and then centrifuged for 15 min at 15,000 rpm. The supernatant was kept at 80 °C until use. PDE activities were measured by radioenzymatic assay as previously described (23) at a substrate concentration of 1 μM CAMP in the presence of 15,000 cpm [3H]CAMP as a tracer. The buffer solution was of the following composition: 50 mM Tris-HCl, pH 7.5, 2 mM magnesium acetate, and 1 mM EGTA. H89 (1 μM) was added to this buffer to prevent phosphorylation of PDEs by exogenous cAMP. Assays of total CAMP hydrolitic activity and isoform-specific PDE activity were run in the same batch of experiments. Sonicated homogenates were diluted in order to have around 15% of hydrolysis in absence of specific inhibitors. PDE isoform-specific activities were determined as the difference between PDE activity in the absence of inhibitor and the residual hydrolitic activity observed in the presence of the selective inhibitor (24). The results were expressed as pmol min⁻¹ mg prot⁻¹. Proteins were determined according to Lowry et al. (25) using BSA as standard.

RESULTS

Functional Expression of Cyclic Nucleotide-gated Channels in Cardiac Myocytes—The E583M mutant of the α-subunit of rat olfactory CNG channels encoded into an adenoviral vector (Ad-CNG) was used as an indicator of CAMP in rat ventricular myocytes. This mutation increases the sensitivity of the chan-
The cell was superfused for 5 min with a control Ringer solution and then challenged with 100 nM forskolin analogue L-858051 (L-85, 100 μM, dotted line) and infected rat ventricular myocytes with an adenovirus encoding the α-subunit (E583M) of rat olfactory CNG channel. A, individual current traces obtained in Ca2+- and Mg2+-free control Ringer (solid line) and in the same solution supplemented with the forskolin analogue L-858051 (L-85, 100 μM, dotted line) during a 200-ms voltage pulse at −50 mV in an NI myocyte and Ad-CNG-infected myocyte (MOI 3000). The current was reversibly activated by L-85 and completely blocked by 10 mM Mg2+. The cell was superfused for 5 min with a control Ringer solution and then challenged with 100 μM L-85 during the period indicated by the solid line. The current was reversibly activated by L-85 and completely blocked by 10 mM Mg2+. CNG current at 50 mV (Fig. 1A) and a linear current-voltage relationship crossed about 400 cells in each condition, this parameter was 14.8 ± 2.2% (non-infected), 10.2 ± 2.7% (MOI 100), 9.4 ± 1.3% (MOI 600) and 10.2 ± 0.3% (MOI 3000). Thus, Ad-CNG infection did not increase mortality at 24 h, even at a MOI of 3000. This virus to cell ratio was used in all subsequent experiments except for co-infection protocols where each adenovirus was used at MOI 600.

Subcellular Localization of Recombinant CNG Channels—In a second series of experiments, CNGA2 expression was investigated by immunofluorescence in non-infected and Ad-CNG-infected myocytes after 24 h of culture. Fig. 2 shows confocal images of representative cells in both groups labeled with the monoclonal anti-CNGA2 or the secondary antibody alone. In non-infected myocytes, a weak longitudinal staining was observed in both conditions, and was thus attributed to nonspecific binding of the secondary antibody. In contrast, in rat ventricular myocytes infected with Ad-CNG, a strong fluorescent signal was observed throughout the cell, with accentuation at the extremities, around the nucleus, and in transversal stripes with a periodicity of ∼1.9 μm. Such pattern was not observed.
when the primary antibody against CNGA2 was omitted. These results suggest that the recombinant channel distributes ubiquitously at the membrane, with preferential localization in particular structures.

