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PAC1hop receptor activation facilitates catecholamine secretion selectively through 2-APB-sensitive Ca^{2+} channels in PC12 cells

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ABSTRACT

PACAP is a critical regulator of long-term catecholamine secretion from the adrenal medulla *in vivo*, however the receptor or pathways for Ca^{2+} entry triggering acute and sustained secretion have not been adequately characterized. We have previously cloned the bovine adrenal chromaffin cell PAC1 receptor that contains the molecular determinants required for PACAP-induced Ca^{2+} elevation and is responsible for imparting extracellular Ca^{2+} influx-dependent secretory competence in PC12 cells. Here, we use this cell model to gain mechanistic insights into PAC1hop-dependent Ca^{2+} pathways responsible for catecholamine secretion. PACAP-modulated extracellular Ca^{2+} entry in PC12 cells could be partially blocked with nimodipine, an inhibitor of L-type VGCCs and partially blocked by 2-APB, an inhibitor and modulator of various transient receptor potential (TRP) channels. Despite the co-existence of these two modes of Ca^{2+} entry, sustained catecholamine secretion in PC12 cells was exclusively modulated by 2-APB-sensitive Ca^{2+} channels. While IP3 generation occurred after PACAP exposure, most PACAP-induced Ca^{2+} mobilization involved release from ryanodine-gated cytosolic stores. 2-APB-sensitive Ca^{2+} influx, and subsequent catecholamine secretion was however not functionally related to intracellular Ca^{2+} mobilization and store depletion. The reconstituted PAC1hop-expessing PC12 cell model therefore recapitulates both PACAP-induced Ca^{2+} release from ER stores and extracellular Ca^{2+} entry that restores PACAP-induced secretory competence in neuroendocrine cells. We demonstrate here that although bPAC1hop receptor occupancy induces Ca^{2+} entry through two independent sources, VGCCs and 2-APB-sensitive channels, only the latter contributes importantly to sustained vesicular catecholamine release that is a fundamental characteristic of this neuropeptide system. These results emphasize the importance of establishing functional linkages between Ca^{2+} signaling pathways initiated by pleotrophic signaling molecules such as PACAP, and physiologically important downstream events, such as secretion, triggered by them.

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1. Introduction

Since the discovery and cloning of the six major splice variants of the pituitary adenylate cyclase activating polypeptide (PACAP) type-1 receptors (PAC1) [1,2] significant progress has been made in understanding the signal transduction mechanisms of these G-protein coupled receptors (GPCRs) in relation to Ca^{2+} signaling. Elucidating the receptor-specific mechanisms of PACAPs action are of particular importance because PACAP participates in both acute and sustained effects at various synapses, and these are likely to have differential effects on regulation of homeostasis *in vivo*. Therefore acute catecholamine release from chromaffin cells *in vivo* [3–5] and in culture evoked by either acetylcholine or PACAP occurs within a few seconds–minutes, via a mechanism that requires Ca^{2+} influx through voltage-dependent Ca^{2+} channels (VGCCS) [6–11]. This mechanism most likely underlies acute responses, such as the flight-or-fight reflex mediated at the adrenomedullary synapse from the so-called primed readily releasable pool (RRP) of vesicles in chromaffin cells [12–14]. A second and longer phase of PACAP-dependent secretion occurs within several minutes to hours, and may involve secretion from a second, RRP-independent releasable pool of vesicles in chromaffin cells [14] that is mechanistically controlled though voltage-independent calcium channels [9]. This second, sustained phase of

Abbreviations: AC, Adenylate cyclase; BCCs, Bovine chromaffin cells; Ca^{2+}, Calcium; $[Ca^{2+}]_i$, Cytosolic calcium concentration; cAMP, Cyclic adenosine monophosphate; DMEM, Dulbecco's Modification of Eagle's Medium; ER, Endoplasmic reticulum; GPCR, G-protein coupled receptor; IC3, Third intracellular loop; ICS, Intracellular stores; InsP3, Inositol triphosphate; KRB, Krebs-Ringer buffer; LDCVs, Large-dense core vesicles; Na^{+}, Sodium; PACAP, Pituitary adenylate cyclase activating polypeptide; PAC1, PACAP-preferring type-1 receptor; PC12, Rat pheochromocytoma; PCR, Polymerase chain reaction; PKA, Protein kinase A; PKC, Protein kinase C; PLC, Phospholipase C; RT-PCR, Reverse transcriptase PCR; SOCE, Store-operated calcium entry; SOCC, Store-operated calcium channel; TRP, Transient receptor potential; VGCCs, Voltage-gated calcium channels.

