DATA SUPPLEMENT TO MANUSCRIPT CIRCRESAHA/2006/124800

Material and Methods

Isolation, Culture and Infection of ARVM - The investigation conforms 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 local ethics committee (CREEA Ile-de-France Sud) guidelines and the French decree n°87/748 of October 19, 1987 (*J Off 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 et de la Forêt (nº7475, May 27, 1997). Male Wistar rats (160-180 g) were subjected to anesthesia by intraperitoneal injection of pentothal (0.1 mg/g) and hearts were excised rapidly. Individual ARVMs were obtained by retrograde perfusion of the heart as previously described.[1] Freshly isolated cells were suspended in minimal essential medium (MEM: M 4780; Sigma, St. Louis, USA) containing 1.2 mmol/L 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 laminin, 2h) at a density of 10^4 cells per dish. The cells were left to adhere for 1 hour in a 95% O_2, 5% CO_2 incubator at 37°C, before the medium was replaced by 400 µL of FBS-free MEM. For experiments with CNG channels, the E583M or the C460W/E583M CNGA2-encoding adenovirus were added to 200 µL of MEM at a multiplicity of infection (MOI) of 3000 pfu/cell. After 2 hours, the same volume of FBS-free medium without adenovirus was added and the cells were placed overnight in incubator. The medium was changed the next morning for adenovirus- and FBS-free MEM. Patch-clamp experiments were performed the same day.

Electrophysiological Experiments - The whole-cell configuration of the patch-clamp technique was used to record the L-type calcium current ($I_{Ca,L}$) and the CNG current (I_{CNG}), as previously described.[2] For $I_{Ca,L}$ measurement, the cells were depolarized every 8 seconds to 0

mV during 400 ms. Fast sodium current was inactivated by holding potential (-50 mV) and potassium currents were blocked by replacing all K^+ ions with external and internal Cs^+. For I_{CNG} measurement, the cells were maintained at 0 mV holding potential and routinely hyperpolarized every 8 seconds to –50 mV test potential during 200 ms. The 0 mV holding potential was chosen because it corresponds to the reversal potential of I_{CNG} under our experimental conditions. I_{CNG} was recorded in the absence of divalent cations in the extracellular solution allowing monovalent cations to flow through the channels in an unspecific manner. All the 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 for Patch-clamp Recording - Control zero-Ca^{2+}/Mg^{2+} extracellular Cs^+ Ringer solution contained (in mmol/L): NaCl 107.1, CsCl 20, $NaHCO_3$ 4, NaH_2PO_4 0.8, D-Glucose 5, sodium pyruvate 5, HEPES 10, adjusted to pH 7.4 with NaOH. For I_{CNG} recording, this solution was supplemented with nifedipine (1 µmole/L) to block nonspecific cation current through L-type Ca^{2+} channels. For $I_{Ca,L}$ recording, nifedipine was omitted and 1.8 mM $CaCl_2$ and 1.8 mM $MgCl_2$ were added to the 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 mmol/L): CsCl 118, EGTA 5, $MgCl_2$ 4, sodium phosphocreatine 5, Na_2ATP 3.1, Na_2GTP 0.42, $CaCl_2$ 0.062 (pCa 8.5), HEPES 10, adjusted to pH 7.3 with CsOH. Isoprenaline (ISO), prostaglandin E_1 (PGE_1), glucagon, ICI 118551 (ICI), CGP 20712A (CGP), rolipram, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) and 3-isobutyl-1-methyl-xanthine (IBMX) were purchased from Sigma. L-858051 (L-85, a hydrosoluble analogue of forskolin) and cilostamide were purchased from France Biochem (Meudon, France). Ro 20-1724 (Ro) was kindly provided by Hoffman-La-Roche (Basel,

Switzerland), piclamilast by Sanofi-Aventis (Paris, France) and RS25344 by Roche (Palo Alto, CA, USA).

