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G $\alpha_{i/o}$ -dependent Ca²⁺ mobilization and G α_q -dependent PKC α regulation of Ca²⁺-sensing receptor-mediated responses in N18TG2 neuroblastoma cells

John S. Sesay^{1,2,4}, Reginald N. K. Gyapong¹, Leila T. Najafi³, Sandra L. Kabler⁴, Debra I. Diz^{4,5}, Allyn C. Howlett^{2,3,4,5}, and Emmanuel M. Awumey^{1,2,4,5}

¹Cardiovascular Disease Research Program, Julius L Chambers Biomedical/Biotechnology Research Institute, North Carolina Central University, Durham, NC 27707

²Department of Biology, North Carolina Central University, Durham, NC 27707

³Department of Pharmacological and Physiological Science, Saint Louis University, St. Louis, MO 63104

⁴Department of Physiology and Pharmacology, Wake Forest University School of Medicine, Winston-Salem, NC 27157

⁵Hypertension & Vascular Research Center, Wake Forest University School of Medicine, Winston-Salem, NC 27157

Abstract

A functional Ca²⁺-sensing receptor (CaS) is expressed endogenously in mouse N18TG2 neuroblastoma cells, and sequence analysis of the cDNA indicates high homology with both rat and human parathyroid CaS cDNAs. The CaS in N18TG2 cells appears as a single immunoreactive protein band at about 150 kDa on Western blots, consistent with native CaS from dorsal root ganglia. Both wild type (WT) and G α_q antisense knock-down (KD) cells responded to Ca²⁺ and calindol, a positive allosteric modulator of the CaS, with a transient increase in intracellular Ca²⁺ concentration ([Ca²⁺]_i), which was larger in the G α_q KD cells. Stimulation with 1 mM extracellular Ca²⁺ (Ca²⁺_e) increased [Ca²⁺]_i in N18TG2 G α_q KD compared to WT cells. Ca²⁺ mobilization was dependent on pertussis toxin-sensitive G $\alpha_{i/o}$ proteins and reduced by 30 μ M 2-amino-ethyl-diphenyl borate and 50 μ M nifedipine to the same plateau levels in both cell types. Membrane-associated PKC α and p-PKC α increased with increasing [Ca²⁺]_e in WT cells, but decreased in G α_q KD cells. Treatment of cells with 1 μ M Gö 6976, a Ca²⁺-specific PKC inhibitor reduced Ca²⁺ mobilization and membrane-associated PKC α and p-PKC α in both cell types. The results indicate that the CaS-mediated increase in [Ca²⁺]_i in N18TG2 cells is dependent on G $\alpha_{i/o}$ proteins via inositol-1,4,5- triphosphate (IP₃) channels and store-operated Ca²⁺ entry

Corresponding Author: Emmanuel M. Awumey, Ph.D, Associate Professor, Cardiovascular Disease Research Program, JLC Biomedical/Biotechnology Research Institute and Department of Biology, North Carolina Central University, 700 George Street, Durham, NC 27707. Phone: (919) 530-7644; Fax: (919) 530-6815. eawumey@nccu.edu.

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channels, whereas modulation of CaS responses involving PKC α phosphorylation and translocation to the plasma membrane occurs via a G α_q mechanism.

Keywords

N18TG2 cells; neuronal CaS; G α_q antisense knock-down; G $\alpha_{i/o}$; Ca $^{2+}$ mobilization; CaS responses; protein kinase C

Introduction

Since the initial cloning of the Ca $^{2+}$ -sensing receptor (CaS; official IUPHAR name) from bovine parathyroid gland (Brown 1999, Brown *et al.* 1993, Brown & MacLeod 2001)), nervous tissue from rat has been found to express a full-length, alternatively spliced form of the receptor, which is concentrated in nerve terminals and involved in the regulation of neuronal cell growth and migration during development, synaptic plasticity and neurotransmission in mature nerve terminals [for review, see (Bouschet & Henley 2005, Bouschet *et al.* 2005), (Ruat & Traiffort 2013)]. In addition to the brain (Ruat *et al.* 1995), the CaS is also expressed in perivascular sensory nerves (Bukoski 1998, Bukoski *et al.* 1997, Wang & Bukoski 1998, Wang & Bukoski 1999), trigeminal ganglia and sensory axons (Heyeraas *et al.* 2008). We reported the cloning and sequencing of the dorsal root ganglion (DRG) CaS message and found significant homology with the rat kidney CaS cDNA (Wang *et al.* 2003b). Expression analysis of a DRG CaS-EGFP fusion protein transfected into HEK293 cells showed that the fusion protein incorporates into the cell membranes and is functionally linked to a transient increase in [Ca $^{2+}$] $_i$ (Awumey *et al.* 2007). Activation of the CaS expressed in DRG and perivascular sensory nerves (Bukoski *et al.* 1997, Ishioka & Bukoski 1999) by extracellular Ca $^{2+}$ (Ca $^{2+}$ $_e$) results in the release of a vasodilator transmitter, possibly an endocannabinoid (Awumey *et al.* 2008, Bukoski 1998) (Bukoski *et al.* 2002, Ishioka & Bukoski 1999).

