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High-throughput real-time monitoring of G_s-coupled receptor activation in intact cells using cyclic nucleotide-gated channels

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Abstract

Cyclic adenosine-monophosphate (cAMP) is one of the major second messenger molecules transmitting extracellular stimuli into short- and long-term changes of intracellular homeostasis. Measurements of cellular cAMP levels are often used to quantify and characterize signaling by G protein-coupled receptors. Current assays for cAMP determination are usually end-point assays involving cell lysis. We have developed a technology to monitor real-time changes of cAMP levels in living cells. This method uses a modified cyclic nucleotide-gated (CNG) Ca^{2+} channel which is opened by intracellular cAMP. Thus, changes in cAMP levels are translated into changes in free Ca^{2+} which can easily be measured using fluorimetric imaging technologies compatible with high-throughput screening formats. The new assay method was used to characterize the pharmacology of various endogenously and heterologously expressed G protein-coupled receptors and allows for the simultaneous study of G_s, G_i and G_q-linked receptors in the same cell population.

Keywords: Cyclic nucleotide-gated channel; cAMP; Ca^{2+} imaging; Second messenger assay

1. Introduction

All eukaryotic cells use second messengers like cyclic adenosine-monophosphate (cAMP) and Ca^{2+} to translate extracellular signals from neurotransmitters or hormones into the intracellular environment. Changes in cAMP and Ca^{2+} produce short-term effects modulating excitability and membrane potentials but can also lead to long-term effects involving gene transcription. All known G protein-coupled receptors are modulating either cAMP or Ca^{2+} levels. G protein-coupled receptors constitute one of the largest gene families in the genome and are targets of a huge number of therapeutically used drugs. Quantitative second messenger assays are not only used in drug screening and pharmacological characterization of G protein-coupled receptor agonists and antagonists, they are also important tools to study fundamental cellular processes and signaling cascades.

Dynamic changes in intracellular Ca^{2+} can be easily monitored and quantified in real-time by fluorimetric techniques using Ca^{2+}-sensitive dyes such as Fura-2 or Fluo-4 (Lipp et al., 2000). However, most commonly used methods for determination of cAMP involve cell lysis and extraction of cAMP followed by radioimmunoassays or similar immunological methods. Also, in vitro labeling techniques of newly formed cAMP are end-point assays which do not allow for observation of temporal changes in living cells. A method involving protein kinase A (PKA) subunits, either chemically labeled with different fluophores (Adams et al., 1991) or genetically fused to different species of green fluorescent protein (Zaccolo et al., 2000), uses fluorescence energy transfer for real-time analysis of cAMP changes but requires sophisticated equipment and has a narrow dynamic signal-to-noise range. In addition, injection of single cells with fluorescently labeled PKA subunits is not amenable to high-throughput screening. Reporter gene assays involving cAMP-mediated gene transcription are also end-point assays (Stratowa et al., 1995). Since the reporter gene is far downstream the signaling cascade and the assays are based on the integration of cAMP concentrations over an extended

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period (typically several hours), they are more prone to yielding false-positive results caused by compounds producing nonspecific effects.

Cyclic nucleotide-gated (CNG) Ca^{2+} channels provide a "link" between the two second messenger systems that can be used to generate a cAMP-regulated form of Ca^{2+} entry (Dhallan et al., 1990). Functionally, the cyclic nucleotide-gated channels belong to the family of ligand-gated cation channels. The retinal CNG channel is gated by cGMP, whereas the olfactory CNG channel is opened by binding either cGMP or cAMP on the intracellular side of the channel. The cyclic nucleotide binding domain resides in the carboxyterminal part of the protein (Varnum and Zagotta, 1997). At physiological concentrations of extracellular Ca^{2+}, the olfactory CNG channel is highly selective for Ca^{2+}. Ca^{2+}/calmodulin binding at the amino-terminal intracellular domain provides a mechanism of channel inactivation (Grunwald et al., 1999). Although wildtype CNG channels are heterooligomers containing multiple subunits, homooligomeric channels comprising only the rat olfactory CNG α subunit (also termed CNG2 or CNGα3) can form a functional Ca^{2+} channel (Frings et al., 1995). Recently, a report demonstrated the use of the olfactory CNG channel to measure cAMP changes indirectly by Fura-2 imaging of intracellular Ca^{2+} (Rich et al., 2001). The study used transient transfection with an adenovirus-based vector and monitored cAMP-induced changes of Ca^{2+} in single cells. We have extended this approach to a high-throughput format in stably and transiently transfected human embryonic kidney (HEK) 293 cells. Using a fluorimetric imaging plate reader (FLIPR) with 96-well format, we have characterized the pharmacological profile of various G_s-coupled receptors. In addition, this method allows for simultaneous detection of G_s-, G_i- and G_q-coupled receptors in the same cell population and could be a useful tool to study cross-talk and G protein coupling diversity of G protein-coupled receptors in general.

