Adenovirus-mediated Expression of an Olfactory Cyclic Nucleotide-gated Channel Regulates the Endogenous Ca\(^{2+}\)-inhibitable Adenylyl Cyclase in C6-2B Glioma Cells*

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Previous studies have established that Ca\(^{2+}\)-sensitive adenylyl cyclases, whether endogenously or heterologously expressed, are preferentially regulated by capacitative Ca\(^{2+}\) entry, compared with other means of elevating cytosolic Ca\(^{2+}\) (Chiono, M., Mahey, R., Tate, G., and Cooper, D. M. F. (1995) J. Biol. Chem. 270, 1149–1155; Fagan, K. A., Mahey, R., and Cooper, D. M. F. (1996) J. Biol. Chem. 271, 12438–12444; Fagan, K. A., Mons, N., and Cooper, D. M. F. (1998) J. Biol. Chem. 273, 9297–9305). These findings led to the suggestion that adenylyl cyclases and capacitative Ca\(^{2+}\) entry channels were localized in the same functional domain of the plasma membrane. In the present study, we have asked whether a heterologously expressed Ca\(^{2+}\)-permeable channel could regulate the Ca\(^{2+}\)-inhibitable adenylyl cyclase of C6-2B glioma cells. The cDNA coding for the rat olfactory cyclic nucleotide-gated channel was inserted into an adenovirus construct to achieve high levels of expression. Electrophysiological measurements confirmed the preservation of the properties of the expressed olfactory channel. Stimulation of the channel with cGMP analogs yielded a robust elevation in cytosolic Ca\(^{2+}\), which was associated with an inhibition of cAMP accumulation, comparable with that elicited by capacitative Ca\(^{2+}\) entry. These findings not only extend the means whereby Ca\(^{2+}\)-sensitive adenylyl cyclases may be regulated, they also suggest that in tissues where they co-exist, cyclic nucleotide-gated channels and Ca\(^{2+}\)-sensitive adenylyl cyclases may reciprocally modulate each other’s activity.

Cation-permeable, cyclic nucleotide-gated channels (CNG channels) have traditionally been considered in terms of their roles in visual and olfactory signal transduction. The retinal channel, which is activated by cGMP, is responsible for the “dark” current (1, 2), while the closely related olfactory channel is gated by cAMP, and is thought to lead to activation of Ca\(^{2+}\)-activated Cl\(^{-}\) currents and membrane depolarization (3). Increasingly, however, a more widespread function for these channels in cell physiology has been envisioned, partially due to the finding that the channels are expressed in a wide range of tissues and cell types. For instance, proteins homologous to the CNG channel have been cloned from such diverse tissues as heart, kidney, and testis, as well as from liver and skeletal muscle (4–6). CNG channels have also been found in various brain regions, namely, the hippocampus, cortex, and Purkinje cells of the cerebellum and other neural derived tissues such as pineal and pituitary gland (5, 7–10). The observation that these channels are widely expressed prompts a reevaluation of their role in signal transduction. Functionally, the CNG channels belong to the family of ligand-gated channels, but, structurally, they are similar to voltage-gated channels. CNG channels also share the important feature of Ca\(^{2+}\) permeation with voltage-gated Ca\(^{2+}\) channels. At physiological [Ca\(^{2+}\)], an expressed, homomeric version of the olfactory CNG channel exhibits a nearly “pure” Ca\(^{2+}\) current (11). In comparison, only ∼5% of the current through the NMDA channel is carried by Ca\(^{2+}\) (12). Therefore, these channels provide a second messenger-regulated form of Ca\(^{2+}\) entry into the cell whose primary function may be to elevate [Ca\(^{2+}\)].

Adenylyl cyclases are regulated by physiological transitions in [Ca\(^{2+}\)], (reviewed in Refs. 13 and 14)). In fact, of the nine currently described isofoms of adenylyl cyclase, Ca\(^{2+}\) directly regulates four. Adenylyl cyclase types I and VIII are stimulated, while types V and VI are inhibited by submicromolar [Ca\(^{2+}\)]. We have previously shown that Ca\(^{2+}\)-sensitive adenylyl cyclases are regulated by capacitative Ca\(^{2+}\) entry (CCE) while they are refractory to [Ca\(^{2+}\)], rises produced by other means, such as release from internal stores or entry mediated by ionophore in nonexcitable cells (15, 16). The dependence of these adenylyl cyclases on Ca\(^{2+}\) entering through CCE channels suggested a functional colocalization of CCE channels and Ca\(^{2+}\)-sensitive adenylyl cyclases. Therefore, it was of interest to determine whether Ca\(^{2+}\) entry through heterologously expressed CNG channels might regulate these enzymes. C6-2B cells, which endogenously express a Ca\(^{2+}\)-inhibitable adenylyl cyclase (type VI) (17), were used to determine whether Ca\(^{2+}\) entry through an olfactory CNG channel could regulate cAMP accumulation. Expression of the rat olfactory CNG channel (18) was accomplished by creating an adenovirus construct containing the channel. Infection with the adenovirus/CNG channel permits efficient expression in a large majority of the cells. The expression of the channel was evaluated by both [Ca\(^{2+}\)], measurements in cell populations and electrophysiological methods. Activation of the CNG channel with the cell-permeant cGMP analog, CPT-cGMP, generated a [Ca\(^{2+}\)], rise that was depend-
Fig. 1. Construction of Ad5dl327CMV-CNGC. The Ad5dl327Bstβ-Gal-TP complex (see "Experimental Procedures") is represented at the top, with the thick line indicating the foreshortened adenovirus chromosome and TP indicated by filled in circles at either end of the chromosome. The inverted terminal repeats (ITR), which act as origins of replication, and the cis-acting packaging sequence (PKG) are indicated. The pACCMV-CNGC plasmid is indicated immediately below the Ad5dl327Bstβ-Gal-TP complex. Adenovirus sequence and the CMV-CNGC cassette are indicated by thick lines, and plasmid vector (of which only the ends associated with adenovirus sequence are shown) is indicated by thin lines. Ad5dl/Bst-β-Gal and pACCMV-CMV-CNGC were restriction-digested as indicated, the restriction digests were ligated together, and the ligated sample was used to transfet HEK 293 cells. A lysate of the transfected cells was used to infect new 293 cells, and plaques that were clear in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside were examined for the presence of the CNGC gene. The recombinant viral chromosome encoding CNGC, Ad5dl327CMV-CNGC, is schematically represented at the bottom.

