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Regulation of exocytosis in chromaffin cells by phosducin-like protein, a protein interacting with G protein βγ subunits

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Abstract Phosducin and related proteins have been identified as ubiquitous regulators of signalling mediated by βγ subunits of trimeric G proteins. To explore a role for phosducin in regulated exocytosis, we have examined the distribution and putative function of phosducin-like protein (PhLP) in adrenal medullary chromaffin cells. The full-length cDNA encoding the short splice variant of PhLP (PhLPs) was cloned from cultured chromaffin cells. Native PhLPs was found associated with plasma membranes and detected in the subplasmalemmal area of resting chromaffin cells by confocal immunofluorescence analysis. Stimulation with secretagogues triggered a massive redistribution of PhLPs into the cytoplasm. When microinjected into individual chromaffin cells, recombinant PhLPs inhibited catecholamine secretion evoked by a depolarizing concentration of K^+ without affecting calcium mobilization. Thus, PhLPs may participate directly in the regulation of calcium-evoked exocytosis.

Key words: Chromaffin; Exocytosis; G protein; Phosducin-like protein

1. Introduction

In addition to their well known role in signal transduction, heterotrimeric G proteins participate in the regulation of intracellular membrane trafficking events (reviewed in [1,2]), including direct control of calcium-evoked exocytosis [3–6]. Although Gα subunits clearly regulate certain aspects of exocytosis [5–8], the Gβγ complex has also been implicated [9–11]. Indeed, Gβγ can interact directly with a variety of effectors displaying a pleckstrin homology (PH) domain [12–15], including phosphatidylinositol 3-kinase [16] involved in the exocytotic process in chromaffin cells [17]. In addition, we recently described that Gβγ complexes mediate the binding of the ADP-ribosylation factor 6 to secretory granule membranes [18], and this may be crucial to the late stages of exocytosis [19,20].

The responsiveness of G protein-regulated pathways may be directly modulated through interactions of Gβγ subunits with intracellular regulatory proteins. Phosducin and related proteins constitute a family of ubiquitous Gβγ regulators. Phosducin (Phd) and a long splice variant of Phd named phosducin-like protein 1, as well as two splice variants from a second gene, PhLPs (short variant) and PhLPl (long variant), directly interact with Gβγ [21] and thereby inhibit the GTPase activity and signaling function of various trimeric G proteins [22,23]. Phosducin-like proteins also impair Gβγ-mediated functions by competing with several Gβγ effectors [24,25]. Phosphorylation of Phd dissociates it from Gβγ, allowing Gα to rapidly reassociate with Gβγ [22,26,27]. Thus, Phd modulation of Gβγ signaling itself may be regulated by a feedback loop involving phosphorylation. The mechanism of interaction of Phd and Gβγ has been clarified by the recent crystal structure of the Phd–Gβγ complex [26,28], in combination with mutational and biophysical analysis [29,30]. The N- and C-terminal ends of Phd appear to envelop two faces of the βγ propeller to form an extensive interaction surface. Although they have not been crystallized yet, PhLP proteins which present 65% amino acid homology with Phd are presumed to have a similar three-dimensional structure [31].

Phd is expressed at high levels in retinal rod cells [32] where it plays an important role in light adaptation [33]. The precise cellular functions of PhLPs and PhLPl remain unknown. Their different tissue distribution suggests that PhLP proteins represent functional counterparts of Phd in the regulation of trimeric G proteins [21,32,34]. Here, we report cloning of PhLPs from cultured bovine chromaffin cells. Microinjection of recombinant PhLPs inhibited secretion from single chromaffin cells at a stage distal from calcium entry. Our findings support the hypothesis that PhLPs may participate in the regulation of calcium-dependent exocytosis from neuroendocrine cells.