**Comparative Effects of ISO and L-85 on I\textsubscript{CNG} and I\textsubscript{Ca,L}**—We next tested whether CNG channels were able to detect cAMP increases elicited by a hormonal-like stimulation. Fig. 3A presents a typical experiment in which the effect of the sympathomimetic amine ISO was tested and compared with the effect of L-85 on \(I\textsubscript{CNG}\) at \(-50\) mV. ISO at 100 nM induced a clear increase of \(I\textsubscript{CNG}\), although \(-5\) times smaller than that elicited by 100 \(\mu\)M L-85. In 7 similar experiments the mean current density was augmented from 4.3 \pm 1.3 pA/pF in control Ringer to 10.9 \pm 2.1 pA/pF with 100 nM ISO and to 51.4 \pm 7.8 pA/pF with 100 \(\mu\)M L-85 (Fig. 3B). Comparable differences between ISO and L-85 were obtained at MOIs of 100 and 600 (data not shown). In order to link these results to a physiological process governed by \(\beta\)-adrenergic receptors in the heart, the regulation of the L-type \(Ca^{2+}\) current \(I\textsubscript{Ca,L}\) was examined with 1.8 mM \([Ca^{2+}]_o\) in the bath. At variance with what was observed on \(I\textsubscript{CNG}\), ISO (100 nM), and L-85 (100 \(\mu\)M) exerted similar, maximal stimulatory effects on \(I\textsubscript{Ca,L}\) in both Ad-CNG-infected myocytes (MOI 3000) and in non-infected cells (Fig. 3C). This indicates: (i) that the \(\beta\)-adrenergic regulation of \(I\textsubscript{Ca,L}\) was apparently not affected by CNG channel overexpression, and (ii) that limited cAMP signals elicited by a \(\beta\)-adrenergic stimulation are sufficient to fully activate L-type \(Ca^{2+}\) channels.

**Comparative Effects of Isoprenaline and L-858051 on I\textsubscript{CNG} Elicted by Two CNGA2 Mutants with Different Sensitivity to cAMP**—The smaller current density obtained with ISO compared with L-85 may result from a weaker activation of the channels, or by the fact that only a given subset of channels is activated by the \(\beta\)-adrenergic agonist. To address this point, we compared the effects of ISO and L-85 on \(I\textsubscript{CNG}\) elicited by the E583M CNGA2 channel with those obtained with another CNGA2 variant, C460W/E583M, with a higher sensitivity to cAMP (12). As expected, the CRC for the effect of L-85 on \(I\textsubscript{CNG}\) in cells expressing C460W/E583M was leftward-shifted compared with E583M (Fig. 4A). The forskolin analog started to activate \(I\textsubscript{CNG}\) significantly at 10 \(\mu\)M with C460W/E583M and at 30 \(\mu\)M with E583M, with a maximal effect obtained at 100 \(\mu\)M for both channels. The Hill fit of the data yielded an apparent half-maximal activation (\(EC_{50}\)) and Hill coefficient (\(n\)) of 12.7 \(\mu\)M and 1.78 for C460W/E583M, and 36.7 \(\mu\)M and 1.53 for E583M, respectively, while the apparent maximal effects (\(E_{\text{max}}\)) were similar with both channels (59.9 pA/pF versus 61.4 pA/pF, respectively). The two curves were statistically different as indicated by Fisher test (\(p < 0.001\)). The \(E_{\text{max}}\) values reflected a saturation of the channels by cAMP since addition of the non-selective PDE inhibitor IBMX (100 \(\mu\)M) to 300 \(\mu\)M L-85 failed to further increase \(I\textsubscript{CNG}\) through E583M CNGA2 (data not shown). As illustrated in Fig. 4B, the results obtained with ISO differed substantially from those obtained with L-85. With both CNGA2 mutants, ISO started to increase \(I\textsubscript{CNG}\) density significantly at 10 \(\mu\)M, and the effect was maximal at 100 \(\mu\)M since a 10-times higher concentration produced no additional effect. The apparent \(EC_{50}\) and \(n\) values for ISO were 12.8 \(\mu\)M and 1.20 for C460W/E583M, and 22 \(\mu\)M and 1.45 for E583M, respectively, and apparent \(E_{\text{max}}\) was 20.5 pA/pF with C460W/E583M and 13.6 pA/pF with E583M. Comparison of the two CRC using the Fisher test indicated that the two curves were significantly different (\(p < 0.05\)).

