Notes & Tips

Functional analysis of endogenous β-adrenergic receptor through fluorimetric monitoring of cyclic nucleotide-gated ion channel

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β-Adrenergic receptors (β₁, β₂, and β₃), coupling either to heterotrimeric G protein G_s solely or to G_s and G_i [1,2], regulate the activity of adenylyl cyclase that controls the intracellular level of cyclic 3′,5′-adenosine monophosphate (cAMP).[1] Currently, many cAMP assays are used to analyze endogenous β-adrenergic receptor function. However, most of the existing cAMP assays for G protein-coupled receptors (GPCRs) are based on competitive antibody binding to cAMP and require cell lysis for the measurement of total cytoplasmic cAMP in cell lysate [3–7]. Consequently, these assays are relatively slow, indirect, and unable to detect physiological relevant changes of cAMP that often are transient, localized, and rapidly reversible as receptors desensitize.

In this report, a cyclic nucleotide gated ion channel (CNGC)-based fluorimetric assay is used to measure cAMP in live cells, enabling us to functionally analyze endogenous β-adrenergic receptors. This new cAMP assay uses a mutated CNGC as the cAMP sensor [8,9]. The fluctuation of intracellular cAMP is coupled to the change of membrane potential through CNGC that conducts the cation influx, depolarizing plasma membrane (see supplementary Scheme 1). The change of transmembrane potential is proportional to the fluorescence change of a membrane potential-sensitive fluorescent dye [10,11].

Antibody-based enzyme-linked immunosorbent assays (ELISAs) are well accepted for measuring cAMP in cells. Among them, HitHunter and CatchPoint cAMP assay kits are widely used in high-throughput screening (HTS) appli-

cations. These two well-known cAMP assay kits were compared with CNGC-based cAMP measurement. The samples with known cAMP concentrations were tested with these two cAMP immunoassay kits to establish the cAMP standard curve and validate the assays (Fig. 1A). Forskolin, a direct adenylyl cyclase activator, is used to stimulate intracellular cAMP generation [12]. The HitHunter and CatchPoint kits determined EC_{50} values of forskolin to be 28.1 and $55.4 \pm 8.5 \, \mu M$, respectively, whereas CNGC-based cAMP assay had an EC_{50} value of forskolin of $0.75 \pm 0.3 \, \mu M$ (Fig. 1B). It is apparent that the CNGC-based cAMP assay is much more sensitive than the antibody-based cAMP assays. In addition, the CNGC-based cAMP assay has a larger dynamic range than do the cAMP immunoassays.

To demonstrate the utility of the CNGC-based cAMP assay, the three cAMP assays were further compared for functional analysis of endogenous β-adrenergic receptors. The HEK293 cell line was reported to have endogenous β₁ and β₂ adrenergic receptors [13,14]. A nonspecific β-adrenergic receptor agonist, isoproterenol, was selected as an activator. The HitHunter and CatchPoint assay kits were unable to detect any cAMP change in response to the activation of isoproterenol at various concentrations, whereas the CNGC-based cAMP assay was sensitive enough to determine the EC_{50} value of isoproterenol to be 14 nM (Fig. 2). The CNGC-based cAMP assay was further used to measure EC_{50} values of other β-adrenergic receptor agonists (Fig. 3A) and antagonists (Fig. 3B).