2. Materials and methods

2.1. Cell culture, vectors and transfection

Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum at 37 °C in an atmosphere containing 5% CO_2. Rat opioid receptor-like receptor ORL-1 (Genbank accession no. NM_031569) and the rat κ opioid receptor KOR (L22536) were cloned in pRcRSV (Invitrogen). The human dopamine D1 receptor (NM_000794) and the rat trace amine TA1 receptor (AF380186) were cloned in pcDNA 3.1 (+) neo (Invitrogen). Transient and stable transfections were done using Lipofectamine (Life Technologies) according to the manufacturer's protocol. Stably transfected clones were obtained after selection with 800 mg/l G418.

2.2. Construction of oCNG mutants

The wildtype rat olfactory CNG channel (GenBank accession no. X55519) was kindly provided by Dr. Randall Reed (Johns Hopkins University). Functional domains of the olfactory CNG channel have been mapped extensively before (Varnum and Zagotta, 1997; Grunwald et al., 1999). We aimed to change the ligand selectivity and inactivation characteristics of the wildtype channel in order to make it more suitable as a cAMP sensor. A Ca^{2+}/calmodulin-insensitive mutant (termed oCNG-N) was generated by introducing two point mutations (F68A and W81A) using synthetic oligonucleotides and the Quik Change Site-Directed Mutagenesis kit (Stratagene). A cAMP-specific mutant (termed oCNG-C) was constructed by introducing point mutations C460W and E583M using the same method. Double mutant channels (termed oCNG-NC) were obtained by fusing the respective fragments using suitable endonuclease restriction sites. Wildtype and mutant channels were subcloned into pcDNA3.1 (+) neo (Invitrogen) and sequenced on both strands.

2.3. Measurement of intracellular Ca^{2+}

Twenty-four hours after transfection, cells were seeded into black clear-bottom 96-well plates at a density of 50,000 cells/well. Following overnight incubation, the cells were loaded for 1 h with 1 μM of the fluorescent calcium indicator dye Fluo4-acetoxymethylester (Molecular Probes) dissolved in assay buffer containing Hank's balanced salts, 20 mM HEPES, 1% fetal bovine serum, 2.5 mM probenecid, pH 7.4. After loading, the cells were washed three times with assay buffer without serum. Addition of drugs and measurement of changes in intracellular calcium were performed using a fluorimetric imaging plate reader (FLIPR, Molecular Devices). Agonist responses were determined using the maximum change in fluorescence over baseline. Drugs and chemicals were obtained from Sigma unless specified differently. Dynorphin A was purchased from Bachem (Bubendorf, Switzerland) and Orphanin FQ/Nociceptin was custom synthesized by Research Genetics (Invitrogen). Dose–response curves were calculated using GraphPad Prism (GraphPad Software) and values are given as halfmaximal effective concentration (EC_{50}) ± standard error of mean (S.E.M.).

2.4. Measurement of cAMP accumulation

HEK 293 cells were transiently cotransfected with dopamine D1 receptor and oCNG-NC channel plasmids and seeded into 96-well plates 24 h later. Following overnight incubation, the culture medium was exchanged for 50 μl/well Opti-MEM (Life Technologies) containing 100 μM 3-isobutyl-1-methylxanthine (IBMX) and increasing concentrations of drugs. Cells were then incubated for 15 min at 37 °C. Cyclic AMP was extracted as described (Reinscheid et

al., 1995) and quantified using a commercial cAMP assay kit (Flashplate, NEN). Dose–response curves were calculated using GraphPad Prism (GraphPad Software) and values are given as halfmaximal effective concentration (EC_{50}) ± standard error of mean (S.E.M.).