EXPERIMENTAL PROCEDURES

Materials—Thapsigargin, forskolin, and Ro 20-1724 were from Calbiochem. [2-3H]Adenine and [α-32P]ATP were obtained from Amersham Pharmacia Biotech. Fura-2/AM and pluronic acid were from Molecular Probes, Inc. (Eugene, OR). Other reagents were from Sigma.

Cell Culture—C6-2B rat glioma cells were maintained in 13 ml of F-10 medium (Life Technologies, Inc.) with 10% (v/v) bovine calf serum (Gemini) in 75-cm² flasks at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Cells were plated at approximately 70% confluence in 100-mm culture dishes for infection with the CNG channel construct. 48 h postinfection, cells were detached with phosphate-buffered saline containing 0.03% EDTA and immediately used for measurement of cAMP accumulation or [Ca²⁺].

Construction of Adenovirus Encoding the CNG Channel (See Fig. 1)—A fragment encoding the rat olfactory CNG channel α-subunit cDNA (18) was ligated between the BamHI and SstI sites in the plasmid pACCMV, which encodes the left end of the adenovirus chromosome with the E1A gene and the 5'-half of the E1B gene replaced by the cytomegalovirus major immediate early promoter, a multiple cloning site, and intron and polyadenylation sequences from SV40 (19) to yield the plasmid pACCMV-CNGC. pACCMV-CNGC was digested with SalI and ligated with a BstBI adaptor in order to create pACCMV-CNGCₜₘ, such that sequences encoding CNGC and the left end of the adenovirus chromosome could be ligated directly to the right arm of the adenovirus chromosome to create a transducing vector using a newly developed protocol.² pACCMV-CNGCₜₘ was digested with BstBI (to provide an end to ligate with adenovirus DNA) and XmnI (to provide a blunt end that would inhibit recircularization of the plasmid as well as the formation of concatamers). The digested plasmid DNA was ligated with BstBI-digested Ad5dl327Bst-β-Gal-TP complex (20). Ad5dl327Bst-β-Gal has a deletion of the fragment between the XbaI sites at 28,593 and 30,471 base pairs and therefore does not encode any of the products of the E3 region. The ligated DNA was used to transfect 293 cells using Ca₆(P0₄)₂ precipitation (21). The transfected cells were incubated for 7 days. A freeze-thaw lysate was prepared from the cells, and dilutions were used to infect 293 plates for plaque purification. The infected 293 plates were overlaid with medium in Noble agar, fed after 4 days, and stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside and neutral red (20). Clear plaques, which are derived from recombination that results in deletion of the lacZ gene present in the parental viral chromosome, were amplified and analyzed by polymerase chain reaction and restriction digestion for the presence of the CNGC cDNA. Plaques that proved positive by polymerase chain reaction and restriction digestion analysis were tested for the ability to direct expression of CNGC. This procedure resulted in efficient production of viruses encoding CNGC. The virus, termed Ad5dl327CMV-CNGC, was grown in large scale, purified by sucrose density banding on step and isopycnic CsCl gradients, and dialyzed versus three changes of 10 mM Tris-HCl, pH 7.9, 135 mM NaCl, 1 mM MgCl₂, 50% glycerol at 4 °C. Virus particle concentration was quantitated by determination of the absorbance at 260 nm. Virus was stored at −20 °C until use.

Measurement of CAMP Accumulation—CAMP accumulation in intact cells was measured according to the method of Evans et al. (22) as described previously (16) with some modifications. C6-2B cells on 100-mm culture dishes were incubated in F-10 medium (60 min at 37 °C) containing [2-3H]adenine (20.0 μCi/dish) to label the ATP pool. The cells were then washed once and detached using phosphate-buffered saline-containing EDTA (0.03%). The cells were then resuspended in a nominally Ca²⁺/Mg²⁺-free Krebs buffer containing 120 mM NaCl, 4.75 mM KCl, 1.44 mM MgSO₄, 11 mM glucose, 25 mM HEPES, and 0.1% bovine serum albumin (fraction V) adjusted to pH 7.4 with 2 M Tris base. The resuspended cells were then aliquotted (approximately 3 × 10⁵ cells/tube) and used for CAMP determination assays in triplicate. All experiments were carried out at 30 °C in the presence of phosphodiesterase inhibitors, 3-isobutyl-1-methylxanthine (500 μM) and Ro 20-1724.