The cAMP compartmentalization during GPCR activation has been widely reported [9,15]. Under these circumstances, the level of local cAMP may increase enough to initiate certain biological events, although the total cytosolic cAMP level does not change significantly. Most of the existing cAMP assays require cell lysis to measure total cytoplasmic cAMP. In the current study,
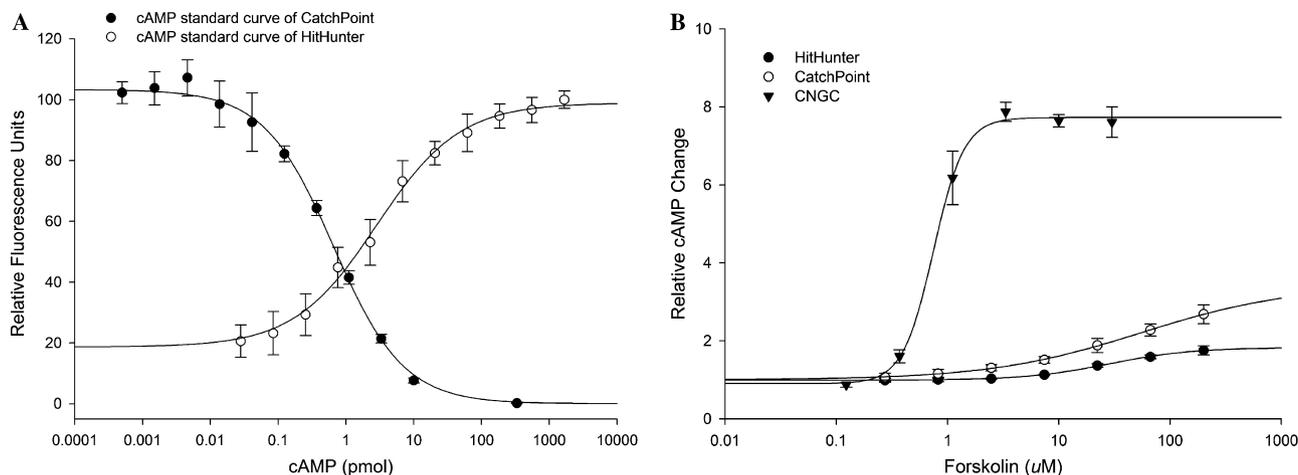

Fig. 1. Measurement of cAMP concentration by three different cAMP assays: HitHunter fluorescence cAMP assay kit, CatchPoint fluorescence cAMP assay kit, and CNGC-based live cell cAMP assay. (A) cAMP standard curve. A series of cAMP solutions with known concentrations were measured by HitHunter and CatchPoint. (B) Forskolin-induced cAMP activation. The HEK293-CNG cells were stimulated with different doses of forskolin for 30 min in the presence of 1 mM IBMX, a nonspecific PDE inhibitor. The cAMP level in cell lysate was measured by using the HitHunter and CatchPoint assays, whereas the cAMP level in whole cells was measured (without cell lysis) by using the CNGC-based assay. Means ± standard deviations, $n = 3$.
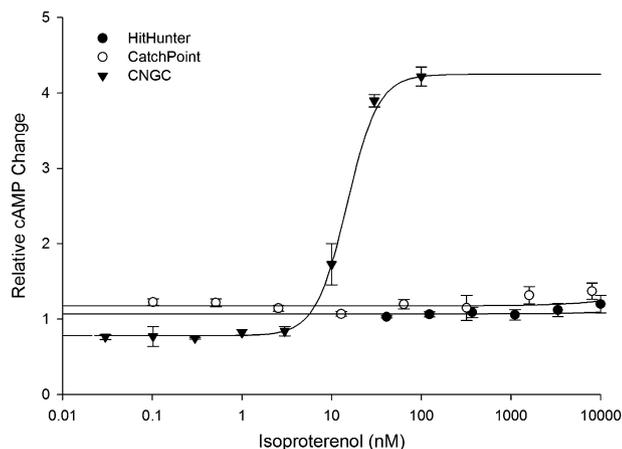

Fig. 2. Isoproterenol-induced cAMP activation was monitored with three different cAMP assays: Hit Hunter fluorescence cAMP assay kit, CatchPoint fluorescence cAMP assay kit, and CNGC-based live cell cAMP assay. The HEK293-CNG cells were stimulated with different doses of isoproterenol for 30 min in the presence of 1 mM IBMX. The cAMP level in cell lysate was measured with the two commercial cAMP assay kits in comparison with the CNGC-based cAMP assay that directly monitors cAMP change in live cells. Means ± standard deviations, $n = 3$.

these antibody-based cAMP assays were able to detect the cAMP increment when cells were stimulated by forskolin but not when cells were stimulated by isoproterenol.

We have described a physiological HTS assay for real-time monitoring of cAMP changes in live cells using CNGC variants as a cAMP reporter. The cAMP-gated CNGC activities are measured by using HLB 021-152, a fluorogenic membrane potential indicator. We demonstrated that the CNGC-based cAMP assay is able to detect cAMP activation by both forskolin and isoproterenol, whereas the HitHunter and CatchPoint assays can detect only the forskolin activation. Forskolin directly activates all types of adenylyl cyclases in whole cells, increasing cyto-

solic cAMP to a level high enough to be detected by cAMP protein-binding assays [16]. However, isoproterenol activates the endogenous β-adrenergic receptors, probably through stimulating the local adenylyl cyclases close to the receptors. Thus, the cAMP increment is restricted to the subcellular domains, whereas the total cytosolic cAMP change remains insignificant. β-Adrenergic receptors have been reported to be present at very low levels (1–3 fmol/mg) in HEK293 cells [17]. The CNGC-based cAMP assay can monitor the change of cAMP concentration more sensitively than can the conventional ELISAs because the CNGCs are in the same three-dimensional compartment as are adenylyl cyclases and β-adrenergic receptors [8,9].