2.5. Analysis of assay variability

Intra-day assay variability and signal-to-noise ratio was calculated by stimulating 48 wells of transiently transfected HEK 293 cells with 50 μM forskolin and measuring the maximal response during 5 min following drug administration. These values were compared with 48 wells receiving only assay buffer to obtain signal-to-noise values.

Inter-day variability was calculated by comparing EC_{50} values for β-adrenergic (endogenous) and dopamine D1 receptor (transiently transfected) activation from independent experiments on separate days.

3. Results

3.1. Expression and functional analysis of mutant olfactory CNG channels

Forskolin stimulation of HEK 293 cells transiently transfected with the rat wildtype olfactory CNG channel produced a dose-dependent increase in intracellular free Ca^{2+}, however, the overall signal intensity was weak (Fig. 1A).

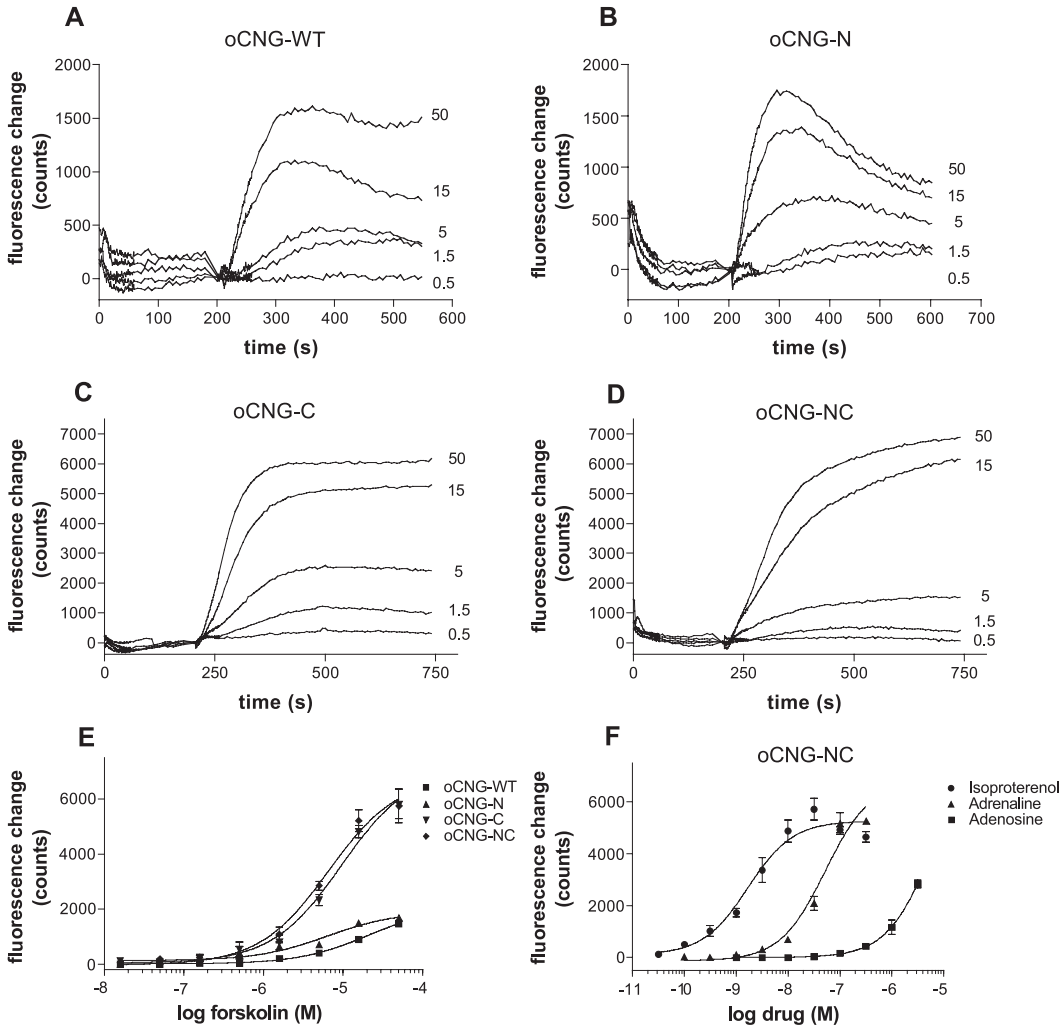

Fig. 1. Expression of wildtype and mutant olfactory CNG (oCNG) channels in HEK 293 cells. HEK 293 cells were transiently transfected with wildtype oCNG channel (A), Ca^{2+}/Calmodulin-insensitive mutant CNG channel (B), cAMP-specific mutant (C), or the double mutant channel (D) and cAMP-mediated changes of intracellular Ca^{2+} were measured after the addition of increasing concentrations of forskolin. Numbers next to traces denote forskolin concentrations (in μM). Cells were incubated with 100 μM IBMX and forskolin was added 180 s later. To compensate for baseline drift, all traces were normalized to the fluorescence measured at 200 s. Traces of representative experiments are shown. (E) Forskolin dose–response of cAMP-mediated Ca^{2+} influx in the four oCNG channel constructs. EC_{50} values were 20.3 ± 2.1 μM for oCNG-WT, 6.3 ± 1.3 μM for oCNG-N, 9.5 ± 1.05 μM for oCNG-N, and 6.6 ± 0.8 μM for oCNG-NC, respectively. (F) Agonist-induced activation of endogenously expressed G_s-coupled GPCRs in HEK 293 cells transiently transfected with oCNG-NC measured as cAMP-mediated increase in intracellular Ca^{2+}. All experiments were performed in triplicate and repeated at least twice.