2. Materials and methods

2.1. RNA isolation, cDNA amplification and sequencing

Total RNA was isolated from cultured chromaffin cells (1.3×10^6 cells/assay). 1 μg of total RNA was transcribed into cDNA with Superscript RNase H^- reverse transcriptase (Gibco-BRL, France) and an oligo(dT) primer. 2-μl aliquots of the cDNA were used as template for PCR (total volume of 50 μl) using Taq polymerase (MBI Fermentas) and specific primers. Expression of bovine PhLP cDNA was tested with primers 5′-CATATGACAAGTTGCTGGGGGAGAAATTG-3′ and 5′-GGATCCTCAATCTATTTCTAGATCGCTGTCTT-3′ for the long variant of PhLP and with primers 5′-CATATGGAGCGGCTGATCAAAAAGCTGTC-3′ and 5′-GGATCCTCAATCTATTTCTAGATCGCTGTCTT-3′ for the short variant of PhLP [35]. The PCR products were purified and directly ligated into the TA cloning vector (pMOSBlue T-vector Kit, Amersham). DNA sequence analysis was done on both strands. The bovine PhLPs sequence reported in this paper has been deposited in the EMBL database (accession number AJ277994) and GenBank database (accession number AF242854).

2.2. Expression and purification of recombinant 6His-PhLPs

PhLPs cDNA was inserted into the pET14b vector (Novagen) using the *Nde*I and *Bam*HI sites. Large-scale production of 6His-PhLPs was

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performed as described previously [36]. Fusion proteins, purified on a nickel affinity column (Novagen), were ~90% pure as estimated by Coomassie staining after SDS–PAGE. Recombinant 6His-PhLPs was dialyzed against 10 mM PIPES, 50 mM K-glutamate.

2.3. Culture of chromaffin cells and subcellular fractionation

Chromaffin cells were isolated from fresh bovine adrenal glands and maintained in primary culture as described previously [6]. Experiments were carried out on 3–7-day-old cultures. Cultured chromaffin cells were collected in buffer A (0.32 M sucrose, 10 mM Tris, pH 7.4), homogenized and then centrifuged at $800\times g$ for 15 min. The post-nuclear supernatant (H1) was subsequently centrifuged at $20\,000\times g$ for 20 min. The supernatant (S1) was used to purify microsomes (P2) from cytosol (S2) after 45 min centrifugation at $100\,000\times g$. The pellet (P1) containing crude membranes was resuspended in buffer A, layered on a continuous sucrose density gradient (sucrose 1–2.2 M), and centrifuged for 90 min at $100\,000\times g$. Twelve 1-ml fractions were collected from top to bottom. The distribution of dopamine-β-hydroxylase (DβH, chromaffin granule marker) and Na^+/K^+ ATPase (plasma membrane marker) in the fractions of the gradient was estimated as described previously [8].

2.4. Microinjection, $[Ca^{2+}]_i$ measurement and electrochemical detection of catecholamine secreted from single cells

Microinjection of PhLPs in 10 mM PIPES, 50 mM K-glutamate, pH 7.4 or buffer alone was performed as described earlier [37]. The calculated injected volume represented 50–100 fl. Single cell $[Ca^{2+}]_i$ assay was performed using the fluorescent Ca^{2+} indicator Indo 1-AM (Molecular Probes) [38]. Electrochemical measurements of catecholamine secretion were performed 30 min after microinjection [37]. Catecholamine secretion was evoked by applying K^+ (100 mM) in Locke's solution without ascorbic acid for 5 s to single cells by means of a glass micropipette positioned at a distance of 100 μm from the cell. The amplitude of secretion was quantified by measuring the area below the current curve using the MacLab system.

2.5. Antibodies and immunocytochemistry

Polyclonal anti-PhLPs antibodies were raised in rabbits against recombinant 6His-PhLPs and affinity-purified as described [39]. Rat polyclonal antibodies against DβH were prepared in our laboratory; their specificity has been previously demonstrated [37]. Secondary antibodies (goat anti-rat conjugated to Cy2 and goat anti-rabbit conjugated to Cy3) were purchased from Amersham (Les Ulis, France). Immunocytochemistry was performed as described previously [37]. Stained cells were monitored using the Zeiss laser scanning microscope (LSM 410 invert) with an oil immersion lens (63×).

2.6. GTPase activity of purified granule membranes

The GTPase activity was assayed by incubating plasma (0.5 μg) or secretory granule (1 μg) membranes for 10 min at 30°C with the indicated concentrations of PhLPs as described earlier [8]. Non-enzymatic hydrolysis of [γ-^{32}P]GTP during the assay was subtracted from all data.