If one assumes that cAMP is uniformly distributed under both L-85 and ISO stimulations, then the lower saturating effect of ISO should be ascribed to its smaller capacity to raise the cAMP level. As indicated in Fig. 4C by the intersection between ISO CRC and L-85 CRC obtained with E583M, this level corresponds to that induced by \(-15\) \(\mu\)M L-85. However,
PKA Inhibition Unmasks Global cAMP Signals—Among the various targets of PKA that could control cAMP levels, PDEs appear as obvious candidates (8, 12, 13, 14). Thus, in a first series of experiments, the effect of the non-selective PDE inhibitor IBMX on cAMP homeostasis reported by I_CNG was investigated. As shown in Fig. 6A, IBMX (100 μM) alone barely affected I_CNG. However, it dramatically potentiated the response of I_CNG to 100 nM ISO, which reached values similar to that obtained with L-85. Indeed, in 8 similar experiments, I_CNG density was 7.9 ± 2.3 pA/pF with 100 nM ISO alone and 53.0 ± 9.0 pA/pF when the β-adrenergic agonist was applied in the presence of 100 μM IBMX (Fig. 6B). In 6 of these myocytes, the effect of 100 μM L-85 was tested, and the stimulation of I_CNG was similar (47.0 ± 10.8 pA/pF). Parallel experiments were conducted to investigate the consequences of PDE inhibition on L-85 activation of cAMP production (Fig. 6C and D). Fig. 6C shows that in the presence of 100 μM IBMX, L-85 at 10 μM induced almost the same stimulation of I_CNG as L-85 at 100 μM. Indeed, in the presence of the PDE inhibitor, the mean I_CNG density was 54.8 ± 6.5 pA/pF for 10 μM L-85 and 72.4 ± 10.3 pA/pF for 100 μM L-85. Thus, PDE blockade resulted in a major potentiation of submaximal doses of L-85, underlining the crucial role of PDE in cAMP homeostasis.

We next examined the respective contribution of PDE3 and PDE4, the two main cAMP-hydrolyzing PDEs in mammalian cAMP Dynamics in Cardiomyocytes

with this hypothesis, the effect of ISO on the double mutant channel C460W/E583M should rise as indicated by the arrow on Fig. 4C, to reach ~37 pA/pF instead of 20.5 pA/pF. Thus, this clearly shows that the difference between ISO and L-85 is not simply caused by a different amount of cAMP generated.

PKA Inhibition Unmasks Global cAMP Signals—The fact that CNG channels are directly opened by cAMP and are not regulated by PKA (26) provides the unique possibility to investigate directly the role of this kinase in cAMP homeostasis. For this, the response of I_CNG to β-adrenergic and L-85 stimulations were compared in the absence and presence of the PKA inhibitor H89 (27) in myocytes infected with E583M Ad-CNG. The inhibitory effect of H89 on PKA was confirmed in separate experiments in which the drug antagonized PKA-mediated stimulation of the L-type Ca^2+ channel current by IBMX. Indeed, 100 μM IBMX increased I_CNL by 139.3 ± 10.7% (n = 7) under control condition, but only by 51.5 ± 11.5% (n = 7) in the presence of 1 μM H89. While H89 antagonized cAMP stimulation of I_CNL, it stimulated cAMP activation of I_CNG. Indeed, Fig. 5A shows a typical experiment in which ISO (100 nm) alone moderately increased I_CNG, but induced a major activation of the current in the presence of 1 μM H89, that reached values close to those obtained upon application of 100 μM L-85. The summary of 9 similar experiments confirmed these findings (Fig. 5B), whereas H89 alone failed to modify I_CNG, it greatly potentiated the effect of 100 nm ISO on I_CNG density (12.5 ± 3.3 pA/pF versus 40.1 ± 9.4 pA/pF in the presence of H89), which became equivalent to that of 100 μM L-85 (41.6 ± 6.7 pA/pF). The effect of submaximal (10 μM) and maximal (100 μM) doses of L-85 in the presence of H89 on I_CNG are depicted in Fig. 5C. This experiment shows that with H89, 10 μM L-85 markedly increased the current, which reached about 50% of the effect obtained at 100 μM. Fig. 5D compares the effect of L-85 at these two concentrations in the absence and in the presence of 1 μM H89. The PKA blocker potentiated the effect of L-85 at 10 μM from 6.3 ± 2.4 pA/pF to 20.8 ± 5.5 pA/pF, but failed to do so at 100 μM (48.2 ± 6.0 pA/pF versus 43.8 ± 7.9 pA/pF in the presence of H89). This lack of effect is likely related to a saturation of CNG channels at this concentration of L-85 (see Fig. 4A).