As a G protein-coupled receptor (GPCR), the CaS can couple to more than one type of G α subunit and influence the properties of G $\beta\gamma$ signaling (Neves *et al.* 2002). Three modes of CaS coupling to G proteins have been reported, namely through: i) G α_i to inhibit adenylyl cyclase (AC) and activate mitogen activated protein kinase (MAPK); ii) G α_q to stimulate phospholipase C (PLC) and phospholipase A $_2$ (PLA $_2$); and iii) G $\beta\gamma$ to stimulate phosphoinositide-3-kinase (Brown & MacLeod 2001). Activation of the CaS by Ca $^{2+}$ $_e$, other polyvalent cations or allosteric regulators stimulates PLC, PLD, or PLA signaling pathways depending on the cell type (for review, see (Conigrave & Ward 2013 Breitwieser 2013). PLC activation results in the generation of inositol-1,4,5-trisphosphate (IP $_3$) and the release of Ca $^{2+}$ from the endoplasmic reticulum (ER). It has been difficult to assess the roles of G $\alpha_{i/o}$ versus G α_q in activation of the neuronal CaS because there have been no established neuronal cell models for studying receptor coupling to intracellular signal transduction events. N18TG2 cells, a mouse neuroblastoma clone, express many properties of neurons (Mukhopadhyay *et al.* 2002), and have been shown to produce the endocannabinoid, 2-arachidonoylglycerol (2-AG) in response to elevations in [Ca $^{2+}$] $_i$ (Bisogno *et al.* 1997). Using this established neuronal cell model, the present study describes the signaling

mechanisms of the endogenously-expressed CaS and its coupling via $G\alpha_{i/o}$ to Ca^{2+} mobilization and $G\alpha_q$ to PKC α phosphorylation, which could account for rapid reduction of CaS responses.

Materials and Methods

Materials

DMEM/F-12 (1:1), Hanks Balanced Salt Solution (HBSS), Fura-2/AM, Pluronic® F-127, penicillin/streptomycin (100X), heat-inactivated bovine serum, TRIzol reagent, SuperScript™ II RT and pCR-XL-TOPO vector were from Invitrogen (Carlsbad, CA). 2-Amino-ethyl-diphenyl borate (2-APB), Gö 6976, ionomycin and phorbol-12-myristate-13 acetate (PMA) were from EMD Biosciences (La Jolla, CA). CaS polyclonal antibody (PA1-37213), raised against a synthetic peptide corresponding to the N-terminus of rat CaS and Halt Protease Inhibitor Cocktail were from Pierce Biotechnology (Rockford, IL). Calindol, rabbit polyclonal PKC α (sc-208) and p-PKC α (sc-12356-R) antibodies were from Santa Cruz Biotechnology, and pertussis toxin (PTX) was from Biomol International (Plymouth Meeting, PA). All other chemicals used were of the purest grade available commercially.

Cell culture

A stable $G\alpha_q$ antisense-knockdown (KD) clone was derived from N18TG2 cells as follows: Cells were transfected (Lipofectamine in Opti-MEM media) with the full-length 1.7 Kb cDNA coding sequence of $G\alpha_q$ that had been ligated into pcDNA3 (Invitrogen, Carlsbad, CA) in an antisense orientation (Gardner *et al.* 2002). Clones were selected by resistance to G418 sulfate (Mediatech, Herndon, VA) and maintained in media containing 250 μ g/ml G418 sulfate in DMEM/F12 (1:1) medium supplemented with heat-inactivated bovine serum (10%) and penicillin/streptomycin (100 U ml⁻¹/100 μ g.ml⁻¹). Cells were grown on glass cover slips for $[Ca^{2+}]_i$ determination.

Expression Analysis of CaS and PKC isoforms in N18TG2 Cells

Reverse transcription-polymerase chain reaction (RT-PCR) was carried out with total RNA extracted from sub-confluent cells to determine whether N18TG2 cells express mRNA that is homologous with the CaS message expressed in DRG neurons. The forward primer sequence (5'>GCT ATA AGC TTC ACT TCT CAG GAC TCG AGG ACC AGC<3') is specific for the exon 1 splice variant that is expressed in DRG but not in the kidney or parathyroid glands, and a reverse primer sequence (5'>GCT ATG GAT CCT AAT ACG TTT TCC GTC ACA GAG C<3') is based on 3'-UTR sequence that is common in the three tissues. *Hind* III and *Bam* H1 sites (underlined) were inserted in the forward and reverse primers, respectively, for cloning. The PCR product was cloned into the pCR-XL-TOPO vector and sequenced with an ABI Prism 373 Genetic Analyzer (Applied Biosystems, Carlsbad, CA) using M13 forward/reverse primers to establish identity.

To determine the expression of PKC isoforms, cells were harvested at 90% confluence with Trizol/10% and β -mercaptoethanol, and lysed using Qiashredder (Qiagen Inc. Valencia, CA). RNA was extracted using the RNeasy kit (Qiagen Inc. Valencia, CA). RNA

concentrations were read on a Nanodrop 2000 (Thermo Scientific) and cDNA was generated from the RNA having a 260/280 ratio > 1.8 using the First Strand RT2 kit (SA Biosciences Frederick, MD). PKC isoform expression levels were determined using the mouse “Human Alzheimer's Disease” RT² Profiler™ PCR Array Cat # PAMM-057 (Qiagen Inc. Valencia, CA). CT values were calculated as an average CT from 3 PCR Array plates minus the mean of the following reference genes, GAPDH, β -actin and Hsp90 α 1 from the same plates. The CT was determined by comparing each CT to that of N18TG2 WT PKC α , and the 2^{-CT} values were normalized to N18TG2 WT PKC α , as 100. The data were analyzed by a 2-way ANOVA and the Holm-Sidak multiple comparisons and Bonferroni tests were used to compare the N18TG2 WT with the G α_q KD cells.