² J. Schaack, unpublished data.
(100 μM), which were preincubated with the cells for 10 min prior to a 1-min assay. Assays were terminated by the addition of 10% (v/v, final concentration) trichloroacetic acid. Unlabeled CAMP (100 μM, 10 mCi), ATP (10 μM, 85 mCi), and [γ-32P]ATP (7000 cpm) were added to monitor recovery of cAMP and ATP. After pelleting, the [γ-32P]ATP and [3H]cAMP content of the supernatant were quantified according to the standard Dowex/alumina methodology (23). Accumulation of cAMP is expressed as the percentage of conversion of [γ-32P]ATP into [3H]cAMP; means ± S.D. of triplicate determinations are indicated.

**Electrical Recording**—Currents through CNG channels were measured using the whole-cell patch clamp technique and an Axopatch-200A patch clamp amplifier (Axon Instruments Inc., Foster City, CA). Pipettes were pulled from borosilicate glass and heat-polished. To ensure adequate voltage control in the whole cell configuration pipette, resistances were limited to 3.5 megohms and averaged 2.8 megohms. Pipettes were then lowered onto the cells, and gigahms seals were formed by applying light suction (12.8 ± 0.9 gigaohms). After achieving whole cell configuration, capacitive transients were elicited by applying 20-mV steps from the holding potential (0 mV), filtered at 10 kHz, and recorded at 40 kHz for calculation of access resistance and input impedance. In all experiments, the voltage error due to series resistance was less than 5 mV. Current records were filtered at 1 kHz, sampled at 5 kHz, and analyzed on an IBM-compatible computer using Pclamp6 software (Axon Instruments). The intracellular pipette filling solution contained 145 mM KCl, 4 mM NaCl, 0.5 mM MgCl₂, 10 mM HEPES, and either 0 or 1 mM cGMP, and pH was adjusted to 7.4 with KOH. The bath solution contained 145 mM NaCl, 4 mM KCl, 10 mM HEPES, 11 mM glucose, and either 10 mM MgCl₂ or 1 mM EGTA, and pH was adjusted to 7.4 with NaOH.

[Ca²⁺]i, Measurements—[Ca²⁺]i was measured in populations of C6-2B cells, using fura-2 as the Ca²⁺ indicator, exactly as described earlier (18).

**Statistics**—Analyses were performed using the Prism statistical software package (version 2.00, GraphPad Software, Inc.)

**RESULTS**

**Effect of Varying CNG Channel Construct Multiplicity of Infection on Ca²⁺ Entry**—Initial experiments were conducted to optimize the multiplicity of infection (m.o.i.) for the adenovirus/CNG channel construct (Ad5/dl32TCMV-CNGC) in C6-2B rat glioma cells, using cytosolic Ca²⁺ rises ([Ca²⁺]i) in response to CPT-cGMP as the functional readout (Fig. 2). Following a 48-h incubation of the cells with the CNG channel construct at an m.o.i. of 10, 100, or 200 (A, B, or C), the cells were loaded with fura-2 (see “Experimental Procedures”) and subsequently resuspended in a nominally Ca²⁺-free Krebs buffer for Ca²⁺ measurements in cell populations. Cells were pretreated with various CPT-cGMP concentrations (0, 100, or 300 μM) 8 min prior to the addition of [Ca²⁺]extr, and the resultant [Ca²⁺]i rise was measured. Infection of the cells with an m.o.i. of 10 (Fig. 2A) showed no increase in [Ca²⁺]i, as a result of CPT-cGMP pretreatment, indicating little or no expression of the CNG channel. The small [Ca²⁺]i rise observed following the addition of [Ca²⁺]extr from a resting level of approximately 60 nM to approximately 180 nM results from the cells being maintained in a nominally Ca²⁺-free buffer for 8 min and reflects limited CCE. When the cells were infected at higher m.o.i. values (100 and 200; Fig. 2, B and C), pretreatment with CPT-cGMP resulted in a greatly augmented [Ca²⁺]i rise, which was dependent on the [CPT-cGMP]. Cells infected with an m.o.i. of 100 (Fig. 2B) gave a maximal [Ca²⁺]i rise to approximately 600 nM following pretreatment with 300 μM CPT-cGMP and the addition of [Ca²⁺]extr. When the cells were not pretreated with CPT-cGMP, the resultant rise in [Ca²⁺]i was similar to that seen in cells infected with an m.o.i. of 10 (approximately 180 nM). Increasing the m.o.i. to 200 slightly augmented the [Ca²⁺]i rise, which reached approximately 700 nM with a [CPT-cGMP] of 300 μM. Again, in the absence of CPT-cGMP pretreatment, the [Ca²⁺]i rise was similar to that observed in cells not expressing the CNG channel (to approximately 170 nM, Fig. 2A). Therefore, an m.o.i. of 100 or larger results in the expression of the CNG channel. All subsequent experiments used an m.o.i. of 100. Electrophysiological experiments were conducted to evaluate channel expression.

**Electrophysiological Determination of CNG Channel Expression**—The effectiveness of using an adenovirus construct to heterologously express the olfactory CNG channel was assessed by monitoring currents in the whole-cell patch clamp configuration. Currents were elicited by 250-ms steps from the holding potential, 0 mV, to membrane potentials between −80 and +60 mV in 10 mV increments, followed by a 100-ms step to −40 mV. To determine if CNG channels were present in the infected cells, the pipette solution contained either 0 or 1 mM MgCl₂, which blocks >95% of inward current and >80% of outward current through the olfactory CNG channel (11). Thus, if CNG channels are present and cGMP is in the patch pipette, only a small outwardly rectifying current should be observed in the presence of 10 mM external Mg²⁺. Removal of Mg²⁺ from the bath solution would be expected to reveal a substantially larger, nonrectifying current.