To further confirm the implication of PKA in the control of β-adrenergic cAMP signals, the effect of ISO and L-85 were assessed in myocytes co-infected with Ad-CNG and Ad-PKI, an adenovirus encoding the substrate inhibitor PKI (21), each at a MOI of 600. In these cells, the basal current density and the effect of L-85 (100 μM) were not significantly different (6.5 ± 1.7 pA/pF and 58.7 ± 9.9 pA/pF, respectively, n = 10) from those obtained in myocytes infected with Ad-CNG alone at MOI 600. However, similar to what was obtained with H89, the effect of ISO on I_CNG density was ~3 times higher in PKI-overexpressing cells (26.4 ± 2.9 pA/pF, n = 10) than in myocytes infected with Ad-CNG alone at MOI 600. Indeed, in the presence of the PDE inhibitor, the mean I_CNG density became equivalent to that of 100 μM IBMX (Fig. 6A). In 6 of these myocytes, the effect of 100 μM L-85 was tested, and the stimulation of I_CNG was similar (47.0 ± 10.8 pA/pF). Parallel experiments were conducted to investigate the consequences of PDE inhibition on L-85 activation of cAMP production (Fig. 6, C and D). Fig. 6C shows that in the presence of 100 μM IBMX, L-85 at 10 μM induced almost the same stimulation of I_CNG as L-85 at 100 μM. Indeed, in the presence of the PDE inhibitor, the mean I_CNG density was 54.8 ± 6.5 pA/pF for 10 μM L-85 and 72.4 ± 10.3 pA/pF for 100 μM L-85. Thus, PDE blockade resulted in a major potentiation of submaximal doses of L-85, underlining the crucial role of PDE in cAMP homeostasis.

We next examined the respective contribution of PDE3 and PDE4, the two main cAMP-hydrolyzing PDEs in mammalian
FIG. 5. PKA inhibition potentiates cAMP signals induced by ISO and L-85. A, time course of $I_{\text{CNG}}$ amplitude in an Ad-CNG-infected myocyte. The cell was superfused for several minutes in control Ringer solution prior to challenge with 100 nM ISO alone and 100 nM ISO in the presence of the PKA inhibitor H89 (1 μM). At the end of the experiment, the effect of 100 μM L-85 was tested. B, summary of the results of several similar experiments as in A. C, typical experiment showing the effect of L-85 (10 and 100 μM) in the presence of H89 (1 μM) on $I_{\text{CNG}}$. D, summary of similar experiments as in C. H89 significantly increased the stimulation induced by L-85 at 10 μM but not at 100 μM. Statistically significant differences are indicated as: *, $p < 0.05$.

FIG. 6. Effect of the non-selective PDE inhibitor IBMX on ISO and L-85 stimulation of subsarcolemmal cAMP. A, effect of IBMX on basal and β-adrenergic stimulation of $I_{\text{CNG}}$ by ISO compared with the effect of L-85 (100 μM) alone. B, summary of several similar experiments as in A. C, effect of L-85 (10 μM and 100 μM) in the presence of IBMX (100 μM). D, summary of several similar experiments as in C. Statistically significant differences are indicated as: ***, $p < 0.005$. 
obtained in a series of experiments as in A. Cilostamide (1 μM) significantly potentiated the effect of ISO (100 nM) on I_CNG, although the overall effect remained weaker than that of L-85 (100 μM). C, Ad-CNG-infected myocyte was superfused for several minutes with a control solution and then challenged with different drugs during the period indicated by the solid lines. D, summary of several similar experiments as in C. In the presence of 10 μM RO 20-1724, the effect of 100 nM ISO became identical to the effect of 100 μM L-85. Statistically significant differences are indicated as *, p < 0.05; ***, p < 0.005.