The Ca^{2+}/calmodulin-insensitive mutant oCNG-N displayed a very similar pharmacology upon forskolin treatment (Fig. 1B). Interestingly, the oCNG-N channel appeared to inactivate faster than the wildtype channel, which became more obvious at higher forskolin concentrations. Increasing the ligand selectivity for cAMP such as in the oCNG-C mutant channel dramatically increased the signal amplitude and thus Ca^{2+} flux through the open channel (Fig. 1C). A combination of both mutations (oCNG-NC) did not increase the signal intensity further (Fig. 1D). In wildtype and mutant olfactory CNG channels, the EC_{50} for forskolin-stimulated increase in intracellular free Ca^{2+} was between 6 and 20 μM. However, the maximum signal obtained after stimulation with 50 μM forskolin increased more than fourfold in oCNG-C and oCNG-NC mutants as compared to wildtype and the oCNG-N mutant channel (Fig. 1E). Forskolin showed no effect on intracellular Ca^{2+} flux in untransfected cells (data not shown).

The double mutant channel (oCNG-NC) was chosen for further experiments to characterize the pharmacological profile in more detail. First, we analyzed whether activation of endogenous G_s-coupled receptors, such as β_2-adrenoceptors or adenosine A_{2B} receptors, can be detected with this assay. As shown in Fig. 1F, cells transfected with oCNG-NC showed a dose-dependent change in intracellular Ca^{2+} after stimulation with the agonists isoproterenol (EC_{50}: 1.68 ± 0.24 nM), adrenaline (EC_{50}: 52 ± 6.3 nM) or adenosine (EC_{50}: 15.7 ± 0.55 μM), respectively. The EC_{50} values for agonist activity in the oCNG channel assay were found to be very similar to classical cAMP assays (isoproterenol: 9 nM (Fraser, 1989); adrenaline: 38 nM (Liapakis et al., 2000); adenosine: 64.6 μM (Cooper et al., 1997)). Untransfected HEK 293 cells showed no change in intracellular Ca^{2+} using the same compounds (data not shown).