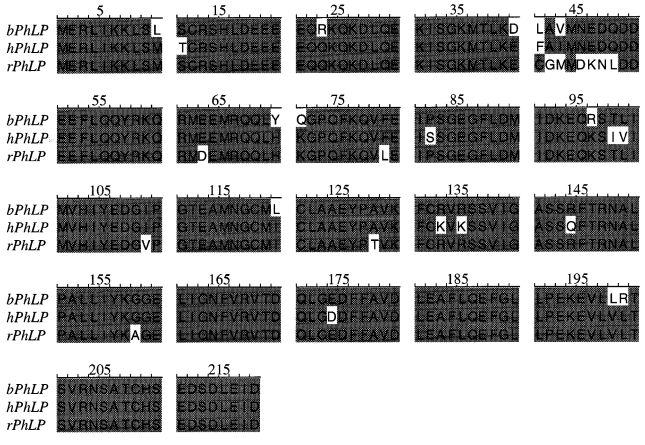

Fig. 1. Comparison of amino acid sequences of PhLPs from bovine, human and rat. The deduced amino acid sequences from human [45] and rat [35] PhLPs were aligned with bovine PhLPs using SeqApp. Identical amino acids are highlighted.

3. Results and discussion

3.1. Presence of PhLPs in the chromaffin cell plasma membrane

We first examined the expression of PhLP in chromaffin cells by RT-PCR using specific primers for PhLPs and PhLPl. A single band of 660 bp was detected with the primers for PhLPs. No band for PhLPl was found in three separate experiments performed with two distinct cDNA preparations. The 660-bp PCR product obtained from chromaffin cells was cloned and sequenced; it presented 86% analogy at the nucleotide level to the rat PhLPs sequence [35]. These results suggest that only the PhLPs mRNA (and not PhLPl) is expressed in chromaffin cells. The putative open reading frame

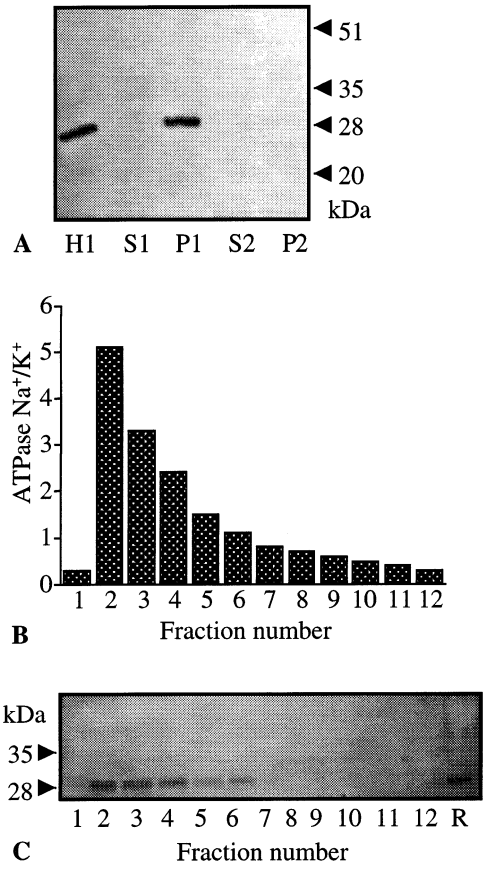

Fig. 2. Immunochemical detection of PhLPs in subcellular fractions from chromaffin cells. A: Cultured chromaffin cells were collected, homogenized and processed for subcellular fractionation. Protein (100 μg) from the total homogenate (H1), the crude membrane pellet (P1), cytosol (S2) and microsomes (P2) was subjected to SDS gel electrophoresis and immunodetection on nitrocellulose using anti-PhLPs antibodies (diluted 1:100). Note the absence of a 45-kDa band corresponding to PhLPl. Position of protein standards (kDa) is on the right. B: Twelve fractions collected from a continuous sucrose density gradient layered with the crude membrane pellet (P1) were assayed for Na^+/K^+-ATPase activity (expressed as arbitrary units). C: Proteins (50 μg) from each fraction were separated by SDS gel electrophoresis, transferred and analyzed with anti-PhLPs antibodies (diluted 1:100). Recombinant PhLPs (250 ng) was used as positive control (R). Position of protein standard (kDa) is on the left. Similar results were obtained in two independent experiments.

