Endothelin-1 Mobilizes Profilin-1–Bound PIP_2 in Cardiac Muscle

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Phosphatidylinositol 4,5-bisphosphate (PIP_2) is a key downstream substrate of the endothelin signaling pathway and plays a role in regulating protein function at the membrane-cytoskeletal interface. However, the dynamic properties of distinct pools of PIP_2 are poorly understood, especially for PIP_2 that is bound to cytoskeletal proteins. We investigated the effects of endothelin-1 (ET-1) stimulation on protein-bound PIP_2 in cardiac muscle. Isolated rat myocytes and homogenized mouse ventricles were exposed to 10 n*M* ET-1 for varying time periods and protein-bound PIP_2 was analyzed using an anti-PIP_2 antibody and Western blotting. Several cytoskeletal proteins were found to contain tightly bound PIP_2, including profilin-1 (~15 kDa), capZ (~32 kDa), gCap39, (~39 kDa) and α-actinin (~106 kDa). Interestingly, ET-1 pretreatment reduced the amount of PIP_2 bound to profilin-1 by 46% after 15 mins, followed by a recovery to near basal levels after 60 mins. ET-1 had no effect on capZ-, gCap39-, or α-actinin–bound PIP_2 levels. To further explore the dynamics of PIP_2 binding, brefeldin-A (BFA) was used to disrupt PIP_2 binding to ADP-ribosylation factors and to impair receptor internalization. Pretreatment with 1 μ*M* BFA increased the PIP_2 signal on profilin-1 × 54% after 15 mins, followed by a decline to subbasal levels after 60 mins. Like ET-1, BFA had no effect on levels of PIP_2 bound to capZ or to α-actinin. Taken together, the data indicate that profilin-1 binds PIP_2 dynamically and may serve as a key regulator of the balance between cytoskeletal integrity and PIP_2 availability for Ca^{2+}/PKC signaling in the heart.
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Key words: brefeldin-A; α-actinin; phosphoinositides

Introduction

Phosphatidylinositol 4,5-bisphosphate (PIP_2) plays multiple roles in a variety of signaling pathways, as well as modulating numerous actin regulatory proteins (1). As an intermediate in the endothelin pathway, PIP_2 is hydrolyzed by phospholipase C (PLC) into the second messengers diacylglycerol and inositol 1,4,5 trisphosphate. Depletion of PIP_2 caused by $G_{\alpha q}$ overexpression in transgenic mice has been shown to increase cardiomyocyte apoptosis and contribute to subsequent heart failure (2). Hamsters suffering from severe cardiomyopathy and congestive heart failure have reduced PIP_2 mass in the sarcolemma, which is thought to jeopardize cardiac function (3). In addition, depletion of PIP_2 has been shown to increase delayed rectifier K^+ currents in atrial myocytes (4). Therefore, regulation of PIP_2 levels appears to be a critical element for proper cardiac function.

PIP_2 has been implicated as a possible membrane-anchoring site for ADP-ribosylation factors (ARFs), a family of small GTP-binding proteins that regulate membrane traffic (5). ARFs can interact with membrane-bound PIP_2, followed by insertion of its N-terminus into the hydrophobic region of the membrane bilayer. PIP_2 may influence the accessibility of ARFs to the catalytic domain of guanine nucleotide exchange factors (GEFs; Ref. 5), or activate a variety of GTPase-activating proteins, which provide additional membrane trafficking regulation (6). Inhibition of ARFs/GEFs with brefeldin A (BFA) would be expected to increase the availability of PIP_2 for other purposes.

Another possible regulator of the PIP_2 pool is the ~15 kDa cytoskeletal protein profilin. Profilin is an actin-binding protein that is essential in actin polymerization, but also binds polyphosphoinositol lipids (7). Three separate profilin proteins have been identified and characterized, though only profilin-1 is present in cardiac tissue. Profilin has also been linked to membrane trafficking, as noted by its presence at budding Golgi vesicles and its recruitment of dynamin 2, a protein necessary for vesicle budding (8).