In a series of experiments, we then analyzed the effect of (i) different extracellular Ca^{2+} concentrations, (ii) sensitivity of the signal to treatment with phosphodiesterase inhibitors, and (iii) a "priming" effect of low forskolin concentrations.

As shown in Fig. 2B, addition of 5 mM EDTA to the extracellular medium almost completely abolished isoproterenol-induced Ca^{2+} flux, thus showing that the cAMP-mediated Ca^{2+} influx is solely dependent on extracellular Ca^{2+}. In contrast, EDTA addition had no effect on a transient increase of intracellular Ca^{2+} elicited by activation of endogenous $P2Y_2$ receptors by 200 μM ADP, since this receptor is coupling via G_q proteins to the phospholipase C pathway (Fig. 2A). On the other hand, increasing the concentration of extracellular Ca^{2+} to 5 mM did not increase the signal, indicating that the channel is opened at maximum capacity in the presence of physiological Ca^{2+} concentrations (Fig. 2C). Pretreatment of the cells with two different phosphodiesterase inhibitors, 3-isobutyl-1-methyl-

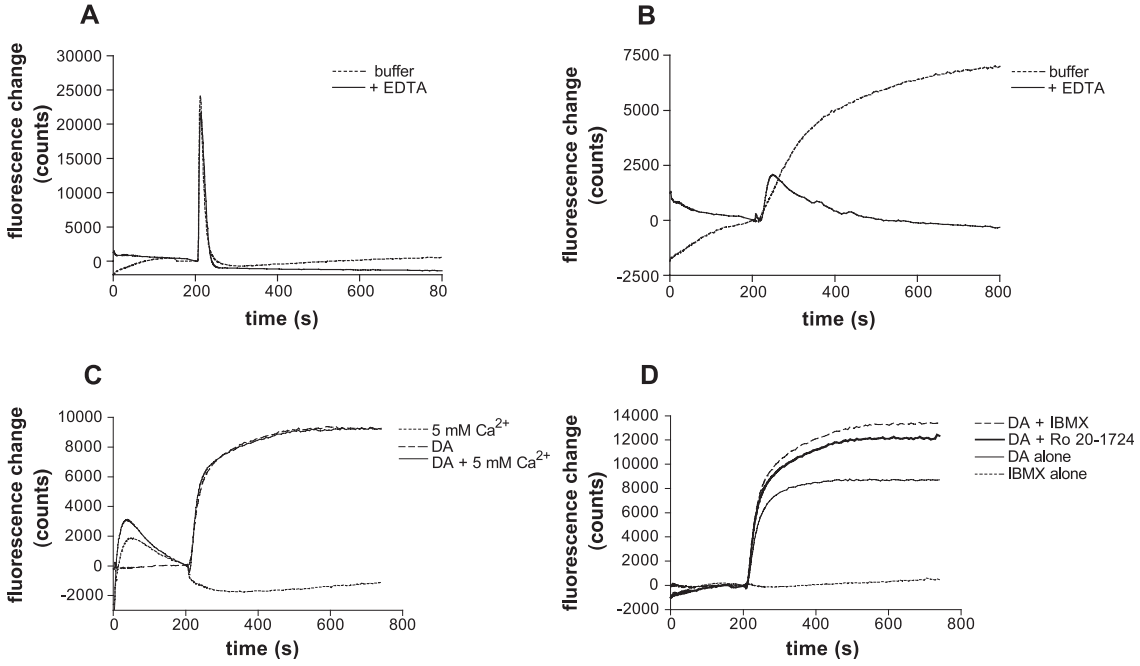

Fig. 2. Characterization of cAMP-mediated Ca^{2+} influx in HEK 293 cells transiently transfected with oCNG-NC. (A) The transient rise in intracellular Ca^{2+} produced by stimulation of the G_q-coupled $P2Y_2$ receptor in HEK cells with ADP is independent of extracellular Ca^{2+}. ADP (200 μM) was added after 180 s. (B) Stimulation of the same cells with 100 nM isoproterenol elicits a cAMP-dependent rise in intracellular Ca^{2+} which is almost completely abolished in the presence of 5 mM extracellular EDTA. Cells were incubated with and without EDTA at 0 s and isoproterenol was added after 180 s. (C and D) HEK 293 cells transiently transfected with dopamine D1 receptor and oCNG-NC. (C) Increasing the extracellular Ca^{2+} concentration to 5 mM does not increase the signal amplitude produced by 100 nM dopamine (DA). Assay buffer containing either normal or 5 mM Ca^{2+} was added at 0 s and dopamine was applied at 180 s. (D) Effect of phosphodiesterase inhibitors. The amplitude of the Ca^{2+}- dependent fluorescence signal produced by 100 nM dopamine (DA) can be increased by both 100 μM IBMX or 10 μM Ro 20-1724. To compensate for baseline drift, all traces were normalized to the fluorescence measured at 200 s. In all figures, representative traces are shown. Experiments were performed in triplicate and repeated at least twice.