When recording from cells infected with the adenovirus en-

![Fig. 2. Infection of C6-2B cells with the CNG channel construct results in functional expression of the channel. C6-2B cells were infected with the CNG channel construct at an m.o.i. of 10 (A), 100 (B), or 200 (C) 48 h prior to Ca²⁺ measurements. [Ca²⁺], was determined in aliquots of 4 × 10⁶ fura-2-loaded C6-2B cells as described under “Experimental Procedures.” Cells in a nominally Ca²⁺-free Krebs buffer were preincubated with CPT-cGMP (either 0, 100, or 300 μM, as indicated) 8 min prior to the addition of [Ca²⁺]extr (2 mM). Activation of the expressed CNG channel by CPT-cGMP results in an increased [Ca²⁺]i, rise following the addition of [Ca²⁺]extr. Data are representative of two similar experiments.](image-url)
coding the CNG channel, only small leak currents (<6 pA at ± 40 mV) were observed in 10 mM external Mg²⁺ when the patch pipette did not contain cGMP (Fig. 3A). Removal of Mg²⁺ from the bath solution caused a reversible, 2–3-fold increase in leak (Fig. 3, B and C). The addition of the membrane-permeant cGMP analogue, CPT-cGMP (100 μM), to the bath solution induced a large current that was subsequently blocked by 10 mM external Mg²⁺ (data not shown). However, when the patch pipette contained 1 mM cGMP, a small outward current was observed in the presence of 10 mM external Mg²⁺ (Fig. 3D). Removal of external Mg²⁺ revealed a substantially larger, non-rectifying current that could be blocked by 10 mM Mg²⁺ (Fig. 3, E and F). The collected results from 39 cells are shown in Fig. 4. Uninfected cells in the presence (n = 9) or absence (n = 10) of cGMP and infected cells in the absence of internal cGMP (n = 9) displayed small inward leak currents, <15 pA at −40 mV, in 0 mM external Mg²⁺. Infected cells that gave a measurable response in the presence of internal cGMP (n = 11) displayed large inward currents, >700 pA at −40 mV in 0 mM external Mg²⁺. The CgMP-dependent current was observed in >70% (13 of 18, including 2 of 3 cells exposed to CPT-cGMP) of infected cells and no (0 of 9) uninfected cells.

**Ability of Ca²⁺ Entry through the CNG Channel to Inhibit ACV1**—The next experiments aimed to determine whether the Ca²⁺ entry through the expressed CNG channel could regulate a Ca²⁺-inhibitable adenylyl cyclase that is endogenously expressed in C6-2B cells. The effect of CPT-cGMP pretreatment followed by the addition of varying [Ca²⁺]_i on cAMP accumulation in uninfected versus infected cells was examined (Fig. 5). cAMP accumulation was measured over a 1-min period following the addition of [Ca²⁺]_i along with forskolin and isoproterenol to stimulate adenylyl cyclase activity (see "Experimental Procedures"). All cells were pretreated with the phosphodiesterase inhibitors 3-isobutyl-1-methylxanthine (500 μM) and Ro 20-1724 (100 μM) 10 min prior to the 1-min assay. In cells expressing the CNG channel, pretreatment with CPT-cGMP (300 μM) caused steadily increasing inhibition in cAMP accumulation as a function of the [Ca²⁺]_i, [Ca²⁺]_i of 1, 2, and 4 mM inhibited cAMP accumulation by 20, 25, and 34%, respectively. This was in contrast to uninfected cells, also pretreated with CPT-cGMP (300 μM), which gave a maximal inhibition of 20% at a [Ca²⁺]_i of 4 mM. The modest degree of inhibition of cAMP accumulation seen with increasing [Ca²⁺]_i in the cells not infected with the CNG channel construct was the result of limited capacitative Ca²⁺ entry (see Fig. 2). The above data support the idea that Ca²⁺ entry through the CNG channel can be sensed by the Ca²⁺-sensitive adenylyl cyclase. To further understand the functional relationship between the CNG channel and the Ca²⁺-sensitive adenylyl cyclase, detailed manipulations of the CPT-cGMP concentration and exposure time, as well as the [Ca²⁺]_i were carried out.

**Effect of Varying [CPT-cGMP] and Exposure Time on Ca²⁺ Entry and Inhibition of ACV1**—The ability of the cGMP analog, CPT-cGMP, to activate the CNG channel partly depends on its ability to cross the plasma membrane and reach an effective concentration at the CNG channel. Permeation of CPT-cGMP was examined by varying the amount of time the cells were exposed to the cGMP analog prior to the addition of [Ca²⁺]_i.

