Methods

Our investigations conform with the European Community guiding principles in the care and use of animals (86/609/CEE), CE Off J n° L358, 18 December 1986), the use of ethics committee (CREEA Ile- de-France Sud) guidelines and the French decree n°87-848 of October 19, 1987 (J of République Française, 20 October 1987, pp. 12245-12248). Authorizations to perform animal experiments according to this decree were obtained from the French Ministère de l'Agriculture, de la Pêche et de l'Alimentation (n°7475, May 27, 1997).

Isolation, Culture and Infection of Adult Rat Ventricular Myocytes

Adult rat ventricular myocytes (ARVMs) were obtained by retrograde perfusion from hearts of male Wistar rats (160-180 g) as previously described.\(^1\) Freshly isolated cells were suspended in minimal essential medium (MEM: M4780; Sigma) containing 1.2 mM Ca\(^{2+}\), 2.5% fetal bovine serum (FBS, Invitrogen, Cergy-Pontoise, France), 1% penicillin-streptomycin and 2% HEPES (pH 7.6) and plated on laminin-coated culture dishes (10 µg/ml, 2h) at a density of 10\(^4\) cells per dish. The cells were left to adhere for 1h in a 95% O\(_2\), 5% CO\(_2\) incubator at 37°C. The medium was replaced by 200 µl of FBS-free MEM containing or not the WT CNGA2 encoding adenovirus (Ad-CNGA2) or the double mutant E583M/C460W CNGA2 encoding adenovirus. Adenovirus was used at a multiplicity of infection (MOI) of 3000 plaque forming units (pfu) per cell. After 2h, the same volume of FBS-free medium without adenovirus was added, and the cells were placed overnight in an incubator.

Immunocytochemistry
Cardiomyocytes attached onto coverslips were fixed, permeabilized and incubated with a mouse monoclonal antibody against CNGA2 as previously described. This antibody was a generous gift from Drs F. Mueller and B. Kaupp (Juelich, Germany). Cells were revealed with Alexa fluor 488 goat antibody, and mounted in Mowiol antifadent mounting medium (France Biochem, Meudon, France) and examined under a laser scanning confocal microscope (Zeiss LSM 510). Optical section 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.

**Electrophysiological Experiments**

The whole cell configuration of the patch-clamp technique was used to record the CNG current ($I_{CNG}$). The cells were maintained at 0 mV holding potential and routinely hyperpolarized every 8 s to -50 mV test potential during 200 ms. Current-voltage relationships were obtained by varying the test potential amplitude to values ranging from -50 to +50 mV. The 0 mV holding potential was chosen because it corresponds to the reversal potential of $I_{CNG}$ under our experimental conditions (Fig. 1C of main article). Indeed, $I_{CNG}$ was recorded in the absence of divalent cations in the extracellular solution (see below) allowing monovalent cations to flow through the channels in a non specific manner. Currents were not compensated for capacitance and leak currents. All experiments were done at room temperature (21-27°C), and the temperature did not vary by more than 1°C in a given experiment.