xanthine (IBMX) and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724), increased the subsequent signal elicited by isoproterenol by about 40% (Fig. 2D), while the phosphodiesterase inhibitors alone had no effect. It had been shown before that pretreatment with a low dose of forskolin and thus slightly elevating intracellular cAMP concentrations could have a so-called "priming" effect on G_s-coupled second messenger responses (Barovsky and Brooker, 1985). However, when we treated cells with 0.1 μM forskolin (100-fold below the EC_{50} for forskolin), we could only observe additive stimulatory effects (data not shown).

It is conceivable that permanent expression of a cAMP-activated Ca^{2+} channel could be detrimental to cells. Therefore, all the above-mentioned experiments were carried out with transiently transfected cells. Nevertheless, we attempted to obtain HEK 293 clones that stably express the oCNG-NC channel. After screening a large number of antibiotic-resistant clones, we finally isolated a single stable clone that displayed the expected pharmacology. This clone (HEK oCNG-NC-5) showed cAMP-mediated Ca^{2+} influx after forskolin and isoproterenol stimulation with EC_{50} values of 7.2 ± 0.87 μM and 4.6 ± 0.41 nM, respectively (Fig. 3). The agonists adrenaline and adenosine activated their respective endogenous receptors with EC_{50} values of 17 ± 2.2 nM and 23.9 ± 4.3 μM, respectively.

3.2. Agonist-induced activation of G_s-coupled GPCRs

As mentioned above, agonist activation of two endogenous G_s-coupled GPCRs could be detected as a cAMP-mediated increase in intracellular Ca^{2+}. Next, we used the human dopamine D1 receptor and the rat trace amine TA1 receptor to analyze whether heterologous expression of G_s-coupled GPCRs could be studied with this assay. In cells transiently coexpressing dopamine D1 receptor and oCNG-NC channel, the agonist dopamine produced a dose-dependent increase in intracellular Ca^{2+} with an EC_{50} of 1.25 ± 0.1 nM. The D1 receptor-specific agonists (±)-6-Chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrobromide (SKF-81297) and (±)-6-Chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrobromide (SKF-82958) displayed EC_{50} values of 0.26 ± 0.08 and 1.08 ± 0.21 nM, respectively (Fig. 4A). No agonist-induced mobilization of intracellular Ca^{2+} was observed in HEK 293 cells expressing only the dopamine D1 receptor (data not shown). When cAMP levels were measured by radioimmunoassay, we obtained slightly lower values for dopaminergic agonist potencies. Dopamine stimulated cAMP accumulation with an EC_{50} value of 8.5 ± 1.23 nM, whereas SKF-82958 displayed an EC_{50} value of 9.4 ± 2.1 nM (Fig. 5). In the same cells, stimulation of endogenous β-adrenoceptors by isoproterenol and adrenaline displayed EC_{50} values of 3.33 ± 1.18 and 49 ± 11 nM, respectively. These values are almost identical to the agonist potencies obtained using the oCNG channel as a cAMP sensor.