In this study we examined the dynamic nature of PIP_2 pools bound to cytoskeletal proteins in the heart. Endothelin-1 was added for G-protein stimulated PLC breakdown of PIP_2. BFA, a macrocyclic lactone synthesized by fungi and an inhibitor of ARF/GEFs, was used to elevate PIP_2 levels as well as to inhibit membrane trafficking (9). Our evidence shows the unique ability of profilin-1 to bind PIP_2 dynamically and suggests roles in regulating the availability

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of PIP_2 for second messenger production and in mediating endothelin-induced cytoskeletal reorganization in the heart.

Materials and Methods

Materials. Reagents were obtained from Sigma Chemical Company (St. Louis, MO), unless otherwise stated. Tris-base and glycine were obtained from Fisher Scientific (Fair Lawn, NJ). Collagenase was from Worthington Enzymes (Freehold, NJ).

Sample preparation. Ventricular myocytes were isolated by enzymatic digestion from 3-month-old male Sprague-Dawley rats using collagenase and hyaluronidase, as described previously (10, 11). Animal handling practices used in this study have been reviewed by and received approval from the Animal Care Committee of the University of Wisconsin. Myocytes were maintained in Ca^{2+} Ringer's solution (in m*M*: 125, NaCl; 5, KCl; 25, HEPES; 2, NaH_2PO4; 1.2, $MgSO_4$; 5, pyruvate; 11, glucose; 0.5, $CaCl_2$; 20, taurine; pH 7.4). Ventricular tissue from wild-type 6- to 8- week-old mice was homogenized and maintained in Ca^{2+} Ringer's solution. Isolated rat myocytes and homogenized mouse tissue were exposed to 10 n*M* endothelin-1 (ET-1), 1 μ*M* BFA, or both for varying time periods and then skinned using 0.5% Triton X-100. Samples were then washed, centrifuged, and resuspended in Ca^{2+} Ringer's solution or relax buffer (see below).

Western blotting. Approximately 2 mg total protein was run on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to polyvinylidene fluoride membranes. PIP_2-binding proteins and profilin-1 were targeted using immunoblot analyses with a monoclonal anti-PIP_2 antibody and a polyclonal antiprofilin antibody from Novus Biologicals (Littleton, CO). α-actinin was detected with a monoclonal antibody from Sigma.

Confocal microscopy. Ventricular myocytes were isolated as described previously (10, 11) and maintained in relax buffer (in m*M*: 5, ATP; 100, KCl; 10, imidazole; 1, $MgCl_2$; 2, ethylene glycol tetraacetic acid; 1, phenylmethylsulfonyl fluoride; 1, benzamidine; 1, dithiothreitol; 20, 2,3 butanedione monoxime; and 1, protease inhibitor cocktail tablet; pH 7.0), followed by skinning in 100 μg/ml saponin. Samples were transferred to relax buffer containing 2% BSA for blocking and incubated with a monoclonal anti–profilin-1 antibody, then with an Alexa Fluor 568 secondary antibody from Molecular Probes (Eugene, OR). Imaging was performed with a Bio-Rad MRC 1024 laser scanning confocal microscope equipped with a mixed gas (Ar/Kr) laser operated by 24-bit Laser-Sharp software (Carl Zeiss, Thornwood, NY).

Results

Western blot analyses were used to determine the presence of PIP_2-binding proteins in cardiac muscle tissue. Primary antibody to PIP_2 detected multiple protein bands in both isolated rat ventricular myocytes and homogenized mouse ventricular tissue (Fig. 1). Four of the proteins were tentatively identified as α-actinin (~106 kDa), gCap39 (~39 kDa), capZ (~32 kDa), and profilin-1 (~15 kDa) on the basis of their mobility in SDS-PAGE (Fig. 1), peptide mass fingerprinting (not shown), and Western blots (below). All four proteins are known to be expressed in cardiac muscle and are thought to bind PIP_2 (7, 8, 12–14).

