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MEMBRANE DEPOLARIZATION COMBINED WITH Gq-ACTIVATED G-PROTEIN-COUPLED RECEPTORS INDUCE TRANSIENT RECEPTOR POTENTIAL CHANNEL 1 (TRPC1)- DEPENDENT POTENTIATION OF CATECHOLAMINE RELEASE

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Abstract—Agonists of the $G_{q/11}$-activated G-protein-coupled receptors (GPCRs) combined with strong membrane depolarization (high KCl) induce a synergistic amplification of transmitter release. The molecular basis for the synergy is unknown. Here, we investigated this potentiated transmitter release (PTR) phenomenon at the single cell level by monitoring catecholamine (CA) release in chromaffin cells using amperometry. We found that the 60 mM KCl (K60)-triggered release in bovine chromaffin cells synergizes with bradykinin (BK) or histamine (Hist) to potentiate CA release. PTR was independent of Ca^{2+} influx through voltage-gated calcium channels (VGCC), but required Ca^{2+} at the extracellular medium and was abolished by inhibitors of phospholipase Cβ (PLCβ). The $\sim$ four-fold PTR induced in mouse chromaffin cells by BK and K60, was not observed in chromaffin cells prepared from TRPC1 KO mice and was restored by expressing hTRPC1. The synergy between strong voltage perturbation (K60) in the presence of VGCC blockers, and the activation of the $G_{q/11}$-PLCβ signal-transduction cascade generates unique and fundamental amplified signaling machinery. The concerted activation of two independent cellular pathways could reinforce physiological signals that impinge on regulation of secretory events during repeated sequence of high-frequency excitation, hyperpolarization, and relief of inhibition such as long-term potentiation (LTP), that lead to neuronal synaptic plasticity.

Key words: Ca^{2+} channel, TRPC channel, Gq/11 GPCR, PLCβ, depolarization-evoked secretion, LTP.

Secretion of neurotransmitters from presynaptic cells is triggered by membrane depolarization during the opening of voltage-gated calcium channels (Katz and Miledi, 1967). Secretion is also induced by the activation of G-protein coupled receptors (GPCRs) (e.g. muscarinic, bradykinin, serotonin, glutamate) acting through the $G_{q/11}$-phospholipase Cβ (PLCβ) pathway (Jhamandas and Marien, 1987; Diamant et al., 1988; Schwartz and Alford, 2000; Weiss and Atlas, 1991; Dunn and Rang, 1990; Schwartz et al., 1991).

The induction of amplified signals by membrane depolarization using high KCl (K60) combined with muscarinic $G_{q/11}$PCR was initially revealed in cortical brain slices (Diamant and Atlas, 1989). In these studies the mutual activation of the GPCR-$G_{q/11}$-PLCβ pathways and K60 enhanced phosphatidyl inositol (PI) turnover more than fivefold (Diamant and Atlas, 1989) and facilitated evoked-secretion more than fourfold (Schwartz and Atlas, 1989). These events were proposed to contribute to synaptic plasticity (Diamant et al., 1990).

In addition, there are ample examples that support the existence of a synergy between membrane depolarization and activation of $G_{q/11}$-coupled GPCRs (Ganitkevich and Isenberg, 1993, 1996; Seyedi et al., 1997; Nakamura et al., 1999; Rae et al., 2000; Martinez-Pinna et al., 2005; Pitt et al., 2005; Hashimotodani et al., 2005; Bauer et al., 2007), yet the molecular basis for the observed synergies remains open.

Voltage perturbation combined with activation of a muscarinic receptor agonist of the $G_{q/11}$GPCR pathway, potentiated Ca^{2+} release from intracellular stores in human neuroblastoma SH-SY5Y cells, and in rat cerebellar granule neurons (Nash et al., 2004; Billups et al., 2006). The concerted action that led to the potentiated Ca^{2+} transient was independent of Ca^{2+} influx through voltage-gated calcium channels (VGCC) (Billups et al., 2006; Pitt et al., 2005; Mahaut-Smith et al., 2008).