PDE Assay - Freshly isolated ARVMs were seeded on 35 mm Petri dishes at a density of 10^5 cells/dish in FBS-free medium. After 1h, cells were washed with PBS and homogenized in ice-cold buffer containing (in mmol/L) NaCl 150, sodium phosphate buffer (pH 7.2) 10, EDTA 2, β-mercaptoethanol 5, sodium pyrophosphate 30, sodium fluoride 50, benzamidine 3, AEBSF 2, NP40 0.1%, leupeptin 5 µg/ml, pepstatin 20 µg/ml, microcystin 1 µM. PDE activity was measured according to a modification of the two-step assay procedure method described by Thompson & Appleman[3] in a total volume of 200 µL including (in mmol/L) Tris-HCl 40, pH 8.0, $MgCl_2$ 10, β-mercaptoethanol 1.25 supplemented with 1 µM cAMP and 10^5 cpm [^{3}H]-cAMP, as detailed previously.[4] PDE family-specific activities were determined as the difference between PDE activity in the absence of inhibitor and the residual hydrolytic activity observed in the presence of the selective inhibitor. Protein concentration was determined by bicinchonic acid assay after addition of deoxycholate and precipitation by trichloroacetic acid (0.1%).

cAMP Level Determination - Freshly isolated ARVMs suspended in FBS-free MEM were plated in laminin-coated 12-well plates at a density of $5x10^4$ cells/well. After 1h, the medium was replaced with control zero Ca^{2+}/Mg^{2+} extracellular Cs^+ Ringer solution in the presence or absence of β-adrenergic antagonists ICI (1µmol/L) or CGP (1 µmol/L), or IBMX (100 µmol/L). After 15 min., the same Ringer solution with or without receptor agonists (ISO, 1 µmol/L; glucagon, 1 µmol/L; or PGE_1, 1 µmol/L) was applied for 3 min at room temperature. The stimulation was stopped by ice-cold trichloroacetic acid (0.1%) in 95% EtOH. After 30 min incubation on ice, samples were scraped and centrifuged for 30 min at 3,000 rpm at 4°C. The pellet was dissolved in 5% SDS in 0.1 N NaOH for protein determination by bicinchoninic acid assay. EtOH in the supernatant was evaporated and the material was

reconstituted with PBS, pH 7.4, then cAMP was measured by RIA after acetylation of the samples and appropriate dilution.[5,6]

Results

For each receptor, identical experiments were performed in myocytes expressing the low affinity cAMP sensor E583M CNGA2, and gave essentially similar results. As shown in supplementary Fig IA and IB, individual inhibition of PDE3 and PDE4 potentiated the β_1-AR response, and these effects were not different from the effect of IBMX. In contrast, simultaneous inhibition of PDE3 and PDE4 was necessary to unmask a substantial and sustained sarcolemmal cAMP accumulation in response to β_2-AR stimulation (supplementary Fig IC and ID), showing that either PDE3 or PDE4 activity is enough to dampen β_2-AR cAMP at the membrane. As noted above, I_{CNG} was not augmented by cell stimulation with glucagon when using the low affinity CNG channel (Fig. IIA and IIB). However, similarly to what observed with the C460W/E583M mutant, selective blockade of PDE3 failed to increase I_{CNG} while PDE4 inhibition induced a major rise in I_{CNG}. Supplementary Fig. IIC and IID present the results obtained when PGE_1 was used to trigger cAMP in cardiac myocytes. While the hormone alone failed to increase I_{CNG}, the additional and concomitant inhibition of PDE3 and PDE4 provoked a slow accumulation of cAMP at the membrane. This accumulation was enhanced when all PDEs were inhibited with IBMX (supplementary Fig. IIC and IID).

Legends to Supplementary Figures

Supplementary Figure I. PDE regulation of cAMP signals from β_1-AR and β_2-AR. A and C, Time course of I_{CNG} in adult rat ventricular myocytes expressing E583M CNGA2. The cells were superfused for a few minutes with a control solution and then challenged with a drug

during the periods indicated with the solid lines. B and D, Summary of the results obtained in a series of experiments as in A and C, respectively. Specific activation of β_1-AR and β_2-AR as in Fig. 1. Cilostamide (Cil, 1 µmol/L) was used for specific inhibition of PDE3 and Ro 20-1724 (Ro, 10 µmol/L) for specific inhibition of PDE4. IBMX (100 µmol/L) was used for non specific inhibition of PDEs. At the end of the experiment, the cell was challenged with a saturating concentration of the forskolin analog L-858051 (L-85, 100 µmol/L) as an internal control for CNG channels expression. The bars show the means±s.e.m. of the number of cells indicated. Statistically significant differences are indicated as **, $p<0.01$ and ***, $p<0.001$.