Fura-2-loaded C6-2B cells were pretreated with varying amounts of CPT-cGMP for either 2 or 5 min prior to the addition of [Ca²⁺]_i. The 2-min exposure to CPT-cGMP (Fig. 6A)
resulted in a \([\text{Ca}^{2+}]_{\text{in}}\) rise following the addition of \([\text{Ca}^{2+}]_{\text{ex}}\) that depended on the \([\text{CPT-cGMP}]\). At the highest \([\text{CPT-cGMP}]\), 100 
\(\mu M\), the \([\text{Ca}^{2+}]\), rise reached a peak of approximately 370 nM
after approximately 2 min. The 50 \(\mu M\) CPT-cGMP condition did
not reach a plateau in the course of the experiment but reached
a slightly lower maximum value (approximately 330 nM) than
the 100 \(\mu M\) condition. At even lower \([\text{CPT-cGMP}]\), 20 
\(\mu M\), the rate of the \([\text{Ca}^{2+}]\), rise was even slower, and the maximum
value achieved was substantially less (approximately 200 nM).
In the absence of CPT-cGMP pretreatment, a very modest
\([\text{Ca}^{2+}]\), rise was observed, due to the fact that the cells were
being maintained in a nominally \(\text{Ca}^{2+}\)-free medium. When the
time of exposure to CPT-cGMP was increased to 5 min, the rate
of the \([\text{Ca}^{2+}]\), rise following the addition of \([\text{Ca}^{2+}]_{\text{ex}}\)
was considerably faster, reaching a plateau within the course of the
experiment (Fig. 6B). Although the rates of the \([\text{Ca}^{2+}]\), rise
increased with longer exposure to CPT-cGMP, the peak values
reached were very similar to the 2-min exposure (see Fig. 6A)
with 20, 50, and 100 \(\mu M\) CPT-cGMP treatments reaching peaks
of approximately 200, 320, and 360 nM, respectively. These
results showed that permeation of the cGMP analog across the
plasma membrane is rather slow, but once maximal activation
of the channel has been reached, the peak \([\text{Ca}^{2+}]_{\text{in}}\), rises are very
similar for a given \([\text{CPT-cGMP}]\).
In order to consolidate the regulatory consequence of \(\text{Ca}^{2+}\)
entry through the CNG channel on the adenyl cyclase, differ-
ent CPT-cGMP exposure times were compared in terms of their
effect on cAMP accumulation and \(\text{Ca}^{2+}\) entry. Fig. 7 shows the
effect of varying both the CPT-cGMP concentration and the
exposure time to the cGMP analog on the cAMP accumulation
in C6-2B cells infected with the CNG channel construct. Fol-
lowing a 2-min exposure to CPT-cGMP, a combination of
\([\text{C}^{2+}]_{\text{ex}}\) as well as forskolin and isoproterenol were added to
the cells with cAMP accumulation measured over the subse-
quent minute. With increasing [CPT-cGMP], there was a step-
wise increase in the inhibition of cAMP accumulation ranging
from 7% inhibition with 20 \(\mu M\) CPT-cGMP to 32% with 300 \(\mu M\)
CPT-cGMP (Fig. 7A). It should be noted that the extent of inhibition
observed with increasing [CPT-cGMP] agrees well with the extent of \(\text{Ca}^{2+}\) entry (Fig. 6A). Without CPT-cGMP
pretreatment of the cells, a minimal inhibition of cAMP accumu-
alization (8%) was observed, which is very similar to the 20 \(\mu M\
CPT-cGMP condition. As seen in Fig. 6A, 0 and 20 \(\mu M\) CPT-
cGMP produce a similar \([\text{Ca}^{2+}]\), rise within the first minute, the
period over which cAMP accumulation is measured. There-
fore, the similarities in the extent of the inhibition seen with 0
and 20 \(\mu M\) CPT-cGMP are consistent with the \(\text{Ca}^{2+}\) data. In
Fig. 7B, the effects of a 5-min exposure to varying CPT-cGMP
concentrations on cAMP accumulation are shown. Again, in-
creasing the CPT-cGMP concentration produced further inhibi-
tion of cAMP accumulation following the addition of \([\text{Ca}^{2+}]_{\text{ex}}\)
with maximal inhibition (31%) observed at 300 \(\mu M\) CPT-cGMP.
The amount of inhibition observed in the absence of CPT-cGMP
was again 8%, which, following a 5-min exposure to CPT-
cGMP, differs greatly from the 20 \(\mu M\) CPT-cGMP condition
(22%). This result was also in good agreement with the corre-
sponding \(\text{Ca}^{2+}\) data (Fig. 6B), where the longer pretreatment
with CPT-cGMP resulted in a faster \([\text{Ca}^{2+}]\), rise and, therefore,
a higher \([\text{Ca}^{2+}]\), level achieved within the 1-min assay period.
It is also noteworthy that the extent of inhibition in cAMP
accumulation appears to reach “maximal” levels at lower CPT-
cGMP concentrations with these longer exposure times. In
other words, the dose-response curve has been shifted to the
left, indicating an increased efficacy in the \(\text{Ca}^{2+}\) entry pro-
Fig. 7. Effect of varying [CPT-cGMP] and exposure time on the ability of CNGC-promoted Ca^{2+} entry to regulate cAMP accumulation. Cells infected with the CNG channel construct were pretreated with varying CPT-cGMP concentrations (0, 20, 50, or 100 μM, as indicated) in a nominally Ca^{2+}-free Krebs buffer either 2 min (A) or 5 min (B) prior to cAMP determination. cAMP accumulation was measured over a 1-min period in the presence of forskolin (10 μM), isoproterenol (10 μM), and added [Ca^{2+}]_{i} (either 0 or 2 mM). The asterisks denote values that differ significantly from the relevant control (0 CPT-cGMP/2 mM Ca^{2+} condition), as judged by Student’s t test (p < 0.01).

Effect of Varying [CPT-cGMP] and Ca^{2+} Entry—The next set of experiments was designed to examine the effect of varying both the [Ca^{2+}]_{i} and the CPT-cGMP on the resultant [Ca^{2+}]_{i}, rise and inhibition of cAMP accumulation in C6-2B cells infected with the CNG channel construct. Fura-2-loaded cells were pretreated with 0, 50, or 100 μM CPT-cGMP by 4 min prior to the addition of either 0 or 2 mM [Ca^{2+}]_{i} (either 0 or 2 min). The asterisks denote values that differ significantly from the relevant control (0 CPT-cGMP/2 mM Ca^{2+} condition), as judged by Student’s t test (p < 0.01).