The aim of the present study was to gain an insight into the molecular basis underlying the mechanism by which the activation of the $G_{q/11}$PCR-PLCβ signaling pathway in a depolarized membrane (K60) amplifies cellular signals. To that end, we investigated catecholamine (CA) release induced by the concerted action of K60 and $G_{q/11}$PCR activation, using bradykinin (BK) or histamine (Hist). Secretion was monitored from single chromaffin cells by amperometry recording, using carbon fiber electrodes. Potentiated transmitter release (PTR) was observed in bovine and wild-type mice chromaffin cells, but not in chromaffin cells prepared from TRPC1$^{-/-}$ mice. It was independent of Ca^{2+} influx through VGCC, and was inhibited by PLCβ inhibitors. We propose that $G_{q/11}$PCR activation during voltage-perturbation of the membrane (e.g. K60; voltage step) represents a novel-signaling pathway, responsible

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Abbreviations: BK, bradykinin; CA, catecholamine; GPCRs, G-protein-coupled receptors; Hist, histamine; IP_3, inositol trisphosphate; K60, 60 mM KCl; LTP, long-term potentiation; PI, phosphatidyl inositol; PLCβ, phospholipase Cβ; PTR, potentiated transmitter release; TMC, total mean charge; TRPC1, transient receptor potential channel 1; VGCC, voltage-gated calcium channels.

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for long-term potentiated synaptic signals. It may underlie the molecular mechanism of amplified cellular responses that modulate several forms of transmission efficacy including synaptic plasticity.

EXPERIMENTAL PROCEDURES

Materials

All materials were purchased from Sigma, Jerusalem, if not otherwise stated. Bradykinin; Histamine; TRI reagent; Papain; Collagenase F; Nifedipine; phospholipase C inhibitor 1-[6-([17β-methoxyestra-1,3,5(10)-trien-17-yl]amino)hexyl]-1*H*-pyrrole-2,5-dione (U73122); 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrollidinedione (U73343), a structurally related but not PLC-inhibiting analog; Ca^{2+} ionophore (A23187); 2-APB (2-aminoethoxydiphenyl borate). Tissue culture serum and medium were from Biological Industries, Kibbutz Beit Haemek, Israel. PC12 cells were a provided by P. Lazarovici.

Bovine chromaffin cell preparation and culture

Bovine adrenal glands were obtained at a local slaughterhouse. The adrenal medulla cells were isolated as described previously (Lerner et al., 2006), plated at a density of 5×10^4 cells/cm^2 on glass cover slips placed in 35 mm plates, and cultured in DMEM (Gibco) supplemented with ITS-X and PenStrep (Sigma, Jerusalem, Israel). Cells were incubated at 37 °C in 5% CO_2 and used for amperometric recordings 2–4 days after preparation at 23 °C.

Mouse chromaffin cell preparation and culture

Chromaffin cells isolated from the adrenal medullae of adult C57BL/4 male mice (4–8 weeks of age) were used in this study. The adrenal medulla cells were isolated as described by (Fulop et al., 2005). Briefly adrenal glands were removed and were placed in an ice-cold dissociation solution that contained (in mM) 80 Na glutamate, 55 NaCl, 6 KCl, 1 $MgCl_2$, 10 HEPES, and 10 glucose, pH 7.0. Glands were trimmed of fat, and the adrenal cortex was dissected from the medullae. The medullae were then incubated in digest solution for 10 min at 37 °C (solution 1: 30 U/ml papain, 1 mM DTT, and 0.5 mg/ml BSA added to 1 ml of dissociation solution). The tissues were transferred into a second digest solution for another 10 min incubation at 37 °C (solution 2: 3 U/ml collagenase F, 0.5 mg/ml BSA, and 100 μM $CaCl_2$ added to 1 ml of dissociation solution). After the second incubation, cells were transferred into a DMEM (Gibco) supplemented with ITS-X and PenStrep (Sigma, Jerusalem, Israel), triturated and plated. Cells were incubated at 37 °C in 5% CO_2 and used for amperometric recordings 1 day after preparation.

Amperometric recordings of catecholamine release from chromaffin cell

Amperometry recordings were carried out using 5 μm thin carbon fiber electrodes (CFE ALA Incorp. Westbury, NY, USA) and a VA-10 amplifier (NPI-electronic, Tamm, Germany) held at 800 mV as described previously (Chow et al., 1992). Cells were rinsed three to four times prior to the experiment and bathed during the recordings in iso-osmotic physiological Ca^{2+} solution (149 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Glucose, 10 mM HEPES, pH 7.3 at ~23 °C (adjusted with NaOH)); La^{3+}solution (all the same as Ca^{2+} solution, except 0.2 mM La^{3+} substituted for 2 mM Ca^{2+}). La^{3+} mediated secretion in chromaffin cells is optimal at a concentration in the range of 0.1–0.2 mM of this trivalent ion (Lerner et al., 2006). Nifedipine (Nif) was used at 5 μM, in DMSO in a final concentration of 0.001%.