To investigate the dynamic nature of cytoskeletal and membrane-bound PIP_2 pools, ET-1 was added to perturb the distribution via PLC breakdown of membrane-bound PIP_2. Exposure to 10 n*M* ET-1 had a pronounced effect on profilin-1–bound PIP_2 after 15 mins (Fig. 1), significantly reducing PIP_2 levels by 46% compared with control samples (Fig. 2). PIP_2 levels returned to near basal levels after 60 mins of ET-1 exposure (Figs. 1 and 2). Conversely, ET-1 had no effect on α-actinin-, gCap39- or capZ-bound PIP_2 (Fig. 1). Therefore, for all subsequent quantitative analyses, profilin-1-bound PIP_2 was normalized to α-actinin–bound PIP_2 within the same lane to serve as an internal reference. This observation suggests that profilin-1 may have a role in maintaining consistent availability of PIP_2 for ET-1 signal transduction.

The pharmacologic agent BFA was used to inhibit GEFs, thereby inhibiting GTP for GDP exchange and subsequent ARF activation. In myocytes and ventricular tissue exposed to 1 μ*M* BFA, a 54% increase in profilin-1–bound PIP_2 was noted after 15 mins, followed by a decline to subbasal levels after 60 mins (Fig. 2). Similar to the results seen with ET-1 alone, BFA had no effect on α-actinin-, gCap39-, or capZ-bound PIP_2 (not shown). Again, profilin-1–bound PIP_2 was normalized to α-actinin–bound PIP_2 within the same lane to serve as an internal reference.

Exposure of myocytes and ventricular tissue to combined treatment with 10 n*M* ET-1 and 1 μ*M* BFA

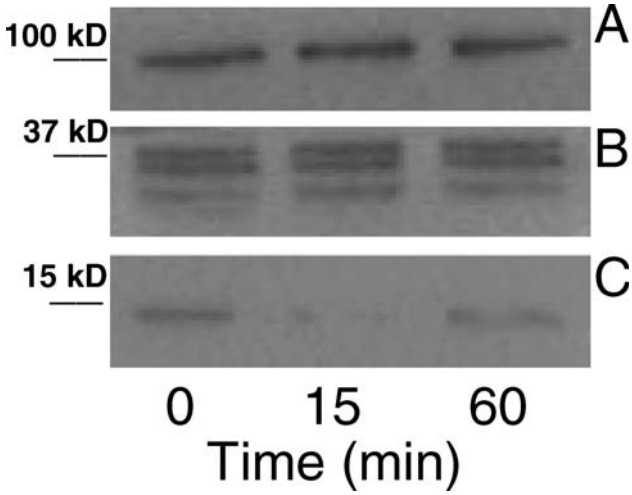

Figure 1. ET-1 affects profilin-1–bound PIP_2, but not α-actinin, gCap39, or capZ. Mouse ventricular tissue was exposed to 10 nM ET-1 and skinned in 0.5% Triton X-100. Samples were blotted with an anti-PIP_2 antibody over a 60-min time course. (A) α-Actinin. (B) gCap39 and capZ (the faint band at ~27 kDa was not identified). (C) Profilin-1.