Intracellular Ca²⁺ measurements

Changes in [Ca²⁺]_i in N18TG2 cells, following stimulation with [Ca²⁺]_e or calindol, were determined by microfluorimetric, dual wavelength [Ca²⁺]_i imaging with Fura-2. Cells grown on glass cover slips in 35 mm dishes for 48 hr. were loaded with 5 μ M Fura-2/AM in HBSS with 0.1% Pluronic for 30 min at 37 °C followed by washing with PSS (mM: NaCl, 150; KCl, 5.4; MgSO₄·7H₂O, 1.2; NaH₂PO₄, 1.2; NaHCO₃, 6.0; CaCl₂, 0.25; glucose, 5.5; HEPES, 20; pH 7.4). Cover slips were then mounted in a stainless steel cell chamber (Attofluor®) in fresh PSS and placed on the stage of an Axiovert 100S inverted microscope equipped with a Zeiss Fluor 40 $\times$ oil-immersion objective. A Dual-wavelength Fluorescence Imaging System (Photon Technology International, Birmingham, NJ) was used to measure changes in [Ca²⁺]_i following stimulation of cells with Ca²⁺ or calindol, a positive allosteric modulator of the CaS, in the presence or absence of PTX, 2-APB, nifedipine, PMA and Gö 6976. Cells loaded with Fura-2 were excited at 340 nm and 380 nm with a xenon light source (75 Watt Xe Compact Arc Lamp) and emissions at 510 nm were captured by an IC-300 intensified CCD or CoolSNAP HQ² cameras. The images were transmitted to a computer and processed using the ImageMaster Pro™ or EasyRatioPro™ Ratio Fluorescence Imaging software, with a macro based on the concentration equation of Grynkiewicz (Grynkiewicz *et al.* 1985) The [Ca²⁺]_i data are reported as emission ratios (F₃₄₀/F₃₈₀). In all experiments, 20-30 cells from a single field were analyzed and the means calculated. Applications of 10 μ M ionomycin followed by 20 mM EGTA were used to determine the maximum (R_{max}) and the minimum (R_{min}) levels of [Ca²⁺]_i, respectively.

Ca²⁺-induced membrane translocation and phosphorylation of PKC α

To determine the activation of PKC following stimulation of cells with increasing concentrations of Ca²⁺, we analyzed PKC α and phosphorylated PKC α (p-PKC α) in crude plasma membrane and supernatant fractions of cell homogenates by Western blotting with PKC α (sc-208; SCBT) and p-PKC α (sc-12356-R; SCBT) antibodies. Immunoblotting was carried out on membrane fractions (100K $\times$ g pellets) from N18TG2 (WT and G α_q KD) cells. Briefly, cells were grown in 100 mm dishes for 48 hr. and stimulated with [Ca²⁺]_e (1 - 5 mM) in the presence or absence of 1 μ M Gö 6976 for 30 min in physiological salt solution (with 0.25 mM Ca²⁺). Cells were then scraped into 12-ml snap-cap tubes and homogenized in Tris buffer (10 mM Tris, pH 7.5; 0.25 M sucrose and 3 mM MgCl₂) with freshly-added Halt Protease Inhibitor Cocktail. The homogenates were passed through a 22-gauge needle and sonicated for 20 sec to complete lysis. Lysates were centrifuged at 800 $\times$ g for 10 min at

4 °C and the supernatants centrifuged at $100,000 \times g$ for 1 hr at 4 °C. The supernatants were removed and pellets suspended in Spiegel's buffer (20 mM Tris, pH 6.8; 150 mM NaCl; and 10 mM EDTA; 1% Triton X-100; 1 mM EGTA) with freshly-added Halt Protease Inhibitor Cocktail. Protein concentrations of the supernatants and pellets were determined by the BCA method and 100 µg samples were separated by electrophoresis on 8% sodium dodecyl sulfate polyacrylamide gel and transferred onto PVDF membranes. The membranes were then blotted with PKCα or p-PKCα antibodies, incubated with horseradish peroxidase-conjugated secondary IgG, and visualized using Enhanced Chemiluminescence (ECL™).

Statistical analysis

Data were analyzed with SigmaPlot 11.0 statistics programs from Systat Software, Inc. (Point Richmond, CA). Comparisons between groups and within groups were done by One Way Analysis of Variance (ANOVA); differences with $p < 0.05$ were considered significant.