Cardiac myocytes (20, 28) to the attenuation of the β-adrenergic response. In the experiment shown in Fig. 7A, selective inhibition of PDE3 with cilostamide further increased I_CNG prestimulated with ISO, although it did not reach the level obtained with L-85 alone. Fig. 7B shows that on average PDE3 inhibition with 1 μM cilostamide exerted no significant effect on basal I_CNG density, but augmented the response to 100 nM ISO about 3-fold (7.8 ± 2.0 pA/pF versus 24.2 ± 6.3 pA/pF, n = 6). This latter value represented about 40% of the current density in the presence of 100 μM L-85 (59.3 ± 3.8 pA/pF, n = 6). Similarly, selective PDE4 inhibition with 10 μM RO 20-1724 had no effect on basal I_CNG density (Fig. 7, C and D). However, application of 10 μM RO 20-1724 in the presence of ISO (100 nM) induced an increase of I_CNG that was comparable to the stimulation obtained with 100 μM L-85 (65.1 ± 11.0 pA/pF and 65.5 ± 9.8 pA/pF, respectively, n = 6). These results emphasize the role of PDE3, and even more of PDE4, in regulating cAMP levels in cardiac myocytes.

**Regulation of cAMP-hydrolyzing PDEs—**Considering the above results, one may wonder whether PKA and PDE represent independent or linked regulators of cAMP dynamics. To answer this question, we examined whether PDE3 and PDE4 were regulated by ISO or L-85 in a PKA-dependent manner in rat ventricular myocytes. As shown in Fig. 8 (panels A and B), in 5 independent experiments ISO (100 nM) stimulated total PDE3 activity by ~70% and total PDE4 activity by ~75%. However, these effects were not blocked by H89 (1 μM). Upon application of L-85 at 10 μM (Fig. 8, panels C and D), total PDE3 activity was increased by ~65%, while total PDE4 activity was augmented by ~60%. As with ISO, this latter effect was insensitive to H89.

**PDE Inhibition Prevents PKA Negative Feedback—**The results above suggest that the potentiating effect of PKA inhibition on ISO- and L-85-stimulated I_CNG (Fig. 5) might not involve PDE inhibition. To check this, we tested the ability of H89 to potentiate the β-adrenergic stimulation of I_CNG when PDEs are inhibited by IBMX (Fig. 9). In the absence of β-adrenergic stimulation, H89 and IBMX, used alone or in combination, had virtually no effect on I_CNG (Fig. 9B). Low concentrations of the β-adrenergic agonist (0.3 nM) did not produce a detectable CNG increase either alone or when PKA was blocked by H89, but CAMP accumulation became apparent in the presence of IBMX (4.1 ± 0.2 pA/pF, n = 5, versus 1.5 ± 0.2 pA/pF for ISO 0.3 nM alone, n = 3). Addition of H89 did not further increase I_CNG augmented by IBMX + ISO (Fig. 9B). These results suggest that PKA inhibition has no consequence on CAMP accumulation when PDEs are blocked. However, the failure of H89 to potentiate the effect of ISO alone may indicate that CAMP synthesis was too small for a retrocontrol inhibition to occur or to be detected. Thus, the same protocol was repeated using a higher concentration of ISO (30 nM). In the representative experiment of Fig. 9A, the activation of the CNG current by 30 nM ISO was enhanced by H89, as well as by IBMX. However, in the presence of the PDE inhibitor, H89 was ineffective in potentiating the β-adrenergic stimulation. On average, ISO (30 nM) increased I_CNG density to 8.1 ± 1.2 pA/pF and to 18.2 ± 3.9 pA/pF in the presence of H89 (1 μM, n = 9). In the presence of IBMX (100 μM) the effect of ISO reached 47.6 ± 4.9 pA/pF (n = 9) and could not be further augmented by simultaneous H89 application (45.4 ± 5.6 pA/pF, n = 6). This was not caused by saturation of the CNG channels with CAMP because a subsequent application of L-85 (100 μM) was still able to further increase the current amplitude (62.6 ± 4.8 pA/pF, n = 5). Thus, PDE inhibition with IBMX eliminated the effect of H89 on ISO-stimulated I_CNG.
DISCUSSION