Prior to recording, spontaneous release during 30 s stimulation period was monitored for each cell and then recoding was initiated. Ten seconds after starting recording, individual cells were stimulated to release by a 30 s application of different buffers-iso-osmotic 60 mM KCl (K60) or by 0.1 μM BK, or by 50 μM Hist, from a ~3-μm tipped micropipette placed 30 μm from the cell in the bath. Amperometric currents were sampled at 10 kHz, using Clampex 9.2 (Axon Instruments) and low pass filtered at 0.2 kHz.

Amperometric data acquisition and analysis

Amperometry records were analyzed with IGOR PRO (Wavemetrics, Lake Oswego, OR, USA). Data were analyzed as described in the text and figure legends. Error bars depict standard errors. Spikes exceeding three times the background noise (>10 pA) were analyzed.

The overall time course of secretion was determined from normalized waiting time distributions constructed by spike counting. In all measurements baseline currents were subtracted. The efficacy of catecholamine release following the different stimuli is presented by two parameters: the averaged spikes number and the total mean charge (TMC). TMC was calculated as the sum of mean charge during 90 s and then averaged (Marom et al., 2007). Spikes/cell was calculated during 90 s for individual cells and then averaged (Archer et al., 2002).

Total RNA isolation and RT-PCR

Total RNA was extracted from PC12 cells, bovine chromaffin cells, or adrenal medulla of adult C57BL/4 male mice (4–8 weeks of age) by TRI REAGENT™ (Sigma) (guanidinium thiocyanate/phenol/chloroform) according to the manufacturer's protocol. For cDNA synthesis, 1 μg of total RNA was reverse transcribed according to the protocol provided by the manufacturer using 200 U of SupercriptTM II reverse transcriptase (Invitrogen, Carlsbad, CA, USA), oligo(dT).

One-tenth of the resulting single strand cDNA (Syntezza Bioscience Ltd, Jerusalem, Israel) or 0.5 μg of cDNA encoded for mTRPC5 as a positive control (kindly provided by Dr. Zhu, Ohio State University, USA) were used for PCR in the presence of 2 μM primers specific for each bovine transient receptor potential channel (TRPC) sequence. The following primers were used: bTRPC1 (GenBank accession number AF012900): 5′-GACAGATGTCAGGTTACC-3′ and 5′-ATTCTTTCAAGGGCTGGC-3′; bTRPC4 (GenBank accession number X99792): 5′-TGGCGTCTCGCTGGTAC-3′ and 5′-AGGACCCACGGTAATATC-3′); bTRPC5 (GenBank accession number AJ271070): 5′-GTACTGCTGGCTTTTGCC-3′ and 5′-TTCAGCAGCACTACCAGG-3′ (Philipp et al., 2000); mTRPC1 (GenBank accession number NM_011643): 5′-GCAACCTTTGCCCTCAAAGTG-3′ and 5′-GGAGGAACATTCCCAGAAATTTCC-3′ (Liu et al., 2007); mTRPC4 (GenBank accession number NM_016984): 5′- TCTGCAGATATCTCTGGGAAGGATG-3′ and 5′ AAGCTTTGTTCGAGCAAATTTCCATTC-3′ (Ohta et al., 2004). The TRPC1/4/5 were amplified by 35 cycles of PCR using an annealing temperature 61° for 30 s then extension for 90 s at 68° with Bio-X-Act polymerase (Bioline).

pSFV virus preparation and infection

Recombinant Semliki Forest virus (SFV) particles were generated as described previously (Ashery et al., 1993). hTRPC1 (kindly provided by Prof. Hamill) was used as a template for the construction of pSFV-hTRPC1. The following primers were used: 5′- AAAAATGATCAATGATGGCGGCCCTGTACCC-3′ and 5′- AAAAAGCGCGCTTAATTTCTTGGATAAAACA-3′ for amplification and insertion of BclI and PauI restriction sites.

Briefly, *in vitro* transcribed RNA from pSFV-hTRPC1 expressing GFP through an internal ribosome entry site (IRES) motif was co-electroporated into BHK-21 cells with pSFV-Helper 2 RNA. Virus stocks were harvested 24 h later and activated with α-chy-