Supplementary Figure II. PDE regulation of cAMP signals from Glu-R and PGE_1-R. A and C, Adult rat ventricular myocytes infected with E583M Ad-CNG for 24h were superfused for a few minutes with a control Ringer solution and then challenged with a drug during the periods indicated with the solid lines. B and D, Summary of the results obtained in a series of experiments as in A and C, respectively. Glu-R activation was achieved by application of glucagon (Glu, 1 µmol/L). Pharmacological inhibition of PDEs as in Fig.2. Experiment was terminated with application of 100 µmol/L L-85. The bars show the means±s.e.m. of the number of cells indicated. Statistically significant differences are indicated as *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$. NS, non significant.

Supplementary references

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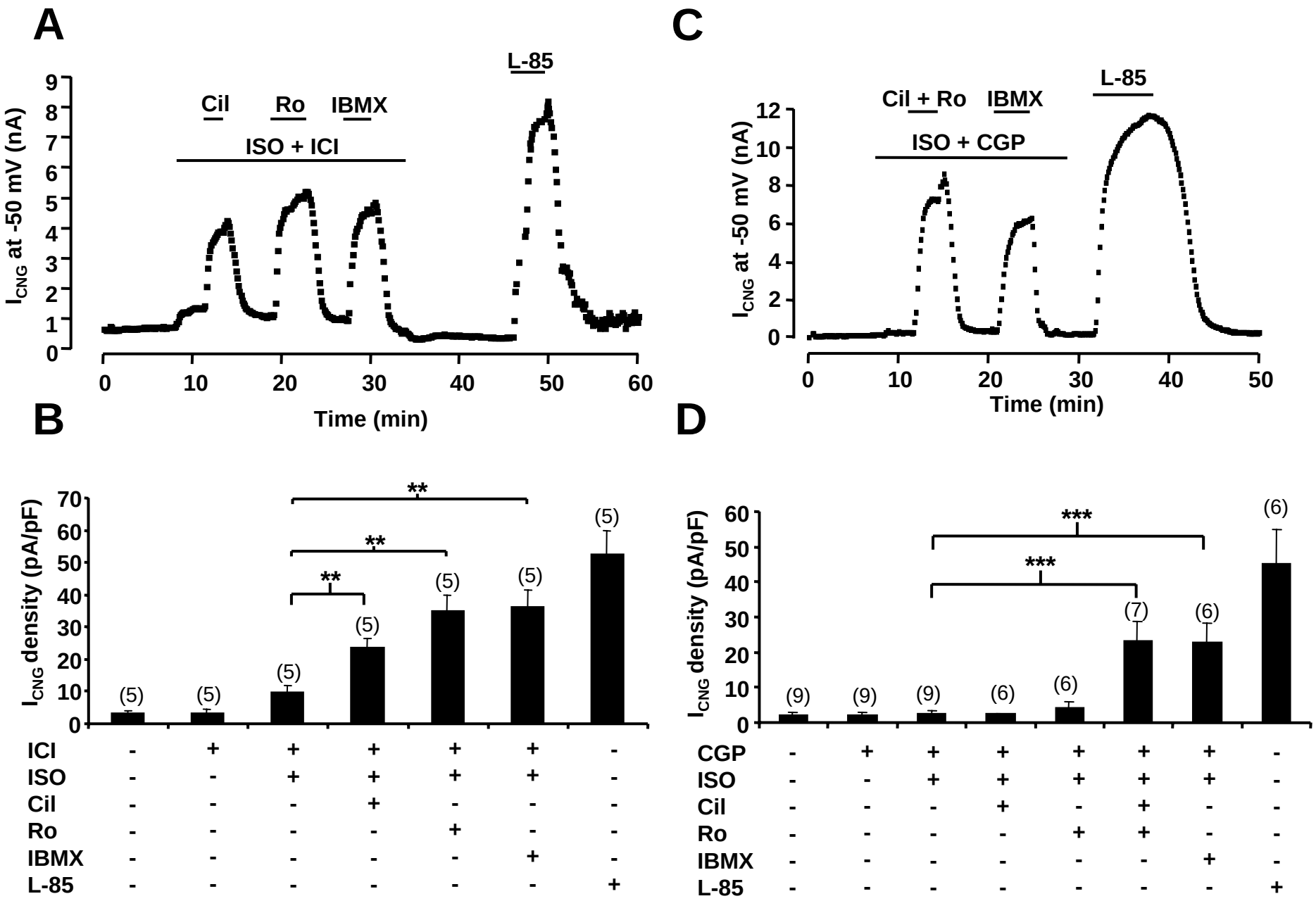
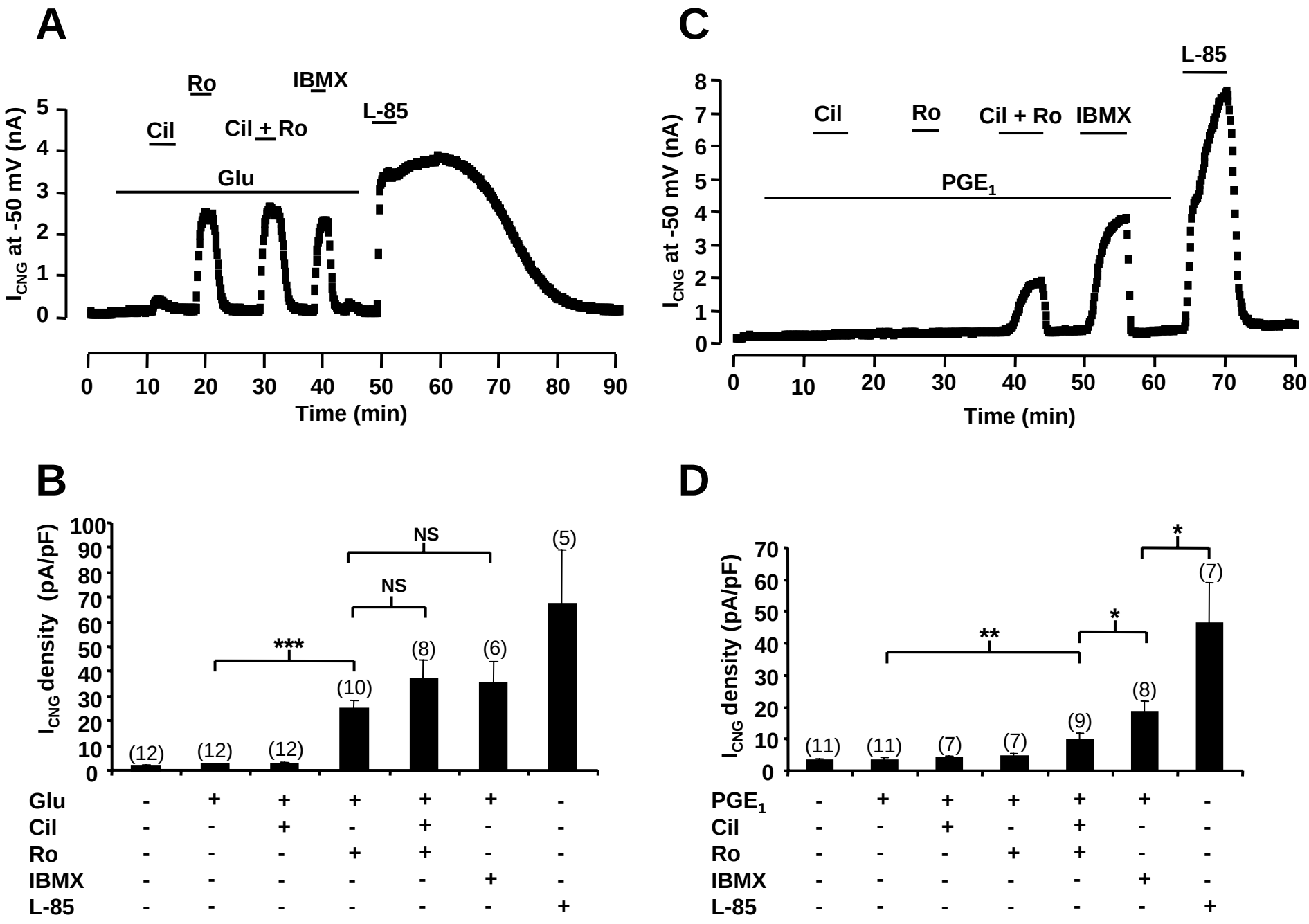


Suppl Figure I



Suppl Figure II