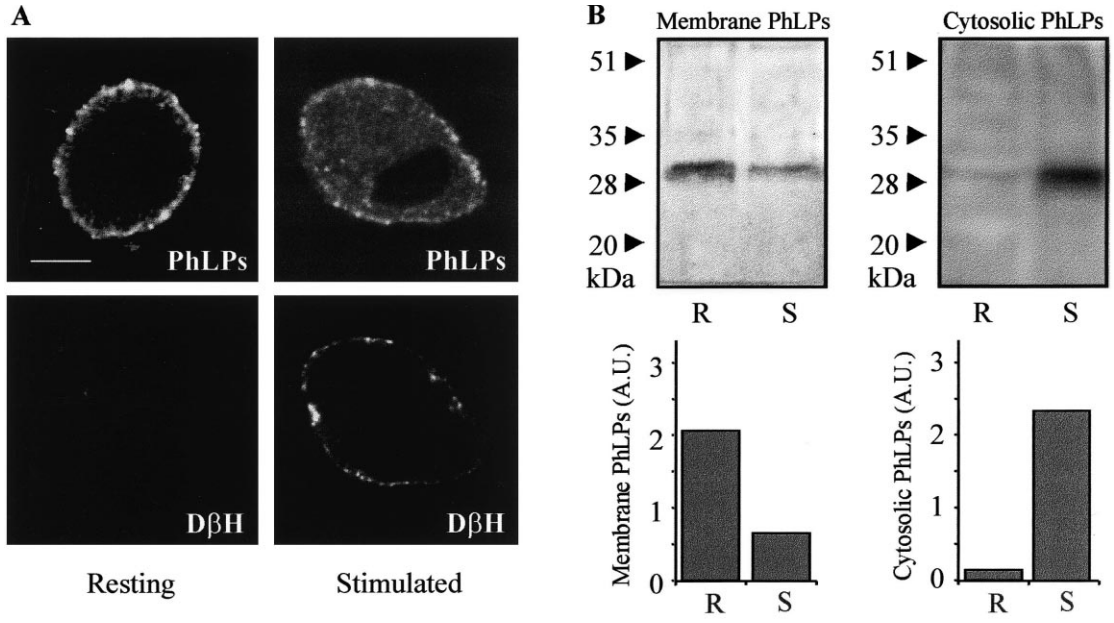

Fig. 3. PhLPs translocates from the cell periphery to the cytosol upon cell stimulation. A: Chromaffin cells were incubated for 10 min in Locke's solution (Resting) or in Locke's solution containing 10 μM nicotine (Stimulated) in the presence of anti-DβH antibodies (diluted 1:50) to visualize exocytotic activity (DβH). Cells were then fixed, washed and stained with affinity-purified anti-PhLPs antibodies diluted 1:5 (PhLPs). DβH immunoreactivity was visualized with anti-rat antibodies conjugated to Cy2 (diluted 1:2000) and PhLPs was revealed with anti-rabbit antibodies conjugated to Cy3 (diluted 1:2000). Note that DβH exocytotic patches were only observed in stimulated cells, which also display a marked increase of cytosolic PhLPs immunoreactivity. Similar observations were made with three different cell preparations. Bar = 5 μm. B: Cultured chromaffin cells (10^6 cells) were incubated for 10 min in Locke's solution (R) or stimulated with 10 μM nicotine (S). The incubation media were collected and secretion of endogenous catecholamine was verified by reverse phase high performance liquid chromatography. Cells were then scraped in 800 μl phosphate buffer, broken by three freeze and thaw cycles, and centrifuged at $100\,000 \times g$ for 60 min. Membrane-bound (pellet) and cytosolic (supernatant) proteins were separated by gel electrophoresis and analyzed for the content of PhLPs by immunodetection on nitrocellulose. The density of the immunoreactive PhLPs band was quantified using the Fuji β phosphorimager and expressed as arbitrary unit (A.U.). Similar results were obtained with two distinct cell preparations.