Results

Expression of a neuronal CaS in N18TG2 cells

The RT-PCR product (Figure 1A), cloned into the pCR-XL-TOPO vector, was sequenced and the BLAST data obtained from sequences in GeneBank database, indicate 99.7% sequence identity (578 of 580 bases; 2 mismatches) between the N18TG2 CaS cDNA sequence and mouse (C57BL/6J strain) genomic CaS sequence (GI: 4731165) in chromosome 1, 97.9% identity (550 of 562 bases; 8 mismatches) with the Norway rat genomic CaS sequence (GI: 8393053) in chromosome 11, and 88.5% identity (323 of 365 bases; 1 mismatch) with the human genomic CaS sequence (GI: 904210) in chromosome 3. Western blot analysis of proteins extracted from N18TG2 cells showed levels of expression of a protein of size (≈ 150 kDa), comparable to the DRG and parathyroid CaS (Fig. 1B). In order to investigate signal transduction that occurs via Gq, we developed a stable anti-sense $G\alpha_q$ KD clone which expresses about 33% (calculated from the Western blot densitometry data) less $G\alpha_q$ protein subunit (Fig 1C). Of note, WT and $G\alpha_q$ KD cells express similar amounts of CaS.

Microfluorimetric analysis of changes in $[Ca^{2+}]_i$ in N18TG2 cells following stimulation with $[Ca^{2+}]_e$ or calindol, a positive allosteric modulator of CaS

To characterize CaS-mediated changes in $[Ca^{2+}]_i$ in N18TG2 cells, 1 mM Ca^{2+}_e or 5 µM calindol was applied to Fura-2-loaded cells on glass cover slips (Fig. 2). The change in $[Ca^{2+}]_i$ consisted of an initial rise followed by a gradual decline to plateau above basal. The rate of increase to peak $[Ca^{2+}]_i$ with 1 mM Ca^{2+}_e was higher in $G\alpha_q$ KD compared to WT cells, but the differences were not statistically significant. The CaS in N18TG2 cells responded to either 5 µM or 10 µM calindol in the presence of 0.25 mM Ca^{2+}_e , a concentration that serves as the background level in the present studies (Fig. 2C-E). Both WT and $G\alpha_q$ KD cells responded with robust, concentration-dependent increases in Ca^{2+} mobilization, which rose at a higher rate than those observed after stimulation of CaS by 1 mM Ca^{2+}_e . The changes in F_{340}/F_{380} ratios for 10 µM calindol were: WT cells, 1.32 ± 0.14 vs. $G\alpha_q$ KD cells, 1.57 ± 0.18 . Compared with the response to Ca^{2+}_e , the response to calindol

continued for a longer period and declined to a plateau that was greater than the background level for both WT and $G\alpha_q$ KD cells.

Pertussis toxin (PTX)-sensitive $G\alpha_{i/o}$ proteins are required for CaS-mediated mobilization of Ca^{2+} in N18TG2 cells

In order to assess contributions of $G\alpha_q$ versus $G\alpha_{i/o}$ in Ca^{2+} mobilization in N18TG2 cells, we examined the effects of inactivating $G\alpha_{i/o}$ proteins with PTX on Ca^{2+} and calindol responses. If $G\alpha_q$ were contributing to the Ca^{2+} mobilization, one would predict a lower peak value in the $G\alpha_q$ KD cells. Rather, Fig. 3 shows that pre-treatment of both N18TG2 WT and $G\alpha_q$ KD cells with 50 ng/ml PTX overnight reduced responses to 1 mM Ca^{2+} by $68 \pm 2\%$ in WT cells ($n = 4$, $p < 0.05$ vs. controls) and by $82 \pm 2\%$ ($n = 4$; $p < 0.01$) in $G\alpha_q$ KD cells (Fig. 3A-C). Calindol-induced peak responses in WT cells were reduced by $35 \pm 2\%$ with no effect on the plateau. In $G\alpha_q$ KD cells, peak Ca^{2+} was reduced by $60 \pm 3\%$ and the plateau by $35 \pm 3\%$ ($n = 4-6$) (Fig. 3D,E). Notably, the diminished levels of $G\alpha_q$ in the $G\alpha_q$ KD cells did not further reduce the rate of rise to peak compared with WT in the $G\alpha_{i/o}$ -inactivated cells. These data demonstrate that Ca^{2+} mobilization is under the dominant regulation of $G\alpha_{i/o}$ proteins.

Ion channels involved in CaS-mediated changes in $[Ca^{2+}]_i$ in N18TG2 cells

To examine the role of the IP_3 -responsive channel in Ca^{2+} signaling by the CaS in N18TG2 cells, we treated the cells with an IP_3 and SOCE channel inhibitor, 2-APB (30 μ M) for 20 min and measured the level of $[Ca^{2+}]_i$. Fig. 4A.-C. shows that 2-APB eliminated the Ca^{2+} -stimulated rise to a peak $[Ca^{2+}]_i$ in WT, and significantly reduced the peak by $78 \pm 2\%$ ($n = 4$; $p < 0.001$ vs. control) in $G\alpha_q$ KD cells. To determine if L-type dihydropyridine-sensitive Ca^{2+} channels play a role in the neuronal CaS-mediated Ca^{2+} influx, we examined the effect of nifedipine on Ca^{2+} mobilization in WT and $G\alpha_q$ KD cells. Cells were treated with 50 μ M nifedipine or vehicle for 20 min and $[Ca^{2+}]_i$ measured in response to a 1 mM Ca^{2+} stimulus. Fig. 4 D.-F. shows that the rate of rise to peak $[Ca^{2+}]_i$ was attenuated following pre-treatment with nifedipine for both WT and $G\alpha_q$ KD cells. The rate of rise, determined as the change in initial slope of tracings over time, was more gradual at 4.7×10^{-3} units/sec for nifedipine-treated cells vs. 15.1×10^{-3} units/sec for control, a 3-fold reduction. The peak $[Ca^{2+}]_i$ heights were reduced by about 50% in both cell types. Plateau levels of $[Ca^{2+}]_i$ were the same in the presence or absence of nifedipine in both WT and $G\alpha_q$ KD cells.