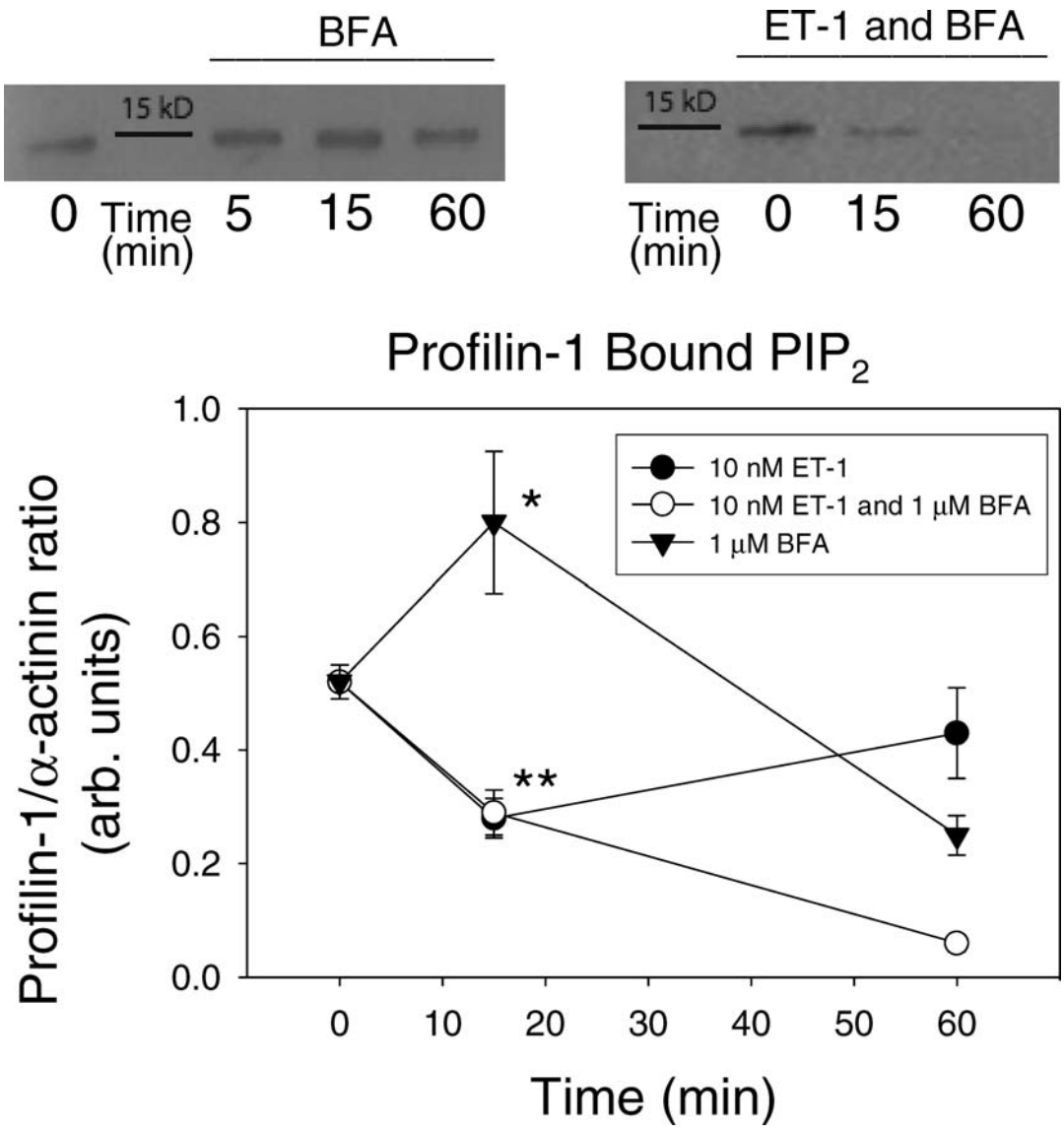

Figure 2. Effects of ET-1, BFA, and combined treatment with ET-1 and BFA on profilin-1–bound PIP_2. Ventricular tissue was exposed to 10 n*M* ET-1, 1 μ*M* BFA, or both for various time periods and skinned in 0.5% Triton X-100. (Upper) Representative blots of mouse ventricular tissue using an anti-PIP_2 antibody. (Lower) Graph summarizes profilin-1–bound PIP_2 band densities standardized to α-actinin–bound PIP_2 and graphed over a 60-min time course. Data from rat and mouse tissues were combined. *1 μ*M* BFA results in a 54% increase in profilin-1–bound PIP_2 after 15 mins, $P < 0.05$. **10 n*M* ET-1 results in a 46% reduction in profilin-1–bound PIP_2 after 15 mins, $P < 0.05$.