Similarly, activation of the rat trace amine TA1 receptor could be measured with this assay system. The agonists tyramine, tryptamine, β-phenylethylamine, and octopamine dose-dependently activated cAMP-mediated Ca^{2+} influx with EC_{50} values of 112 ± 21, 360 ± 42, 279 ± 37 nM and 2.8 ± 0.31 μM, respectively (Fig. 4B). Cells expressing only the rat TA1 receptor displayed no increase in intracel-

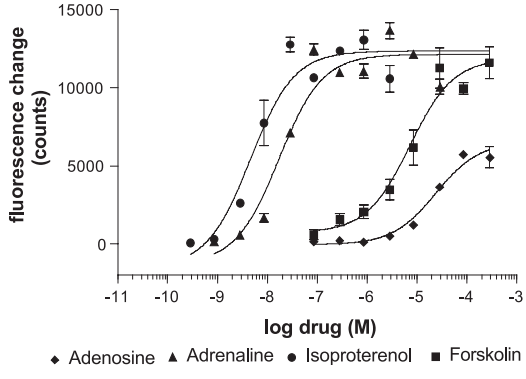

Fig. 3. Agonist dose–response of cAMP-mediated Ca^{2+} influx in the stable clone HEK oCNG-NC-5. Note that the amplitude of the signal is almost twofold higher than in transiently transfected cells. All experiments were performed in triplicate and repeated three times.

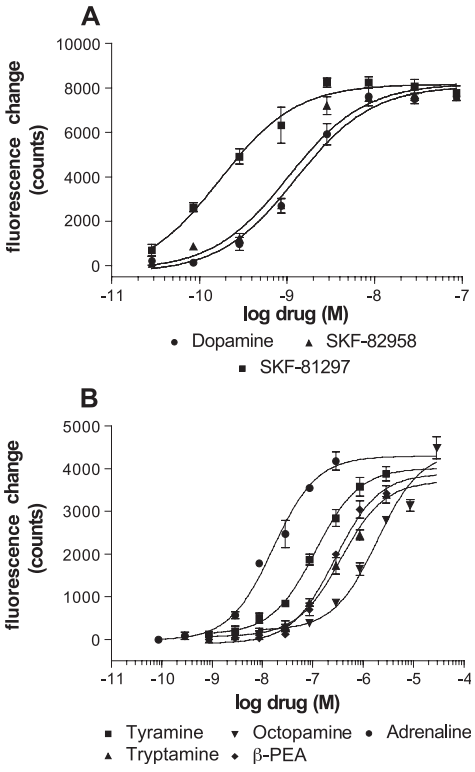

Fig. 4. Pharmacology of G_s-coupled receptors in HEK 293 cells expressing oCNG-NC. (A) Activity of dopaminergic agonists in HEK 293 cells transiently expressing dopamine D1 receptor and oCNG-NC. (B) Activity of aminergic compounds in cells transiently expressing rat trace amine TA1 receptor and oCNG-NC. β-PEA: β-phenylethylamine. All experiments were done in triplicate and repeated three times. Results from a representative experiment are shown.

signaling; whereas at higher concentrations, the G_s-mediated effects could not be attenuated anymore (data not shown). Using 5 nM dopamine for G_s stimulation, the EC_{50} value for orphanin FQ/nociceptin to inhibit cAMP-mediated Ca^{2+} influx was 4.6 ± 0.29 nM (Fig. 6B). This value is in good agreement with the EC_{50} of orphanin FQ/nociceptin at stably expressed ORL-1 receptors ($EC_{50} = 1$ nM) when measuring inhibition of forskolin-stimulated cAMP accumulation by radioimmunoassay (Reinscheid et al., 1995). Similarly, in cells cotransfected with oCNG-NC, dopamine D1 receptor and the rat κ opioid receptor (KOR), dynorphin A was able to reduce 5 nM dopamine-induced Ca^{2+} influx with an EC_{50} value of 0.51 ± 0.07 nM (Fig. 6B). As shown before, dynorphin A can inhibit forskolin-stimulated cAMP formation with an EC_{50} around 0.1 nM as measured by radioimmunoassay of cellular cAMP (Reinscheid et al., 1998). These results demonstrate that HEK 293 cells expressing the olfactory CNG channel can be a useful tool to study simultaneous G_s and G_i-linked GPCR signaling in real-time.