of the bovine PhLPs consists of 654 nucleotides encoding a protein of 218 amino acids. The deduced amino sequence of the bovine PhLPs is strikingly similar to human and rat sequences with 91% and 90% identity and with 96% and 95% homology, respectively (Fig. 1). Bovine PhLPs contains potential phosphorylation sites for protein kinase A at Ser^9 and for protein kinase C at Thr^{37}; the latter is equivalent to Ser^{73} in Phd. The major difference between bovine and rat PhLPs is localized between amino acids 40 and 48 (Fig. 1). Although this region is unlikely to affect the overall structure of bovine PhLPs because it makes a loop between the Gβ-interacting H2 and H3 helices, it could be important for interaction with effectors. Interestingly, the carboxy-terminal region, which is known to contain contact sites between PhLP and Gβ, is highly conserved.

Expression of PhLP in chromaffin cells was assessed by Western blotting using a polyclonal antibody raised against the recombinant bovine PhLPs. A single immunoreactive band of 29 kDa was detected in homogenates prepared from cultured chromaffin cells (Fig. 2A), confirming that PhLPs was indeed expressed, but not PhLPl. To evaluate the portion of cytosolic and membrane-bound PhLPs, the homogenate was separated into a crude membrane fraction (P1), the cytosol (S2), and microsomes (P2). PhLPs was detected only in the crude membrane fraction (Fig. 2A). This observation is in marked contrast with the reported cytosolic localization of Phd in retinal rod cells [32].

The association of PhLPs with a distinct membrane compartment was further probed by separating the crude membrane preparation on a sucrose density gradient. We found that PhLPs was predominantly localized in fractions 2–4 corresponding to the plasma membranes as indicated by the ATPase Na^+/K^+ activity (Fig. 2B,C), and absent in chromaffin granule-enriched fractions 10–12 [8].

3.2. *PhLPs translocates from plasma membrane to the cytosol upon cell stimulation*

The intracellular distribution of PhLPs was next characterized by immunofluorescence and confocal microscopy in resting and stimulated chromaffin cells. Exocytosis was visualized by stimulating cells in the presence of anti-DβH antibodies [37]. DβH is localized on the inner surface of secretory granules and becomes exposed on the external surface of the plasma membrane during secretion, thereby revealing exocytotic sites by the appearance of fluorescent patches at the cell surface. Cytosolic PhLPs was virtually undetectable in resting chromaffin cells (Fig. 3A), confirming the results obtained by subcellular fractionation and immunoreplica analysis. PhLPs immunoreactivity was almost exclusively detected in the subplasmalemmal region. In marked contrast, stimulation with 10 μM nicotine triggered both the formation of DβH fluorescent patches at the cell surface, and significant immunostaining for PhLPs within the cytosol (Fig. 3A). To quantify this apparent translocation of PhLPs, we compared the amount of PhLPs in membrane and cytosolic fractions prepared from resting and nicotine-stimulated chromaffin cells. As illustrated in Fig. 3B, immunoreplica analysis revealed a significant reduction in the level of membrane-bound PhLPs

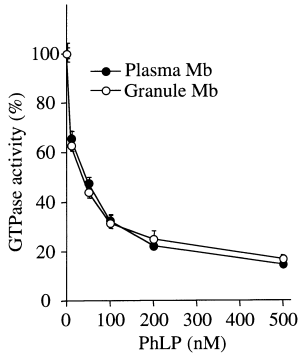

Fig. 4. PhLPs inhibits the intrinsic GTPase activity associated with purified plasma or secretory granule membranes from chromaffin cells. GTPase activity associated with purified chromaffin granule membranes (open circles) or plasma membranes (closed circles) was assayed by measuring the release of free ^{32}P phosphate from [γ-^{32}P]-GTP in the presence of the indicated concentrations of recombinant PhLPs. The intrinsic GTPase activity of the secretory granule membrane was 1.2 nmol/mg/min and of the plasma membrane was 2.1 nmol/mg/min. Assays were performed in triplicate and results are given as the mean ± S.E.M. This experiment was repeated twice.

and a 12-fold increase of soluble cytosolic PhLPs in stimulated cells. Overall, these results indicate that a portion of the plasma membrane-associated PhLPs translocates to the cytosol during the exocytotic reaction, thus suggesting that PhLPs may be involved in the molecular machinery underlying secretion in chromaffin cells.