resulted in a reduction in profilin-1–bound PIP_2 similar to ET-1 exposure alone after 15 mins. However, the rise in PIP_2 levels to near-basal levels after 60 mins was no longer observed. Instead, treatment with ET-1 and BFA resulted in a continuous drop in profilin-1–bound PIP_2 throughout the 60-min duration (Fig. 2). Again, the combined treatment had no effect on α-actinin-, gCap39- or capZ-bound PIP_2 (not shown). Combined treatment of 1 μ*M* BFA and 10 n*M* ET-1 eliminated the transient increase in profilin-1–bound PIP_2 seen with BFA exposure alone (Fig. 2). One possibility for this response could stem from BFA inhibition of an endothelin receptor internalization pathway, leading to sustained PLC activation and PIP_2 hydrolysis. In support of this interpretation, pretreatment of cardiac myocytes with hypertonic sucrose to block clathrin-dependent receptor internalization also inhibited recovery of profilin-1–bound PIP_2 levels at 60 mins (not shown).

Next, we focused our attention on localizing profilin-1 in cardiac muscle using saponin-skinned rat ventricular myocytes. Localization experiments using confocal images with an anti–profilin-1 antibody resulted in a distinct striated pattern (Fig. 3A). This demonstrated that profilin-1 binds with a periodicity similar to that of the transverse tubules and could be strongly associated with the cardiac transverse tubule and/or Z-line. Secondary antibody alone resulted in only faint binding throughout the myocytes (Fig. 3B). Further work was carried out to localize the PIP_2 signal in saponin-skinned rat ventricular myocytes and mouse