Regulation of CaS-mediated changes in $[Ca^{2+}]_i$ by PKC in N18TG2 cells

Ca^{2+}_i mobilization is likely to activate PKC as a primary mechanism of signaling. As shown in Fig. 5, N18TG2 cells express abundant mRNA for PKC α , PKC δ , PKC ϵ , PKC τ , and PKC ζ isoforms (for review of PKC sub-families and their activation, see (Olive & Newton 2010, Wu-Zhang & Newton 2013). Of the conventional PKC isoforms that translocate to the plasma membrane in response to $[Ca^{2+}]_i$ and diacylglycerol, PKC α was expressed in 100-fold greater abundance than PKC β or γ . The PKC α mRNA levels were not changed by knock-down of $G\alpha_q$ (Fig. 5) or by the over-night treatment with PTX to block $G_{i/o}$ stimulation (data not shown). The 2-way ANOVA indicated significant differences between the PKC subtypes as well as between the cell lines ($p < 0.0001$), with a significant

interaction between these factors ($p < 0.002$). Significant differences ($p < 0.05$) were observed between cell lines for δ , τ and ζ , PKC subtypes.

To determine the role played by conventional PKC in CaS-mediated Ca^{2+}_i mobilization in N18TG2 cells, we incubated the cells with PMA, an activator of PKC that mimics the diacylglycerol required for membrane association (Fig. 6). Pre-treatment of cells with 100 nM PMA for 20 min reduced CaS-mediated Ca^{2+}_i mobilization by $73 \pm 2\%$ ($n = 4$, $p < 0.01$ vs. control) in N18TG2 cells (Fig. 6A,C) and $79 \pm 4\%$ ($n = 4$; $p < 0.01$) in $\text{G}\alpha_q$ KD cells (Fig. 6B,C). The plateau $[\text{Ca}^{2+}]_i$ was not affected. We also determined the effect of the Ca^{2+}_i -specific PKC inhibitor, Gö 6976 (a cell-permeable, reversible and $\text{ATP}\beta$ -competitive inhibitor of $\text{PKC}\alpha$ and $\text{PKC}\beta$) on Ca^{2+} mobilization. In both cell types, 100 nM Gö 6976 had no effect on peak Ca^{2+} but reduced the amplitudes (Fig. 7A, B). This concentration also reduced the plateau $[\text{Ca}^{2+}]_i$ in $\text{G}\alpha_q$ KD cells (Fig. 7B). However, 1 μM Gö 6976 reduced the peak and plateau $[\text{Ca}^{2+}]_i$ in both cell types. The plateau was completely eliminated in $\text{G}\alpha_q$ KD cells. In the current study, we focused on $\text{PKC}\alpha$ to investigate activation and translocation in response to CaS signaling.

To further investigate the mechanism by which PKC regulates the neuronal CaS response, we determined the effects of increasing $[\text{Ca}^{2+}]_e$ on PKC phosphorylation and translocation. Western blot analysis indicated that membrane-associated $\text{PKC}\alpha$ (Figs. 9A,B) and p- $\text{PKC}\alpha$ (Figs. 9C,D) levels increased when $[\text{Ca}^{2+}]_e$ was raised from the basal level of 0.25 mM to 5 mM in WT cells. In contrast, $\text{G}\alpha_q$ knockdown increased $\text{PKC}\alpha$ (Fig. 8B) and p- $\text{PKC}\alpha$ (Fig. 8D) levels in basal conditions (0.25 mM Ca^{2+}) and reversed the $\text{PKC}\alpha$ phosphorylation and translocation patterns in response to increased $[\text{Ca}^{2+}]_e$. Upon pretreatment with Gö6976, Ca^{2+}_e failed to increase membrane-associated $\text{PKC}\alpha$ and p- $\text{PKC}\alpha$ levels. These findings indicate that $\text{G}\alpha_q$ signaling directs the $\text{PKC}\alpha$ cellular response. Inhibition of PKC by Gö 6976 reduced the $[\text{Ca}^{2+}]_e$ -dependent increase and decrease in membrane-associated $\text{PKC}\alpha$ and p- $\text{PKC}\alpha$ levels.

Discussion

CaS activation leads to signal transduction through cyclic AMP and phospholipases depending on the cell type (Awumey et al. 2007, Brown & MacLeod 2001, Wang *et al.* 2003a, Wang et al. 2003b). Activation of PLC leads to production of IP_3 -mediated release of stored Ca^{2+} from the ER causing a transient rise in $[\text{Ca}^{2+}]_i$. ER Ca^{2+} store emptying also opens plasma membrane SOCE channels that allow influx of Ca^{2+} resulting in a sustained plateau as shown in the present study.