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catecholamine secretion is of great importance physiologically, as it is this phase of secretion that is presumably responsible for survival during prolonged hypoglycemia [15] and for the long-term, PACAP-dependent adrenomedullary catecholamine response to psychogenic stressors [16].

In addition to PACAP-evoked extracellular Ca^{2+} influx, PACAP modulates Ca^{2+} release from intracellular endoplasmic reticulum (ER) stores [1,6,7,15]. Early characterization studies of the PAC1 receptor variants potentially responsible for evoking PACAP-dependent Ca^{2+} signaling suggested that the PAC1hop and PAC1null, but not the PAC1hip variant of this receptor, were coupled to both adenylate cyclase activation through Gs, and activation of phospholipase C presumably through Gq. This assumption was based on the ability of PACAP to increase total inositol phosphate accumulation in heterelogous cell lines expressing the receptor variants, which in turn predicted regulation of Ca^{2+} release from $InsP_3R$-sensitive ER stores by PACAP [2]. Tanaka and colleagues additionally revealed using real-time fluorometric monitoring of cytosolic Ca^{2+}, the ability of PACAP to regulate Ca^{2+} release mainly from ryanodine receptor-gated intracellular Ca^{2+} stores [7] despite simultaneous generation of inositol phosphates in adrenal chromaffin cells [17].

The complexity of PACAP-mediated Ca^{2+} signaling has been an obstacle to understanding how PACAP-mediated Ca^{2+} entry and subsequent stimulation of secretion through its various receptors actually occurs *in vivo*. Despite earlier reports of a specific PAC1 isoform mediating calcium influx distinct from the PAC1hop isoform found in chromaffin cells [18], we have since demonstrated that the PAC1hop isoform alone can support Ca^{2+} influx when transfected into a PC12 cell line that is otherwise not competent for sustained PACAP secretion [11].

In this study we employed this cell line, PC12+bPAC1hop, to study reconstituted PACAP-mediated intracellular Ca^{2+} mobilization and extracellular Ca^{2+} influx to study the functional links among these various modes of intracellular calcium elevation, and their role in mediating sustained catecholamine secretion. We demonstrate for the first time the ability of PACAP to trigger extracellular Ca^{2+} influx through 2-APB-sensitive, Ca^{2+} channels, and that this mode of calcium entry is responsible for sustained catecholamine secretion mediated through the PAC1hop receptor, the major form of PAC1 expressed in chromaffin cells, sympathetic neurons, and in the central nervous system.

2. Materials and methods

2.1. Reagents

PACAP-38 was purchased from Phoenix Pharmaceuticals (Mountain View, CA). 2-aminoethoxydiphenyl borate (2-APB), U73122, U73343, ryanodine and ET-18-OCH_3 (Edelfosine), were obtained from Calbiochem-EMD Biosciences. ω-Conotoxin MVIIC, ω-Conotoxin GVIA, nimodipine, mibefradil dihydrochloride hydrate, ATP and cinnarizine were purchased from Sigma-Aldrich. All tissue culture reagents were obtained from Invitrogen unless specified otherwise.

2.2. Cell culture

PC12-G rat pheochromocytoma cells [19] were cultured in high glucose DMEM supplemented with 7.5% heat-inactivated fetal calf serum (Hyclone, UT), 7.5% horse serum (Bio-Whittaker-Cambrex, MD), 25 mM HEPES, 100 U/ml penicillin-streptomycin and 2 mM glutamine. PC12-G cells stably expressing the bPAC1hop receptor (PC12 + bPAC1hop) were created as previously described [11]. Briefly, lipofectamine2000™ (Invitrogen, CA) was used to transfect PC12-G cells with the bovine PAC1 receptor (bPAC1hop) and stably transfected cells were selected and maintained in media supplemented with 500 μg/ml G418. All cells were used between passages 6 and 25.