**Solutions and Drugs**

Control zero Ca$^{2+}$/Mg$^{2+}$ extracellular Cs$^+$-Ringer solution contained (in mM): HEPES 10, NaCl 107.1, CsCl 20, NaHCO$_3$ 4, NaH$_2$PO$_4$ 0.8, D-glucose 5, sodium pyruvate 5, adjusted to
pH 7.4 with NaOH. This solution was supplemented with nifedipine (1 µM) to block non-specific cation current through L-type Ca\(^{2+}\) channels. 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 tube. Patch electrodes (0.7-1.2 MΩ) were made of soft glass (Drummond, Broomall, PA, USA) and filled with control internal solution containing (in mM): HEPES 10, CsCl 118, EGTA 5, MgCl\(_2\) 4, sodium phosphocreatine 5, Na\(_2\)ATP 3.1, Na\(_2\)GTP 0.42, CaCl\(_2\) 0.0062 (pCa 8.5), adjusted to pH 7.3 with CsOH. 2-(N,N6Diethylamino)-diazenolate-2-oxide sodium salt (DEANO) and SpermineNONOate (SPNO) were from Alexis Corp. (La Jolla, CA); S-nitroso-N-acetylpenicillamine (SNAP) was from Tocris-Cookson (Bristol, UK); HMR1766 was a generous gift from Dr. Ursula Schindler, Aventis Pharma Deutschland GmbH (Frankfurt am Main, Germany); 3-morpholinosydnonimine (SIN1) was a generous gift from Dr J. Winicki (Hoechst Houdé Laboratories, Paris La Défense, France); 8-(4-chlorophenylthio) guanosine- 3', 5'- cyclic monophosphorothioate, Sp- isomer (Sp-8) was from Biolog L.S.I. (Bremen, Germany); L-858051 (L-85, a hydrosoluble analogue of forskolin) and cilostamide were from France Biochem; Sildenafil was a generous gift from Dr Claire Lugnier (Strasbourg, France); 2-(3,4-dimethoxybenzyl)-7-{(1R)-1-[(1R)-1-hydroxyethyl]-4-phenylbutyl}-5-methyllimidazo[5,1-f][1,2,4]triazin-4(3H)-one (Bay 60-7550, a PDE2 inhibitor) was a generous gift from Bayer Healthcare AG (Wuppertal, Germany); all other drugs were from Sigma-Aldrich (Saint Quentin Fallavier, France).

**Data Analysis**

\(I_{\text{CNG}}\) amplitude is time-independent and was measured at the end of the 200 ms pulse. In a total of 93 cells, mean membrane capacitance (\(C_m\)) was 152.6 ± 2.8 pF. \(I_{\text{CNG}}\) density (\(dI_{\text{CNG}}\)) was calculated for each experiment as the ratio of current amplitude to \(C_m\). In each experimental condition, the response of \(dI_{\text{CNG}}\) to a drug was expressed relative to the ‘basal
current’ obtained in control extracellular solution following the relation: ‘response’=(I_{CNG}−
basal current’)/C_m. When applicable, the response of I_{CNG} was also normalized to the
maximal current amplitude obtained on the same cell with 100 µM Sp-8 (a membrane permeant cGMP analog) following the relation: ‘normalized response’=(I_{CNG}−basal current’)/(I_{Sp-8}−basal current’), where I_{Sp-8} is the current obtained at 100 µM Sp-8. The
concentration-response curve (CRC) for the effects of ANP on I_{CNG} was fitted to the Hill
equation: \( \frac{dI_{CNG}}{d \text{ANP}} = E_{\text{max}} / \left(1 + (E_{C50}/[\text{ANP}])^n\right) \), where E_{C50} is the ANP concentration required to
produce half-maximal stimulation, E_{max} is the maximal effect, and n the Hill coefficient. All
data are expressed as mean ± S.E.M.. When appropriate, the Student’s \( t \) test was used for
statistical evaluation of I_{CNG} variation induced by the different drugs and a \( p \) value of <0.05
was considered statistically significant.

Results

Subsarcolemmal Localization of Recombinant CNGA2 Channels in ARVMs

CNGA2 expression was investigated by immunofluorescence in ARVMs after 24 h of culture.
Native or Ad-CNGA2 myocytes were labeled with primary antibody against CNGA2 and
visualized with fluorophore conjugated secondary antibody (Alexa 488). Supplemental Fig. 1
shows confocal images of representative cells in Ad-CNGA2-infected or non infected cardiac
myocytes. Infected cells elicited a strong fluorescent signal, mainly around the nucleus and in
transversal T-tubules. Such a pattern was not observed in non-infected cells or when the
primary antibody against CNGA2 was omitted. These results revealed the selective
expression of recombinant WT CNGA2 channels at the sarcolemmal structures in Ad-
CNGA2 infected cells.
**Supplementary Figure Legends**

**Supplementary Figure 1.** Immunocytochemical detection of the recombinant WT-CNGA2 channels in ARVMs. Myocytes were either infected (Ad-CNGA2, B) or not (A) with an adenovirus encoding the wild type (WT) α-subunit of rat olfactory CNG channels and labeled with anti-CNGA2 antibody after 24h of culture. Images were produced using a laser scanning confocal microscope. Each image represents one optical slice of a stack of images collected every 0.5 µm.
Supplementary References