In the present study we investigated the effects of $[\text{Ca}^{2+}]_e$ on $[\text{Ca}^{2+}]_i$ signal transduction via $\text{G}\alpha_{i/o}$ and $\text{G}\alpha_q$ in mouse N18TG2, a neuronal cell model that endogenously express a CaS having > 90% homology with the rat and human neuronal CaS receptors. In other tissues, the CaS exerts its effects mainly by interacting with $\text{G}\alpha_q$, $\text{G}\alpha_{i/o}$ and $\text{G}\alpha_{12/13}$ (Brown & MacLeod 2001, Ward 2004). Our studies indicate that the neuronal CaS requires a signaling pathway mediated by PTX-sensitive $\text{G}_{i/o}$ proteins for Ca^{2+} mobilization. In parathyroid tissue, CaS was reported to transduce the mobilization of Ca^{2+}_i through $\text{G}\alpha_{i/o}$ proteins, with PI-PLC as the major downstream effector (Fitzpatrick *et al.* 1986, Brown et al. 1993).

Stimulation of PI-PLC in AtT-20 pituitary cells (Emanuel *et al.* 1996), and *Xenopus* oocytes (Brown *et al.* 1993) expressing exogenous CaS also occurred via PTX-sensitive proteins. In contrast, bovine parathyroid cell CaS couples to PI-PLC through $G_{q/11}$ (Brown 1999). The CaS exogenously expressed in HEK293 cells is also coupled to $G_{q/11}$ (Kifor *et al.* 1997).

Our data also indicate that the CaS-mediated Ca^{2+} mobilization is sensitive to nifedipine suggesting involvement of dihydropyridine-sensitive Ca^{2+} channels. This is consistent with a report indicating the presence of nifedipine-sensitive Ca^{2+} channels and N-type Ca^{2+} channels in a similar neuronal cell line (Schneider *et al.* 1995). However, the plateau was not affected, indicating that nifedipine may affect the kinetics of release of Ca^{2+} from ER and influx. In neutrophils, nifedipine inhibits Ca^{2+} release from intracellular stores (Rosales & Brown 1992). Furthermore, dihydropyridine-sensitive L-type Ca^{2+} channels influenced IP_3 -induced Ca^{2+} release in angiotensin II-stimulated glomerulosa cells (Spat *et al.* 1996), and nifedipine completely blocked slow Ca^{2+} transients in primary cultures of rat myotubes (Araya *et al.* 2003). ER Ca^{2+} stores play an important role in increasing $[Ca^{2+}]_i$ in neuronal cells (Berridge 2004, Putney 2005), although voltage-dependent Ca^{2+} channels, plasma membrane Ca^{2+} -ATPase, SOCE and the Na^+/Ca^{2+} exchanger also play critical roles in the influx and recovery of resting $[Ca^{2+}]_i$ in rat sensory neurons. Thus, the effect of nifedipine may be much more complex than is apparent in the present study.

The present data demonstrate that in N18TG2 cells, Ca^{2+} mobilization is up-regulated in $G_{q\alpha}$ KD cells suggesting that $G_{q\alpha}$ is not intrinsic to the Ca^{2+} mobilization mechanism, but is important to the modulation of the response. The CaS has numerous phosphorylation sites in the extracellular domain thus, our findings that PMA reduced the CaS-mediated responses, and that the reduced response is accompanied by PKC phosphorylation and mobilization, as well as the reduction of Ca^{2+} mobilization response and membrane translocation by Gö 6976, provide evidence for an alternative mechanism to regulate the sensitivity of the neural CaS. The reduction in Ca^{2+} mobilization (Ca^{2+} peak, amplitude and plateau) by the higher concentration of Gö 6976 in both cell types suggests the involvement of other Ca^{2+} mobilization pathways, such as the ER Ca^{2+} -ATPase and store-operated Ca^{2+} entry channels. The effect of PKC inhibition on Ca^{2+} mobilization in the present study is similar to that reported for other PKC inhibitors (Bis-I, Ro 31-8220, Ro 32-0432) and Gö 6976 in HEK293 cells expressing the DRG CaS-EGFP fusion protein (Awumey *et al.* 2007). The Ca^{2+} influx pathway (the plateau) was blocked in $G_{q\alpha}$ KD cells, thus, PKC inhibition shortened the duration of Ca^{2+} mobilization in these cells suggesting that the inhibitor effect is on both the rate of Ca^{2+} release from ER and Ca^{2+} influx that appears to be much more complex and requires further investigation.

These studies suggest that activated PKC can suppress the Ca^{2+}_i mobilization response to CaS stimulation in both WT and $G_{q\alpha}$ KD cells. Presumably, PMA treatment of cells resulted in the activation of PKC and subsequent phosphorylation of the CaS leading to reduced Ca^{2+} mobilization. PKC is known to phosphorylate a critical threonine in the human parathyroid CaS, and this phosphorylation modulates the functional interaction with G proteins (Bai *et al.* 1998) critical to the regulation of Ca^{2+} release from internal stores (Awumey *et al.* 2007, Jiang *et al.* 2002). The present findings demonstrate a role for $[Ca^{2+}]_e$ -induced translocation and phosphorylation of PKC in modulation of the neural CaS

response, which was protected by diminished $G\alpha_q$ levels in the cell, as was the phosphorylation and mobilization of PKC to the membrane, indicating that $G\alpha_q$ is intrinsic to the modulation of CaS signaling, rather than the Ca^{2+} mobilization mechanism.