Next, the effect on cAMP accumulation of the incremental increases in [Ca^{2+}]_{i}, caused by increasing [Ca^{2+}]_{i} following CPT-cGMP treatment was examined. Fig. 8 depicts the effect of Ca^{2+} entry through CNG channels promoted by treatment with either 50 or 100 μM CPT-cGMP on cAMP accumulation in C6-2B cells infected with the CNG channel construct. Increasing the [Ca^{2+}]_{i} from 0 to 4 mM resulted in an increased inhibition in cAMP accumulation with both CPT-cGMP. The cells pretreated with 100 μM CPT-cGMP yielded the largest inhibition, maximally 32% (Fig. 9). Once again, experimental conditions that alter the [Ca^{2+}]_{i}, rise produced by Ca^{2+} entry through the CNG channel achieve corresponding changes in the inhibition of cAMP accumulation. The data above clearly show that Ca^{2+} entry through an expressed CNG channel regulates the endogenously expressed Ca^{2+}-inhibitable adenyllyl cyclase. Finally, it was of interest to compare regulation of cAMP accumulation by the CNG channel with the normal physiological mode of Ca^{2+} regulation of cAMP accumulation in these cells, i.e. capacitative Ca^{2+} entry.

Comparison of the Efficacy of CNG Channel-promoted Ca^{2+} Entry Versus Capacitative Ca^{2+} Entry—We had previously established the exclusive ability of capacitative Ca^{2+} entry to...
regulate the endogenously expressed Ca\(^{2+}\)-inhibitable adenylyl cyclase in C6-2B cells (16). Other modes of inducing [Ca\(^{2+}\)]\(_{\text{cyt}}\), rises, such as release from intracellular stores or an extremely robust [Ca\(^{2+}\)]\(_{\text{cyt}}\), rise produced by ionophore treatment, were ineffective (16). Therefore, the ability of a [Ca\(^{2+}\)]\(_{\text{cyt}}\) rise emanating from expressed CNG channels to regulate the cyclase was somewhat unexpected. The next set of experiments was designed to examine the relative efficacy of these two forms of Ca\(^{2+}\) entry (endogenous CCE versus heterologously expressed, CNG channel-promoted Ca\(^{2+}\) entry) to regulate cAMP accumulation in C6-2B cells. Fig. 10A depicts the [Ca\(^{2+}\)]\(_{\text{cyt}}\) rise in cells infected with the CNG channel construct treated with CPT-cGMP (100 \(\mu\)M) 4 min prior to the addition of varying [Ca\(^{2+}\)]\(_{\text{cyt}}\). The addition of 1, 2, or 4 mM [Ca\(^{2+}\)]\(_{\text{cyt}}\) produced peak [Ca\(^{2+}\)]\(_{\text{cyt}}\) rises of approximately 220, 280, or 330 nM within 1 min. These [Ca\(^{2+}\)]\(_{\text{cyt}}\) rises were modest in comparison with CCE (cf. Fig. 10B). CCE was promoted by treating the cells with thapsigargin (TG; 100 nM), which inhibits the Ca\(^{2+}\)-ATPase responsible for pumping Ca\(^{2+}\) into the stores (24), leaving the endogenous Ca\(^{2+}\) leak to deplete the stores. The depletion of the intracellular Ca\(^{2+}\) stores promotes the subsequent CCE. Following depletion of intracellular Ca\(^{2+}\) stores, varying [Ca\(^{2+}\)]\(_{\text{cyt}}\) rises in the media resulted in a rapid rise in [Ca\(^{2+}\)]\(_{\text{cyt}}\). The addition of 1 mM [Ca\(^{2+}\)]\(_{\text{cyt}}\) to the TG-treated cells resulted in a [Ca\(^{2+}\)]\(_{\text{cyt}}\) rise to approximately 520 nM, while the addition of 2 and 4 mM evoked peak [Ca\(^{2+}\)]\(_{\text{cyt}}\), rises of approximately 680 and 860 nM, respectively (Fig. 10B). Next, the effects of these two modes of Ca\(^{2+}\) entry were compared with respect to their ability to regulate cAMP accumulation (Fig. 11). In both Ca\(^{2+}\) entry protocols, an increase in the amount of inhibition in cAMP accumulation was observed with increasing [Ca\(^{2+}\)]\(_{\text{cyt}}\). CCE produced greater inhibition of the cyclase, with a maximal inhibition of 40%, using a [Ca\(^{2+}\)]\(_{\text{cyt}}\) of 4 mM. In comparison, CNG channel-promoted Ca\(^{2+}\) entry inhibited cAMP accumulation by 32% at the same [Ca\(^{2+}\)]\(_{\text{cyt}}\). At first glance, it may appear that CCE is more effective at regulating cAMP accumulation, but when the [Ca\(^{2+}\)]\(_{\text{cyt}}\) rise produced by these two Ca\(^{2+}\) entry methods is compared (see Fig. 10), it may be argued that qualitatively they are very similar in their efficacy. CNG channel-promoted Ca\(^{2+}\) entry gave a maximal [Ca\(^{2+}\)]\(_{\text{cyt}}\) rise of approximately 330 nM with a [Ca\(^{2+}\)]\(_{\text{cyt}}\) of 4 mM, and CCE produced a [Ca\(^{2+}\)]\(_{\text{cyt}}\) rise of approximately 860 nM under the same conditions (Fig. 10). Therefore, there is a rough correlation between the [Ca\(^{2+}\)]\(_{\text{cyt}}\) levels reached and the amount of inhibition of cAMP accumulation observed. This finding argues that both of these Ca\(^{2+}\) entry methods are equally efficacious in regulating adenylyl cyclase activity.

