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$G\alpha_{i/o}$-dependent Ca^{2+} mobilization and $G\alpha_q$-dependent PKCα regulation of Ca^{2+}-sensing receptor-mediated responses in N18TG2 neuroblastoma cells

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ABSTRACT

A functional Ca^{2+}-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 $G\alpha_q$ antisense knock-down (KD) cells responded to Ca^{2+} and calindol, a positive allosteric modulator of the CaS, with a transient increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), which was larger in the $G\alpha_q$ KD cells. Stimulation with 1 mM extracellular Ca^{2+} ($Ca^{2+}{}_e$) increased $[Ca^{2+}]_i$ in N18TG2 $G\alpha_q$ KD compared to WT cells. Ca^{2+} mobilization was dependent on pertussis toxin-sensitive $G\alpha_{i/o}$ proteins and reduced by 30 μM 2-amino-ethyldiphenyl borate and 50 μM nifedipine to the same plateau levels in both cell types. Membrane-associated PKCα and p-PKCα increased with increasing $[Ca^{2+}]_e$ in WT cells, but decreased in $G\alpha_q$ KD cells. Treatment of cells with 1 μM Gö 6976, a Ca^{2+}-specific PKC inhibitor reduced Ca^{2+} mobilization and membrane-associated PKCα and p-PKCα in both cell types. The results indicate that the CaS-mediated increase in $[Ca^{2+}]_i$ in N18TG2 cells is dependent on $G\alpha_{i/o}$ proteins via inositol-1,4,5-triphosphate (IP_3) channels and store-operated Ca^{2+} entry channels, whereas modulation of CaS responses involving PKCα phosphorylation and translocation to the plasma membrane occurs via a $G\alpha_q$ mechanism.

1. 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 and 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 and Henley, 2005; Bouschet et al. 2005), (Ruat and Traiffort, 2013). In addition to the brain (Ruat et al. 1995), the CaS is also expressed in perivascular sensory nerves (Bukoski, 1998; Bukoski

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; IP_3, 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 chemiluminiscence; BSA, bovine serum albumin; PMA, phorbol myristate acetate; SOCE, store-operated calcium entry.

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et al. 1997; Wang and Bukoski, 1998; 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. 2003). 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 and 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 and Bukoski, 1999).

As a G protein-coupled receptor (GPCR), the CaS can couple to more than one type of Gα subunit and influence the properties of Gβγ signaling (Neves et al. 2002). Three modes of CaS coupling to G proteins have been reported, namely through: i) $G\alpha_i$ to inhibit adenylyl cyclase (AC) and activate mitogen activated protein kinase (MAPK); ii) $G\alpha_q$ to stimulate phospholipase C (PLC) and phospholipase A_2 (PLA_2); and iii) Gβγ to stimulate phosphoinositide-3-kinase (Brown and 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 and Ward, 2013; Breitwieser, 2014). 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 $G_{i/o}$ versus G_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 PKCα phosphorylation, which could account for rapid reduction of CaS responses.

2. Materials and methods

2.1. 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-ethyldiphenyl 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 PKCα (sc-208) and p-PKCα (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.

2.2. 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 μg/ml G418 sulfate in DMEM/F12 (1:1) medium supplemented with heat-inactivated bovine serum (10%) and penicillin/streptomycin (100 U ml^{-1}/100 μg ml^{-1}). Cells were grown on glass cover slips for $[Ca^{2+}]_i$ determination.

2.3. 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 AT**A AGC TT**C 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 AT**G GAT CC**T 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" RT^2 Profiler™ PCR Array Cat # PAMM-057 (Qiagen Inc. Valencia, CA). ΔCT values were calculated as an average CT from 3 PCR Array plates minus the mean of the following reference genes, GAPDH, β-actin and Hsp90ab1 from the same plates. The ΔΔCT was determined by comparing each ΔCT to that of N18TG2 WT PKCα, and the $2^{-\Delta\Delta CT}$ values were normalized to N18TG2 WT PKCα, 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 $G\alpha_q$ KD cells.

2.4. Intracellular Ca^{2+} measurements

Changes in $[Ca^{2+}]_i$ in N18TG2 cells, following stimulation with $[Ca^{2+}]_e$ or calindol, were determined by microfluorimetric, dual wavelength $[Ca^{2+}]_i$ imaging with Fura-2. Cells grown on glass cover slips in 35 mm dishes for 48 h were loaded with 5 μM 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; $MgSO_4.7H_2O$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 6.0; $CaCl_2$, 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 Fluar 40× oil-immersion objective. A Dual-wavelength Fluorescence Imaging System (Photon Technology International, Birmingham, NJ) was used to measure changes in $[Ca^{2+}]_i$ following stimulation of cells with Ca^{2+} 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 HQ^2 cameras. The images were transmitted to a computer and processed using the ImageMaster Pro™ or EasyRatioPro™ Ratio Fluorescence Imaging software, with a macro