We conclude that in N18TG2 neuroblastoma cells, which endogenously express the CaS with native $G\alpha_{i/o}$ and $G\alpha_q$, stimulation with extracellular Ca^{2+} and the allosteric modulator, calindol promotes rapid increases in $[Ca^{2+}]_i$, which is mediated by $G\alpha_{i/o}$. Furthermore, Ca^{2+} and diacylglycerol-activated PKC α pathway modulates the CaS as a result of PKC phosphorylation and translocation to the membranes following activation by agonist. Our finding that this process is down-regulated in the $G\alpha_q$ KD cells indicates a functional requirement for $G\alpha_q$ in mediating the PKC influence on CaS responses. The current study provide the impetus to seek novel agonists or positive allosteric regulators biased toward coupling the CaS to either $G\alpha_{i/o}$ to sustain an activated state, or $G\alpha_q$ to promote reduced responses. That an established neural-derived cell line expresses a CaS, provides a potentially useful model system to screen for compounds that are selective in cellular regulation of CaS-mediated responses.

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Nonstandard abbreviations

AEA	anandamide
2-APB	2-aminoethyldiphenyl borate
DRG	dorsal root ganglion
EGFP	Enhanced Green Fluorescent Protein
GPCR	G protein-coupled receptor
KD	knock-down
IP₃	inositol-1,4,5-trisphosphate
MAPK	mitogen-activated protein kinase
PLC	phospholipase C
PMA	phorbol-12-myristate-13 acetate
PTX	pertussis toxin
RT-PCR	reverse transcriptase polymerase chain reaction
ECL	Enhanced Chemiluminescence
BSA	bovine serum albumin
PMA	phorbol myristate acetate
SOCE	store-operated calcium entry

Figure 1. Expression analysis of CaS in N18TG2 neuroblastoma cells

A. Total RNA was extracted from cells and subjected to RT-PCR with primers specific for the full length dorsal root ganglia CaS and analyzed on agarose gels followed by staining with ethidium bromide. **M**, 1 kb DNA ladder; **WT**, Wild type N18TG2 cells; **Gα_q KD**, N18TG2 cells in which Gα_q protein was knocked down by anti-sense nucleotide expression. **B.** Western blot analysis of plasma membrane proteins from (i) N18TG2 cells, and (ii) total proteins from DRG and parathyroid (PT) isolated from rats with the polyclonal anti-CaS antibody and horseradish peroxidase (HRP)-conjugated IgG and developed with ECL. The CaS protein from N18TG2 cells, DRG and PT migrated as single bands of ≈ 140 kDa. **C.** Western blot analysis showing reduced expression of Gα_q N18TG2 cells. Data are expressed as means ± SEM (n = 4 experiments).

Figure 2. Pharmacologic properties of the CaS-mediated Ca^{2+} mobilization in N18TG2 WT and $\text{G}\alpha_q$ KD cells

Cells grown on glass cover slips were loaded with 5 μM Fura-2/AM and responses to 1 mM Ca^{2+} and calindol were determined by microfluorimetry. **A.** Responses of N18TG2 WT and N18TG2 $\text{G}\alpha_q$ KD cells to 1 mM Ca^{2+} are shown. After the experimental treatment, the maximum fluorescence in response to 10 μM ionomycin was determined, followed by addition of 20 mM EGTA to determine the Ca^{2+} -free minimum fluorescence. **B.** A histogram showing changes in the peak heights of $[\text{Ca}^{2+}]_i$ in cells following stimulation. Data are expressed as mean $\pm$ SEM ($n = 4$ experiments). **C.-E.** Responses to 5 μM and 10 μM calindol determined by microfluorimetry. **C.** N18TG2 WT cells, **D.** N18TG2 $\text{G}\alpha_q$ KD cells. **E.** Histogram showing changes in the peak heights of $[\text{Ca}^{2+}]_i$ in cells following stimulation with calindol. Data are expressed as means $\pm$ SEM ($n = 5$ experiments).

*Significantly different from WT cells ($p < 0.05$).

Figure 3. Effect of blocking $G_{i/o}$ with PTX on CaS -mediated mobilization of Ca^{2+} in N18TG2 WT and $G\alpha_q$ KD cells

Cells, grown on glass cover slips and treated with PTX (50 ng/ml) or vehicle for 16 hr. were loaded with 5 μM Fura-2/AM for 30 min before measurement of responses to 1 mM Ca^{2+} (A-C) and 5 μM calindol (D, E). A. N18TG2 WT cells, B. N18TG2 $G\alpha_q$ KD cells, C. Histogram showing changes in the peak height of $[Ca^{2+}]_i$ following stimulation with 1 mM Ca^{2+} . Responses of cells to 5 μM calindol following PTX treatment; D. N18TG2 WT cells; E. N18TG2 $G\alpha_q$ KD cells. Data are expressed as means $\pm$ SEM ($n = 4-6$ experiments).

*Significantly different from controls ($p < 0.05$).