The use of recombinant CNG channels as cAMP biosensors was developed in a series of elegant studies in model cell lines (7, 12, 13, 29). Here, we have applied this methodology to differentiated adult cardiomyocytes in primary culture. The results obtained confirm and extend the suggestions made in a previous study of ours (8). We show that whole cell patch-clamp recording of $I_{\text{CNG}}$ provides a reliable readout of subsarcolemmal cAMP fluctuations in a single cardiac myocyte. The use of two CNGA2 variants with different affinities for cAMP indicates that the $\beta$H9252-adrenergic cAMP signal is compartmentalized in these cells. PKA activation of plasma membrane PDE negatively regulate cAMP increases triggered by $\beta$H9252-AR or direct AC activation. This negative feedback controls global cAMP homeostasis beneath the membrane and contributes to the maintenance of restricted hormonal cAMP signals.

Although molecular evidence exists for the presence of CNG channels in the heart (30–33) our immunofluorescence experiments did not reveal a significant expression of CNGA2 in native rat ventricular myocytes. Moreover, in patch-clamp experiments, a cAMP-activated current was never observed, either in native cells or in Ad-GFP-infected myocytes. These results exclude that the signal measured in Ad-CNG-infected myocytes was contaminated by an endogenous CNG-like current. In these cells, immunolocalization attested expression of CNGA2. The observed perinuclear localization could reflect an ongoing maturation process in the Golgi apparatus, and the staining at the ends of the cells, an accumulation near intercalated disks. The striated pattern may represent a facilitated access to less constrained regions of the sarcomere, such as the I-bands. However, the important point is that although not totally homogeneous, CNGA2 distributed throughout the plasma membrane and was thus expected to detect cAMP variations occurring anywhere below.

Because CNG channels are blocked by external and internal Ca$^{2+}$, the results presented in this study were obtained at nominal external [Ca$^{2+}$] and internal pCa 8.5. Since Ca$^{2+}$ inhibits CAMP synthesis by cardiac AC (34), the cAMP signals reported herein are certainly more robust than at physiological [Ca$^{2+}$]. Nevertheless, a larger cAMP accumulation with forskolin than with ISO was also shown to occur in intact cardiac myocytes in the presence of external Ca$^{2+}$ (35, 36). Similarly, it seems very unlikely that the regulatory mechanisms described in this study are caused by the low Ca$^{2+}$ context. Indeed, overexpression of PKI was shown to potentiate the effect of ISO on total cAMP in the presence of external Ca$^{2+}$ (35).

**Fig. 8.** Regulation of cellular cAMP-hydrolyzing PDE activities by ISO and L-85. Mean total cAMP-hydrolyzing activity of PDE3 and PDE4 were determined as described under “Experimental Procedures.” Panels A and B, effect of ISO (1 μM, 5 min) on PDE3 and PDE4 in five independent primary cultures of rat ventricular myocytes infected with Ad-CNG and preincubated or not with H89 (1 μM, 30 min). Panels C and D, same experiments as in A and B, but where L-85 (10 μM, 5 min) was used to stimulate cAMP production. The results are expressed as pmoles min$^{-1}$ mg prot$^{-1}$. Statistically significant differences are indicated as: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.005$.  