4. Discussion

In this study, we have demonstrated that olfactory cyclic nucleotide-gated channels can be used in a high-throughput format to monitor activation of G_s-coupled GPCRs in real time by linking intracellular cAMP levels to Ca^{2+} influx. The assay shows dose-dependency with a variety of receptor-mediated responses and has a high dynamic range. It also allows for real-time monitoring of G_i-linked events that inhibit adenylyl cyclase. Thus, cells transfected with an appropriate olfactory CNG channel are able to simultaneously report G protein-coupled receptor-mediated signaling through all three major G protein pathways, including G_s, G_i, and G_q, which can be quantified as changes in intracellular free Ca^{2+}.

We observed good correlation of agonist potencies between the oCNG channel assay and published data using traditional cAMP assays. Our own experiments gave almost identical EC_{50} values for endogenous G_s-coupled receptors when comparing the oCNG channel assay with a cAMP radioimmunoassay, however, we detected apparently higher agonist potencies for the transiently transfected dopamine D1 receptor using oCNG channels as cAMP sensors. This difference might reflect variations in transfection efficiency. Alternatively, the artificially high expression of both the dopamine D1 receptor and the oCNG channel (both are driven by cytomegalovirus promoters) might render the system supersensitive in that channel activation causing Ca^{2+} influx is already triggered at substantially lower cAMP concentrations. Although the EC_{50} of a given receptor is not an absolute value since it is dependent on the stochiometry of each component of the signaling cascade as well as the cellular environment, we obtained values for various G_s and G_i-coupled receptors that are very similar to published studies in which heterologously expressed receptors were used. This indicates that the oCNG channels are not rate-limiting and the subsequent quantification of changes in intracellular Ca^{2+} correlates well with actual cAMP levels, thus reflecting the respective potencies of agonists at various G_s-coupled receptors to stimulate adenylyl cyclases.

The pharmacological characteristics of the oCNG channel have been modified to optimize its suitability as a cAMP detector in a high-throughput format. Thus, we aimed to increase signal-to-noise ratio and prolong the signal duration. We did not observe regulation of oCNG channels because as mentioned above, the oCNG-NC construct had been engineered to lack the Ca^{2+}/calmodulin regulatory binding site that is normally involved in channel closing. We did not study long-term effects of chronically elevated cAMP levels on expression levels or posttranslational regulation of oCNG channels since most second messenger assays are performed over only a short time range. Therefore, the mutant oCNG-NC construct is primarily a research and screening tool to detect G_s-linked receptor activation and does not reflect the actual in vivo dynamics of cAMP accumulation. The FLIPR technology itself does not allow for single cell resolution since it measures average Ca^{2+} -dependent fluorescence changes in a large population of cells. This enables the use of transiently transfected cells in which only a fraction of cells are expressing the relevant genes. However, in order to study cAMP-dependent changes of intracellular Ca^{2+} in a physiologically more natural system, one might use the wildtype oCNG channel in a single cell recording configuration.

In theory, this assay can be used in any cell type able to express the olfactory CNG channel. So far, we have tested only a few other cell lines and found that HEK 293 cells gave the most robust response. In addition, we have obtained data showing that also Chinese hamster ovary (CHO) cells and COS-7 cells can be successfully transfected with oCNG-NC. Using adenovirus-mediated transfection, it was demonstrated before that olfactory CNG channels can be expressed in GH4C1 pituitary cells and C6 rat glioma cells (Rich et al., 2001; Fagan et al., 1999).

To date, no high-throughput technology for monitoring cAMP responses in real-time has been available. Taking advantage of commercially available reagents and equipment for measuring changes in intracellular Ca^{2+}, this new technique should be a versatile tool to study G_s-coupled receptors on existing technological platforms like the FLIPR. One transient transfection of a 10-cm dish of HEK 293 cells yields enough cells for a 96-well or 384-well plate suitable for Ca^{2+} imaging in the FLIPR. This illustrates that the assay can easily be scaled up to generate thousands of data points per day. The oCNG-based cAMP assay also allows for new approaches to investigate temporal and stochiometric interactions between G_s- and G_i-linked receptor signaling.

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