Figure 4. Effect of Ca^{2+} channel blockers on CaS -mediated Ca^{2+} bilization in WT and $\text{G}\alpha_{\text{q}}$ KD N18TG2 cells

Responses of N18TG2 WT (A, D) and N18TG2 $\text{G}\alpha_{\text{q}}$ KD (B, E) cells loaded with 5 μM Fura-2/AM were pre-incubated with 30 μM 2-APB (A.- C.) or 50 μM nifedipine (D.-F.) for 20 min in PSS containing 0.25 mM Ca^{2+} before measurement of responses to 1 mM Ca^{2+} . Tracings are representative of 4 separate experiments carried out under similar conditions. C, F. Histograms showing changes in the peak heights of $[\text{Ca}^{2+}]_{\text{i}}$ in cells following stimulation with 1 mM Ca^{2+} . Data are expressed as means $\pm$ SEM ($n = 4$ experiments).

*Significantly different from controls ($p < 0.05$).

Figure 5. Expression of multiple isoforms of PKC in N18TG2 WT, $G\alpha_q$ KD, and PTX-treated N18TG2 cells

Gene expression profiles of PKC isoforms from N18TG2 cells, as indicated, were determined using a Qiagen qPCR gene expression array. CT values were calculated, converted to the antilog and normalized to N18TG2 WT PKC α as 100. Data are means ($\pm$ SEM) from three separate experiments compared by 2-way ANOVA, which indicated significant differences between cell types/treatment groups as well as for the indicated PKC isoforms. Differences due to cell type/treatment determined by a Bonferroni post-hoc test are indicated. The average Coefficient of Variation ($n = 3$ gene expression arrays) of the CT values for the data shown was 0.038. *Significantly different from WT ($p < 0.05$).

Figure 6. Effect of activation of PKC with PMA on CaS-mediated Ca^{2+} mobilization in WT and $\text{G}\alpha_q$ KD N18TG2 cells

N18TG2 WT (A) and N18TG2 $\text{G}\alpha_q$ KD (B) cells loaded with 5 μM Fura-2/AM were pre-incubated with 100 nM PMA for 20 min before measurement of responses to 1 mM Ca^{2+} . Tracings are representative of 4 separate experiments. C. Histogram showing changes in the peak heights of $[\text{Ca}^{2+}]_i$ (means $\pm$ SEM) in cells following stimulation with 1 mM Ca^{2+} . Data are expressed as mean $\pm$ SEM ($n = 4$ experiments). *Significantly different from control ($p < 0.05$).

Figure 7. Effect of PKC inhibition on Ca^{2+} mobilization by calindol in N18TG2 WT and $\text{G}\alpha_q$ KD cells
 N18TG2 WT (**A**) and N18TG2 $\text{G}\alpha_q$ KD (**B**) cells loaded with 5 μM Fura-2/AM were pre-incubated with 100 nM or 1 μM of the Ca^{2+} -specific PKC inhibitor, Gö 6976 and responses to 5 μM calindol determined. Tracings are representative of separate experiments; insets are histograms showing changes in the peak heights of $[\text{Ca}^{2+}]_i$ in cells following stimulation. Data are expressed as means $\pm$ SEM ($n = 4-9$ experiments). *Significantly different from control and 100 nM calindol ($p < 0.05$).

Figure 8. [Ca²⁺]_e-dependent membrane translocation of PKCα in N18TG2 cells

Cells were stimulated with the indicated $[Ca^{2+}]_e$ for 30 min and isolated plasma membrane and supernatant fractions (100 μ g/lane) analyzed by SDS-PAGE followed by blotting with polyclonal PKC α antibody. β -Actin was used as the loading control. **A.** N18TG2 WT cells: i) Western blot; ii) Histogram showing densitometric analysis of PKC α bands. **B.** N18TG2 G α_q KD cells: i) Western blot; ii) Histogram showing densitometric analysis of PKC α bands. **C.** N18TG2 WT cells: i) Western blot; ii) Histogram showing densitometric analysis of p-PKC α bands. **D.** N18TG2 G α_q KD cells: i) Western blot; ii) Histogram showing densitometric analysis of p-PKC α bands. Bands were normalized to β -actin (loading control) and calculated as means ($\pm$ SEM). Blots shown are representative of 4 separate experiments carried out under similar conditions. *Significantly higher than basal (0.25 mM Ca^{2+} ; $p < 0.05$).

A. N18TG2 WT cells

i. Western blot

ii. Densitometry

Figure 9. Effect of PKC inhibition on $[Ca^{2+}]_e$ -dependent translocation of PKC α in N18TG2 cells
Cells were stimulated with the indicated $[Ca^{2+}]_e$ in the presence of 1 μ M Gö 6976 for 30 min and isolated plasma membrane and supernatant fractions (100 μ g/lane) analyzed by SDS-PAGE followed by blotting with polyclonal PKC α or p-PKC α antibodies. β -Actin was used as the loading control. **A.** N18TG2 WT cells: i) Western blot; ii) Histogram showing densitometric analysis of PKC α bands. **B.** N18TG2 G_{α_q} KD cells: i) Western blot; ii) Histogram showing densitometric analysis of PKC α bands. **C.** N18TG2 WT cells: i) Western blot; ii) Histogram showing densitometric analysis of p-PKC α bands. **D.** N18TG2 G_{α_q} KD cells: i) Western blot; ii) Histogram showing densitometric analysis of PKC α bands. Bands were normalized to β -actin (loading control) and calculated as the means ($\pm$ SEM). Blots shown are representative of 4 separate experiments carried out under similar conditions.