It has been reported that the CNGC can be inhibited by intracellular PIP3, modulated by calcium-calmodulin [18], and blocked by APPA-tetracaine [19], pseudechetoxin [20], or pseudecin [21]. In the current study, we observed that the EC_{50} value of isoproterenol is significantly right-shifted in the absence of a PDE inhibitor that is commonly used to stabilize intracellular cAMP concentration in many assays. In addition, we noted that β-adrenergic receptor antagonists eliminate the effect of isoproterenol (Fig. 3B). Thus, we believe that the signal of the CNGC-based assay is related mainly to cAMP change induced by isoproterenol.

Unlike the disruptive cAMP immunoassays and reporter gene-based cAMP assays, both of which require prolonged receptor stimulation, the CNGC-based cAMP assay allows real-time detection of receptor activation. Real-time assay allows a kinetic determination of receptor efficacy, being completed within a few minutes after stimulation, during which time receptor desensitization is still negligible. Thus, the CNGC-based assay provides a more accurate biometry of the receptor activations. In addition, the CNGC-based cAMP assay is homogeneous and easy to be adapted for HTS given that the assay does not require cell lysis, antibody binding, and washing steps.
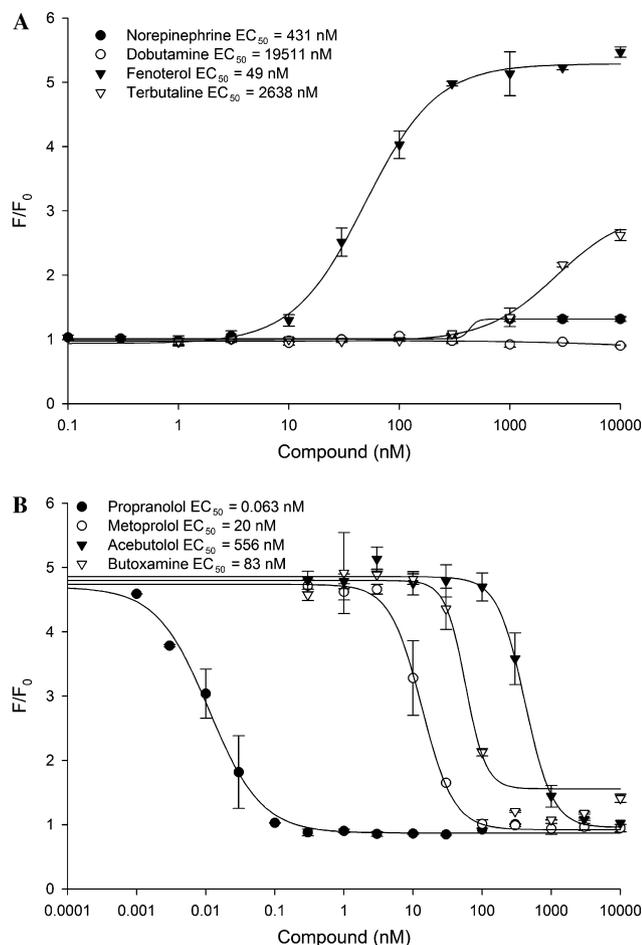
Fig. 3. Functional analysis of β-adrenergic receptor agonists and antagonists by CNGC-based cAMP assay. The HEK293-CNG cells were loaded with HLB 021-152 assay solution for 2 h in the presence of 100 μM Ro-20-1724, a PDE type IV inhibitor. The cells were stimulated with different doses of agonists for agonist assays, whereas the cells were first treated with different doses of antagonists for 15 min and then stimulated with 30 nM isoproterenol for 30 min for antagonist assays. The baseline fluorescence signal (F_0) was recorded before the compound addition (at time 0 min), and the induced fluorescence signal (F) was taken at time 30 min. The F/F_0 was plotted against the concentration of the stimulation compound. (A) β-Adrenergic receptor agonists. (B) β-Adrenergic receptor antagonists. Means ± standard deviations, $n = 3$.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2006.04.051.

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