2.3. Measurement of single-cell $[Ca^{2+}]_i$

Measurement of $[Ca^{2+}]_i$ in PC12 cells was performed by monitoring fura-2 fluorescence as previously described [11]. Briefly, 400,000 PC12-G or PC12 + bPAC1hop cells were seeded onto 1.5 cm diameter glass coverslip slides (Assistent, Germany) coated with 0.5 mg/ml poly-L-lysine 24 h prior to imaging. Fura-2 loading was carried out by incubating cells with 4 μM Fura-2-AM (Invitrogen, Molecular Probes, OR) in Krebs-Ringer buffer (KRB) containing (in mM) 125 NaCl, 5 KCl, 1 Na_2HPO_4, 1 $MgSO_4$, 1 $CaCl_2$, 5.5 glucose and 20 HEPES, pH 7.3 for 22 min at room temperature followed by a further 22 min wash in Fura-2-free KRB prior to imaging. Cells on coverslips mounted onto a custom built perfusion chamber were alternatively excited at 340 nm and 380 nm, and emitted light was collected at 510 nm every 2 s. The ratiometric fluorescence of Fura-2 served as a measure of $[Ca^{2+}]_i$ as previously described [11]. All experiments were conducted following a standard experimental paradigm, as follows. Cells were perfused with KRB for 60 s to establish a stable baseline, and then with KRB containing PACAP or other secretagogues at the specified concentrations followed by a final 60 s KRB wash unless specified otherwise. Pharmacological inhibitors were administered 30 min prior to secretagogue treatment and included in all subsequent washes and treatments at the specified concentrations. Ca^{2+}-free experiments were conducted utilizing Ca^{2+} free KRB buffer supplemented with 100 μM EGTA. The collected data were obtained as averages from 3 to 15 experiments, each comprising 2–3 slides from which approximately 30 cells were selected for individual $[Ca^{2+}]_i$ measurements.

2.4. Total inositol phosphate measurements

Labeling and detection of total inositol phosphates in PC12 cells was based on a previously described procedure [20] with the following modifications. Briefly PC12-G cells or PC12 + bPAC1hop cells were plated at a density of 180,000 cells/well in 0.1 mg/ml poly-L-lysine-coated 24 well plates and allowed to adhere overnight. Inositol phospholipids were labeled by incubating cells in the presence of 1 μCi myo-[^{3}H]-inositol (30 Ci/mmol) (Amersham) in complete culture media for 24 h. Unincorporated radioactivity was then removed by washing cells with KRB containing 0.1% BSA supplemented with 20 mM $LiCl_2$, to prevent metabolism of newly formed inositols, then pre-incubated for an additional 30 min at 37 °C in the same buffer prior to agonist stimuation for 60 min at 37 °C. Treatments were terminated and cells lysed by incubation in ice-cold KRB containing 6% perchloric acid and 20 mM $LiCl_2$ for 60 min under constant agitation at 4 °C. The cell lysates were then neutralized with a pre-determined volume of 1 N KOH and 20 mM HEPES and the ^{3}H-labeled inositols in the aqueous phase were extracted with an equal volume of pre-prepared Dowex AG 1X1-8 (100–200 formate form) resin (Bio-Rad). Total inositol phosphates eluted from the resin with 1.2 M ammonium formate and 0.1 M formic acid solution were then counted in a liquid scintillation counter. Values are the ± S.E.M of triplicate wells performed over at least 3 independent experiments. The statistical difference between control and treatment groups was determined using *T*-test using the GRAPHPAD PRISM program (GraphPad Software 4.0).

2.5. [^{3}H]-Norepinephrine uptake and release studies

The uptake and release of [^{3}H]-norepinephrine in PC12-G or PC12 + PAC1hop cells was carried out as previously described [11]. Cells loaded with 1 μCi/well of Levo-[7-^{3}H]-norepinephrine (1 mCi/ml, Perkin Elmer, USA) for 4 h in complete media at 37 °C were washed with PBS and pre-incubated for 30 min at 37 °C in the presence or absence of pharmacological inhibitors or vehicle in KRB prior to addition of 100 nM PACAP-38 or 55 mM KCl. Following stimulation for 30 min at 37 °C the level of radioactivity in secretion buffer and