![Diagram](http://www.jbc.org/Downloaded from http://www.jbc.org)
Our initial observation that L-85 had a much stronger effect than ISO on $I_{\text{CNG}}$, while both agents activated $I_{\text{Ca,L}}$ maximally, can intuitively be explained in two ways. The first is that $\beta$-adrenergic stimulation produces a modest [cAMP] elevation that activates CNG channels only partially while maximally activating Ca$^{2+}$ channels. This could result from the lower affinity of E583M CNGA2 for cAMP (10 nM) compared with the affinity of PKA (100 nM) (7). The other possibility is that $\beta$-adrenergic stimulation activates only a subset of CNG channels located next to the $\beta$-adrenoreceptor-coupled adenylyl cyclases and Ca$^{2+}$ channels, whereas L-85 activates all cyclases and thus a larger population of CNG channels.

Comparison of the CRC obtained for each agonist using two CNGA2 mutants whose cAMP sensitivities differ by a factor of $\sim$10 (12) suggests that both possibilities might occur (Fig. 4). Indeed, the stronger activation of C460W/E583M than E583M by each concentration of ISO (Fig. 4B) means that the [cAMP] produced by the $\beta$-adrenergic stimulation does not fully activate the channels. However, this difference is too small to involve a large number of channels. This is attested by the larger variation observed between both CNGA2 variants with L-85 (Fig. 4C). The data are consistent with ISO generating discrete microdomains of high [cAMP] where a limited number of channels are saturated. In this scheme, the dose dependence...
of I$_{CNG}$ to the β-adrenergic agonist may be viewed as an increased CAMP microdomain number with increasing concentrations of the agonist. Because maximal concentrations of forskolin and G$_s$ similarly activate AC (37, 38), the ratio of the current densities of the E583M CNGA2 channel at maximal forskolin and G$_s$ similarly activate AC (37, 38), the ratio of the concentrations of the agonist. Because maximal concentrations of forskolin and G$_s$ similarly activate AC (37, 38), the ratio of the fraction of CNG channels, and thus the fraction of membrane area that is activated by the β-adrenergic agonist. By doing so, we can estimate that the maximal β-adrenergic response spreads over ~25% of the total sarcolemmal area. This experimental value is in agreement with the prediction of a recently developed model for β-adrenergic control of cardiac myocyte contractility (39).

Our results also underline the importance of regulatory mechanisms that prevent the spreading of small and diffusible CAMP molecules upon β-adrenergic receptor or direct AC stimulation. In this study, we focused on postreceptor mechanisms, which could presumably apply to ISO and L-85. This of course does not exclude that β-adrenergic receptor desensitization, in particular mediated by β-adrenergic receptor kinase, participates in the differential effect of ISO and L-85 on I$_{CNG}$.

First, we show that PKA exerts a tonic inhibition of CAMP accumulation upon ISO or submaximal concentration of L-85. A similar result was reported by Cui and Green (35) in avian embryonic ventricular myocytes, whereas such regulation was not observed in frog cardiomyocytes, suggesting species differences (40). Second, in agreement with a number of previous studies, we identified PDE3 and PDE4 as critical regulators of CAMP signals in cardiac myocytes. This finding is also consistent with the predominant impact of PDE3 and PDE4 activities for the β-adrenergic regulation of I$_{Ca,L}$ in rat ventricular myocytes (20) and the positive isotropic effect of PDE3 and PDE4 inhibitors in rats (41, 42).

These regulations can be overcome by maximal stimulation of all AC with 100 μM L-85 but not by saturation of the β-adrenergic receptor with ISO (Fig. 4). However, when disrupted by H89, IBMX, or Ro 20-1724, ISO and L-85 stimulations cannot be distinguished. This strongly suggests that these regulations ensure global homeostasis of cAMP but are also fundamental to confine receptor-triggered CAMP signals.