**DISCUSSION**

The present study has established that Ca\(^{2+}\) entry through a heterologously expressed CNG channel can regulate the endogenous Ca\(^{2+}\)-inhibitable adenylyl cyclase of C6-2B cells. Electrophysiological measurements showed that infection using the novel adenovirus construct coding for the olfactory CNG channel-a subunit achieved expression in more than 70% of the cells, which is remarkable, given the refractory nature of these cells to transient transfection. The expressed channel behaved normally, based on cyclic nucleotide dependence, conductance, and Mg\(^{2+}\) block. Subsequently, substantial Ca\(^{2+}\) entry was observed in populations of cells, which was dependent not only on the [CPT-cGMP] and [Ca\(^{2+}\)]\(_{\text{cyt}}\) but also on the time of exposure to CPT-cGMP. The Ca\(^{2+}\) entry through the CNG channel inhibited the endogenous adenylyl cyclase activity of C6-2B cells. The degree of inhibition mirrored the magnitude of the [Ca\(^{2+}\)]\(_{\text{cyt}}\) rise generated by the various experimental conditions. For instance, a relatively small [Ca\(^{2+}\)]\(_{\text{cyt}}\) rise generated either by

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**Fig. 9. Effect of varying [CPT-cGMP] and [Ca\(^{2+}\)]\(_{\text{cyt}}\) on the ability of CNGC-promoted Ca\(^{2+}\) entry to regulate cAMP accumulation.** Cells expressing the CNG channel were pretreated with CPT-cGMP (50 \(\mu\)M, open bars; 100 \(\mu\)M, hatched bars) in nominally Ca\(^{2+}\)-free Krebs buffer 4 min prior to cAMP determination. cAMP accumulation was measured over a 1-min period in the presence of forskolin (10 \(\mu\)M), isoproterenol (10 \(\mu\)M), and added [Ca\(^{2+}\)]\(_{\text{cyt}}\) (0, 1, 2, or 4 mM, as indicated). The asterisks denote values that differ significantly from the relevant controls, as judged by Student’s t test (\(p < 0.005\)).

**Fig. 10. Comparison of CNGC-promoted and capacitative Ca\(^{2+}\) entry in C6-2B cells.** [Ca\(^{2+}\)]\(_{\text{cyt}}\), was measured in cells expressing the CNG channel construct in nominally Ca\(^{2+}\)-free Krebs buffer as described under “Experimental Procedures.” In A, CNGC-promoted Ca\(^{2+}\) entry was stimulated in C6-2B cells expressing the CNG channel by pretreatment with CPT-cGMP (100 \(\mu\)M) 4 min prior to the addition of [Ca\(^{2+}\)]\(_{\text{cyt}}\) (1, 2, or 4 mM, as indicated). Capacitative Ca\(^{2+}\) entry is depicted in B, where C6-2B cells were pretreated with TG (100 \(\mu\)M), which produces a [Ca\(^{2+}\)]\(_{\text{cyt}}\) rise due to Ca\(^{2+}\) release from intracellular stores. Following depletion of Ca\(^{2+}\) stores, [Ca\(^{2+}\)]\(_{\text{cyt}}\) (1, 2, or 4 mM, as indicated) was added to the media, with the resultant capacitative Ca\(^{2+}\) entry depicted.
The present findings not only point to a functional colocalization between CNG channels and the Ca$^{2+}$-sensitive adenylyl cyclase, they also strengthen the notion that CNG channels may function as a pathway for Ca$^{2+}$ entry that is not dependent on Ca$^{2+}$ store depletion or membrane depolarization. It has been clear for some time that Ca$^{2+}$ entry through CNG channels plays an important role in transduction and adaptation in visual and olfactory receptors (Refs. 26–28; reviewed in Refs. 29 and 30). In the cone synapse, it has been shown that CNG channels, as well as voltage-gated Ca$^{2+}$ channels, are involved in exocytosis of synaptic vesicles (31). Furthermore, it has been shown that exocytosis in cone synapses can be modulated by NO, by affecting cGMP production and altering CNG channel activity (32).

CNG channels have also been postulated to play a role in synaptic plasticity, a process that is dependent on Ca$^{2+}$. In the hippocampus, an olfactory-like CNG channel has been found in cell bodies and processes of CA1 and CA3 neurons (8), which express high levels of two Ca$^{2+}$-stimulable adenylyl cyclases, types I and VIII (33). Based on these observations, it has been suggested that modulation of adenylyl cyclase activity by Ca$^{2+}$ entry through the CNG channel in CA1 neurons may participate in maintenance of long term potentiation (8). Evidence in support of this proposal is that hippocampi isolated from an olfactory CNG channel null mouse were impaired in their ability to produce long term potentiation in response to burst stimulation (34). Another tissue in which CNG channels have been detected is the heart (4, 9). The heart is also one of the most abundant sources of Ca$^{2+}$-inhibitable adenylyl cyclases, types V and VI (35, 36). We had earlier proposed that the existence of feedback loops between cAMP-controlled Ca$^{2+}$ entry and Ca$^{2+}$-inhibitable adenylyl cyclases could give rise to oscillations in both [cAMP] and [Ca$^{2+}$] (14). The present finding that Ca$^{2+}$ entry through a CNG channel can inhibit a Ca$^{2+}$-inhibitable adenylyl cyclase may provide a molecular basis for such a proposal.