3.3. Recombinant PhLPs inhibits exocytosis in single chromaffin cells

To study the potential role of PhLPs in the exocytotic process, we expressed and purified recombinant 6His-PhLPs. Phd and related proteins are known to inhibit intrinsic GTPase activity of several trimeric G proteins. Phd is not a direct inhibitor of the G protein GTPase but, via its interaction with G$\beta\gamma$, it slows the release of GDP from Gα. This results in an increase of the fraction of GDP bound and ultimately leads to an inhibition of steady-state GTPase activity [40]. We used this assay to determine the activity of recombinant PhLPs. In chromaffin cells, Go is preferentially associated with the membrane of secretory granules, whereas Gi3 is essentially concentrated at the plasma membrane [6,8]. In purified chromaffin granule and plasma membrane preparations, PhLPs inhibited GTPase activity in a similar dose-dependent manner (Fig. 4), suggesting that recombinant PhLPs was indeed able to modulate both the granule-bound Go and the plasma membrane-associated Gi3. These results are in agreement with PhLP and $\beta\gamma$ subunits being in competition for Gαo and Gαi3 [41].

The secretory granule-associated Go protein has been proposed to control the priming of exocytosis [6], whereas the plasma membrane-bound Gi3 protein is probably involved in the late calcium-dependent fusion step [8]. Because recombinant PhLPs was apparently active on both GTPases in vitro (Fig. 4), we investigated whether PhLPs might participate in the regulation of the exocytotic machinery in chromaffin cells. A robust rise in the cytosolic calcium concentration $[Ca^{2+}]_i$ is a prerequisite for exocytosis. To measure the transient increase of $[Ca^{2+}]_i$ in response to a depolarizing concentration of K^+, individual chromaffin cells were microinjected with buffer, then stimulated 10–15 min later by a local application of 59 mM K^+, and $[Ca^{2+}]_i$ was recorded. As shown in Fig. 5A, K^+-evoked stimulation induced a rapid rise of $[Ca^{2+}]_i$ that lasted for about 60 s. When the same cell was allowed to recover for 30 min and microinjected with 10 μM PhLPs, the $[Ca^{2+}]_i$ signal in response to a second K^+ stimulation was identical (Fig. 5A). Thus, PhLPs did not affect the rise of cytosolic calcium triggered by cell depolarization. In parallel, we estimated the secretory response to K^+ by electrochemically recording catecholamine release. As illustrated in Fig. 5B, microinjection of 10 μM PhLPs inhibited by approximately 75% the release of catecholamines compared to the first secretory response obtained after buffer injection (Fig. 5B), and this inhibitory effect was dose-dependent (Fig. 5C). Thus PhLPs blocked exocytosis without interfering with the mobilization of calcium, suggesting that PhLPs might directly play a function in the exocytotic machinery in chromaffin cells.