These findings raise the question of whether PKA acts through cAMP-PDE or through other targets. PKA activation of PDE3 (43–46) and PDE4 (17, 46–48) has been well characterized in vitro, although the evidence for this in the heart remains scarce (49, 50). Here, while ISO and L-85 clearly enhance the total PDE3 and PDE4 activities, these effects were not blocked by H89 (Fig. 8). This result is at variance with studies by Oki et al. (51) and MacKenzie et al. (52) showing that H89 prevents the stimulatory effect of CAMP-elevating agents on PDE4. However, these were performed in cell lines and, in the case of MacKenzie et al. (52), after overexpression of long PDE4 isoforms. PKA regulation of endogenous cAMP-PDE in adult cardiomyocytes is likely to be more difficult to prove since only certain PDE isoforms are phosphorylated by PKA (17, 53, 54), and PDEs can be regulated by other mechanisms, for instance involving other kinases (17, 55, 56).

Thus, while our electrophysiological data suggest that PDE activities controlling CAMP beneath the membrane are regulated by PKA, this was not apparent when total cellular activities of PDE3 and PDE4 were measured. These results may be reconciled by two kinds of explanations. Membrane-associated cAMP PDEs represent ~20–30% of the total CAMP hydrolyzing activity in guinea pig heart (57) and rat ventricular myocytes (preliminary results from this study, data not shown), an estimation that includes PDEs associated to intracellular organelles such as SR (58). Thus, one could imagine that a PKA-independent activation of “deep” pools of PDE might mask a PKA-dependent activation of the minor PDE fraction associated to the plasma membrane. On the other hand, one should not really expect to be able to mimic in an in vitro assay a regulation that presumably requires much cellular integrity. Indeed, it is possible that recruitment of the phosphorylated PDEs by a scaffold protein is part of the activation process (see below). Upon breaking the cells, such a complex would fall apart and the overall stimulation would be underestimated and might thus not be readily inhibitable by H89.

Alternatively, another mechanism but PDE activation could be involved in PKA-mediated negative feedback of CAMP signals revealed by CNG channels. In particular, adenylyl cyclases type V and VI, the major isoforms present in the adult heart (59), can be phosphorylated and inhibited by PKA (46, 60). In order to check this hypothesis, the effect of H89 on ISO-stimulated I$_{CNG}$ was assessed in the presence of IBMX. Since IBMX completely prevented the effect of H89 (Fig. 9), AC inhibition by PKA was unlikely to play a role. This result demonstrates that cAMP-PDEs are the relevant PKA targets for the control of subsarcolemmal CAMP gradients in cardiac myocytes.

This study documents a typical functional consequence expected from multimolecular signaling complexes of PKA and PDEs such as recently observed for the isoform PDE4D3 (50, 61). We provide a direct validation of the negative feedback model suggested by such a molecular organization and prove that it applies to subsarcolemmal CAMP dynamics. Dodge et al. (50) only found a modest PDE activity associated with AKAP15/18, the AKAP thought to associate with L-type Ca$^{2+}$ channels at the membrane (62, 63). This fits well with the fact that limited CAMP increases induced by ISO fully activate I$_{Ca,L}$ and suggests that the feedback loop identified here is essentially useful to prevent broadcast of CAMP outside of microdomains where it is needed (8, 14). This suggests that other proteins are responsible for targeting PDE3 and PDE4 around these signaling microdomains. Other AKAPs expressed in heart are known to associate to PDE4, such as AKAP450 (61, 64) or myomegalin (65). Alternatively, β-arrestin could be responsible for PDE4 translocation to the β-adrenergic receptors (66–68), but this deserves further investigation in adult cardiac cells.

In conclusion, we show that PKA activation of cAMP-PDE limits subsarcolemmal CAMP increases in intact cardiac cells. This mechanism can be triggered by β-adrenergic receptor stimulation or nonspecific activation of cellular AC and thus appears to control global CAMP homeostasis and to be required for compartmentation of β-adrenergic receptor CAMP signals. Further experiments are required to identify the PDE subtype(s) involved and to examine whether CAMP signals elicited by other G$_s$-coupled receptors are similarly regulated. The methodology presented here should contribute to a better understanding of cyclic nucleotides pathways in healthy and diseased heart cells.

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