For the present, the ability of Ca$^{2+}$ entry through a heterologously expressed CNG channel to regulate a Ca$^{2+}$-sensitive adenylyl cyclase extends earlier observations that endogenous CCE mechanisms could regulate heterologously expressed adenylyl cyclases (15). This finding may suggest that Ca$^{2+}$-sensitive adenylyl cyclases and Ca$^{2+}$ entry mechanisms are endowed with common characteristics, such as preferential solubility in cholesterol-rich domains (37), that ensure their coincidence in microdomains of the plasma membrane.

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REFERENCES

1. Fesenko, E. E., Kolesnikov, S. S., and Lyubarsky, A. L. (1985) Nature 313, 310–313.
2. Finn, J. T., Grunwald, M. E., and Yau, K. W. (1996) Annu. Rev. Physiol. 58, 395–426.
3. Lowe, G., and Gold, G. H. (1993) Nature 366, 283–286.
4. Ruiz, M. L., London, B., and Nadal-Ginard, B. (1996) J. Mol. Cell. Cardiol. 28, 1453–1461.
5. Ding, C., Potter, E. D., Qiu, W., Coss, S. L., Levine, M. A., and Guggino, S. E. (1997) Am. J. Physiol. 273, C1335–C1344.
6. Feng, L., Subbaraya, I., Yamamoto, N., Basehr, W., and Kraus-Friedmann, N. (1990) FEBS Lett. 285, 77–81.
7. Kingston, P. A., Zuffal, F., and Barnstable, C. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10440–10445.
8. Bradley, J., Zhang, Y., Bakin, R., Lister, H. A., Ronnett, G. V., and Zinn, K. (1993) J. Neurosci. 13, 3823–3833.
9. Biel, M., Zong, X., Distler, M., Bosse, E., Klugbauer, N., Murakami, M., Fischeri, V., and Hofmann, F. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3505–3509.
10. Weyand, I., Godde, M., Frings, S., Weiner, J., Muller, F., Altenhofen, W., Hatt, H., and Kaupp, U. B. (1994) Nature 368, 859–863.
11. Frings, S., Seifert, R., Godde, M., and Kaupp, U. B. (1995) Neuron 15, 169–179.
12. Seifeggner, R., Zhou, Z., Kenzorh, A., and Neher, E. (1993) Neuron 11, 133–143.
13. Cooper, D. M. F., Mons, N., and Fagan, K. (1994) Cell. Signal. 6, 823–840.
14. Cooper, D. M. F., Mons, N., and Karpen, J. W. (1995) Nature 374, 421–424.
15. Fagan, K. A., Malhey, R., and Cooper, D. M. F. (1996) J. Biol. Chem. 271, 12438–12444.
16. Fagan, K. A., Mons, N., and Cooper, D. M. F. (1998) J. Biol. Chem. 273, 9297–9305.
17. Debernardi, M. A., Munshi, R., Yoshimura, M., Cooper, D. M. F., and Brooker, G. (1993) Biochem. J. 293, 325–328.
18. Dhallian, R. S., Yau, K. W., Schruder, K. A., and Reed, R. R. (1990) Nature 347, 184–187.
19. Gomez-Foix, A. M., Coates, W. S., Baque, S., Alam, T., Gerard, R. D., and Newgard, C. B. (1992) J. Biol. Chem. 267, 25129–25134.
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20. Schaack, J., Langer, S., and Guo, X. (1995) J. Virol. 69, 3920–3923
21. Jordan, M., Schallhorn, A., and Wurm, F. M. (1996) Nucleic Acids Res. 24, 596–601.
22. Evans, T., Smith, M. M., Tanner, L. I., and Harden, T. K. (1984) Mol. Pharmacol. 26, 395–404.
23. Salomon, Y., Londos, C., and Rodbell, M. (1974) Anal. Biochem. 58, 541–548.
24. Thastrup, O., Cullen, P. J., Drobak, B. K., Hanley, M. R., and Dawson, A. P. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2466–2470.
25. Chiono, M., Mahey, R., Tate, G., and Cooper, D. M. F. (1995) J. Biol. Chem. 270, 1149–1155.
26. Matthews, H. R., Murphy, R. L., Fain, G. L., and Lamb, T. D. (1988) Nature 334, 87–99.
27. Nakatani, K., and Yau, K.-W. (1988) Nature 334, 69–71.
28. Kurahashi, T., and Menini, A. (1997) Nature 385, 725–729.
29. Baylor, D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 560–565.
30. Anholt, R. R. (1993) Crit. Rev. Neurobiol. 7, 1–22.
31. Riese, F., and Schwartz, E. A. (1994) Neuron 13, 863–873.
32. Savchenko, A., Barnes, S., and Kramer, R. H. (1997) Nature 390, 684–698.
33. Mons, N., Harry, A., Dubourg, P., Premont, R. T., Iyengar, R., and Cooper, D. M. F. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8478–8477.
34. Parent, A., Schrader, K., Munger, S. D., Reed, R. R., Linden, D. J., and Ronnett, G. V. (1998) J. Neurophysiol. 79, 3295–3301.
35. Yoshimura, M., and Cooper, D. M. F. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6716–6720.
36. Ishikawa, Y., and Homcy, C. J. (1997) Circ. Res. 80, 297–304.
37. Simons, K., and Ikonen, E. (1997) Nature 387, 569–572.