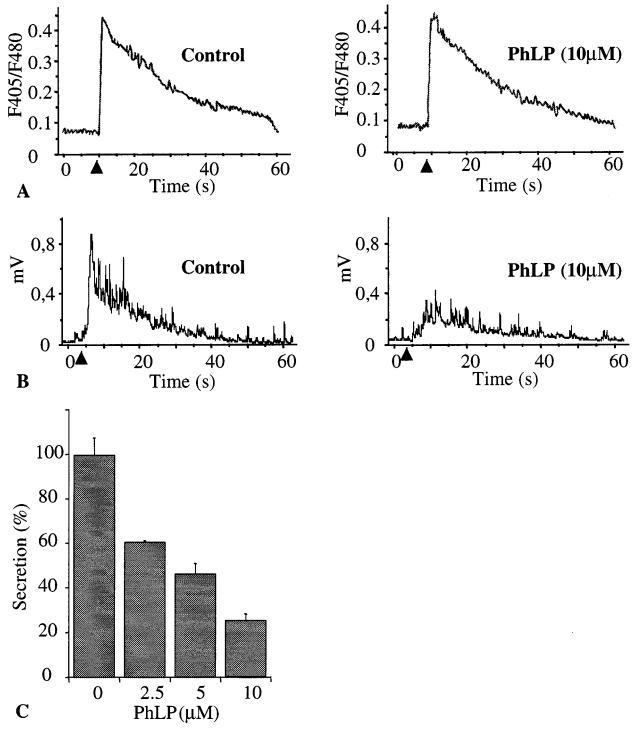

Fig. 5. Effect of PhLPs on high K^+-evoked catecholamine secretion from single chromaffin cells. Indo-1-loaded chromaffin cells were microinjected with buffer, incubated for 10 min in Locke's solution and then stimulated by a local application of 59 mM K^+ (Control). The same cell was then allowed to recover for 30 min in Locke's solution, microinjected with PhLPs (PhLP 10 μM), incubated for 10 min and stimulated for a second time with 59 mM K^+. A: Variations in cytosolic $[Ca^{2+}]_i$ after the first and the second stimuli were recorded on-line as a voltage signal (F_{405}/F_{480} ratio). Representative traces of $[Ca^{2+}]_i$ transients in response to a 5-s 59 mM K^+ pulse (arrowhead) are shown. B: Catecholamine secretion was recorded in parallel by amperometry using a carbon fiber electrode. Microinjection of 10 μM PhLPs did not significantly modify the $[Ca^{2+}]_i$ transient but strongly inhibited the exocytotic release of catecholamines as revealed by the reduced amperometric response. C: Cells were microinjected with buffer ($n = 33$), 2.5 μM ($n = 25$), 5 μM ($n = 20$) or 10 μM ($n = 10$) recombinant PhLPs and after 20 min stimulated with high K^+. The amperometric response was integrated to obtain the total catecholamine secretion expressed in pA/s. In the control group (buffer-injected), secretion was 868 ± 67 pA/s. Results are expressed relative to the response recorded from control cells and are given as means ± S.E.M. Identical results were obtained in three different cell preparations.

3.4. Conclusion

The present results indicate that the short variant of PhLP could play a role in the secretory process in chromaffin cells. PhLPs translocates from the subplasmalemmal region to the cytosol following cell stimulation. Although the functional significance of this observation remains to be elucidated, it is tempting to propose that a phosphorylation reaction may dissociate PhLPs from the plasma membrane-bound Gβγ subunits since it has been reported that the affinity of phosphorylated phosducin for Gβγ is reduced. Increased levels of PhLPs in chromaffin cells inhibited catecholamine secretion, suggesting that PhLPs acts as a negative clamp on the molecular machinery underlying exocytosis. At this stage, the partners for PhLPs in the exocytotic pathway remain to be identified. It appears reasonable to assume that the effects of PhLPs on secretion are mediated via the Gβγ subunits. Indeed, recent observations in mast cells indicate that Gβγ subunits are capable of enhancing the extent of secretion [10]. By inhibiting the regulatory effects of βγ subunits on α, PhLPs may affect the role of G proteins in the exocytotic pathway. We have previously reported that the late ATP-independent fusion step of exocytosis requires the activation of a plasma membrane-associated Gαi3 protein [8]. PhLPs may act in this late phase of exocytosis, by inhibiting the activity of Gαi3. The high affinity of PhLPs for the βγ subunits of G proteins may also have effects that go beyond the alterations of G protein activation. Hence, Gβγ are important not only for the GTPase activity of the α subunit but also for the regulation of effectors. βγ subunits are able to regulate the activity of phosphatidylinositol 3-kinase [16], and Rac-dependent pathways [42] possibly by interacting with nucleotide exchange factors with PH domains [43]. Since both phosphatidylinositol 3-kinase and Rac1 are present in the subplasmalemmal region of chromaffin cells and are probably involved in calcium-regulated catecholamine secretion [17,44], they represent additional putative downstream pathways for PhLPs in the exocytotic reaction. Further understanding of the mechanisms by which PhLPs acts in the secretory machinery now awaits the identification of the plasma membrane partners for PhLPs and the elucidation of the role of calcium and phosphorylation reactions in the cellular localization of PhLPs in chromaffin cells.

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