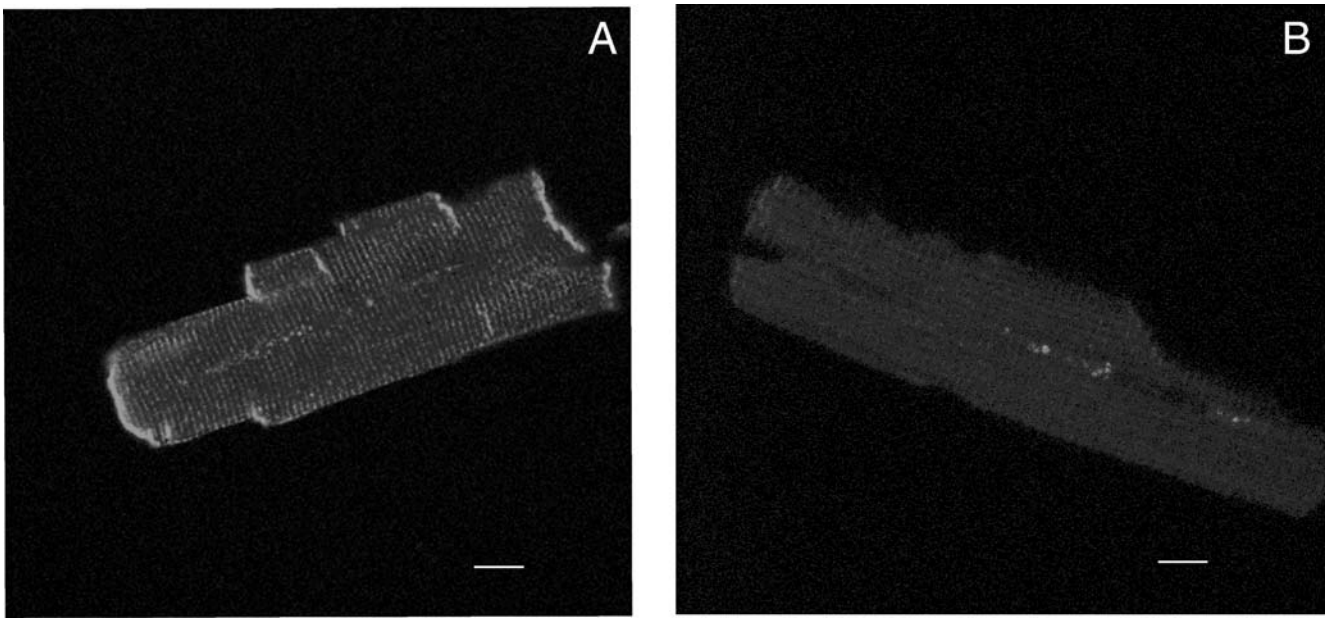

Figure 3. Localization of profilin-1 in rat ventricular myocytes. (A) Myocytes were skinned in 100 μg/ml saponin, incubated with an anti–profilin-1 antibody, then with an Alexa Fluor 568 secondary. (B) Myocytes were skinned in 100 μg/ml saponin and incubated in secondary antibody only. Bar, 10 μm.

ventricular tissue. Using the same anti-PIP_2 antibody, PIP_2 was found to have a similar transverse tubule or Z-line periodicity (not shown). Secondary antibody alone again resulted in only diffuse binding throughout the myocytes.

To dispel the possibility that the observed changes in PIP_2 levels on blots were dependent upon protein up- or downregulation or loading error, Western blotting analysis with an anti–α-actinin antibody or an anti–profilin-1 antibody was performed. Blots demonstrated protein stability and an equivalent protein load in all sample lanes, as indicated by similar band densities at 106 kDa for α-actinin and 15 kDa for profilin-1 (Fig. 4). Thus, ET-1 treatment for up to 60 mins did not stimulate profilin-1 upregulation or protein degradation, but affected the level of PIP_2 bound to profilin-1.

Discussion

This study provides evidence for dynamic binding of PIP_2 to a cytoskeletal protein in cardiac tissue. Specifically, we showed that treatment of rat ventricular myocytes or mouse ventricular tissue with either the G-protein coupled receptor ligand ET-1 or the ARF/GEF inhibitor BFA reversibly altered the amount of PIP_2 bound to profilin-1. The results suggest a potential role for profilin-1 in regulating the availability of PIP_2 as a substrate for endothelin-stimulated PLC. Profilin-1–bound PIP_2 is also likely to play an important role in maintaining cytoskeletal integrity at the transverse tubule/Z-line interface, and in its regulation by endothelin. A recent study in adipocytes reported regulation of insulin stimulated GLUT4 translocation by endothelin via a PIP_2- and actin-dependent mechanism (15), although the role of profilin-1 was not examined.

Importantly, profilin-1 alone displayed this unique ability to regulate PIP_2 levels in cardiac tissue as compared with other known PIP_2-binding cytoskeletal proteins including α-actinin, gCap39, and capZ. We suggest that profilin-1 plays a homeostatic role in maintaining membrane-bound PIP_2 levels at a consistent level, which may be essential for proper endothelin-induced signal transduction. Maintenance of PIP_2 levels appears to be required for normal cardiac function, as indicated by studies that show a link between PIP_2 depletion and cardiomyopathy (1–3). Such a mechanism also provides a possible link between GPCR-mediated PIP_2 turnover and reorganization of the profilin-regulated actin cytoskeleton.

Another finding of this study suggests a possible relationship between endothelin receptor function, membrane trafficking, and PIP_2 levels. When ET-1 is added to myocytes, our results suggest that membrane-bound PIP_2 is transiently depleted, followed by its replenishment (buffering) by profilin-1–bound PIP_2. This would account for the initial drop in profilin-1–bound PIP_2. The ensuing recovery over 60 mins is presumably because of desensitization and/or internalization of ET-1 receptors and resynthesis of PIP_2. When ET-1 was combined with BFA, profilin-1–bound PIP_2 dropped continuously over 60 mins with no recovery. One possibility is that BFA inhibited receptor desensitization and/or internalization and experiments with hypertonic sucrose reinforced this interpretation. With the endothelin receptor in a constantly "turned-on" state, membrane PIP_2 levels would be dramatically depleted, as would profilin-1–bound PIP_2.

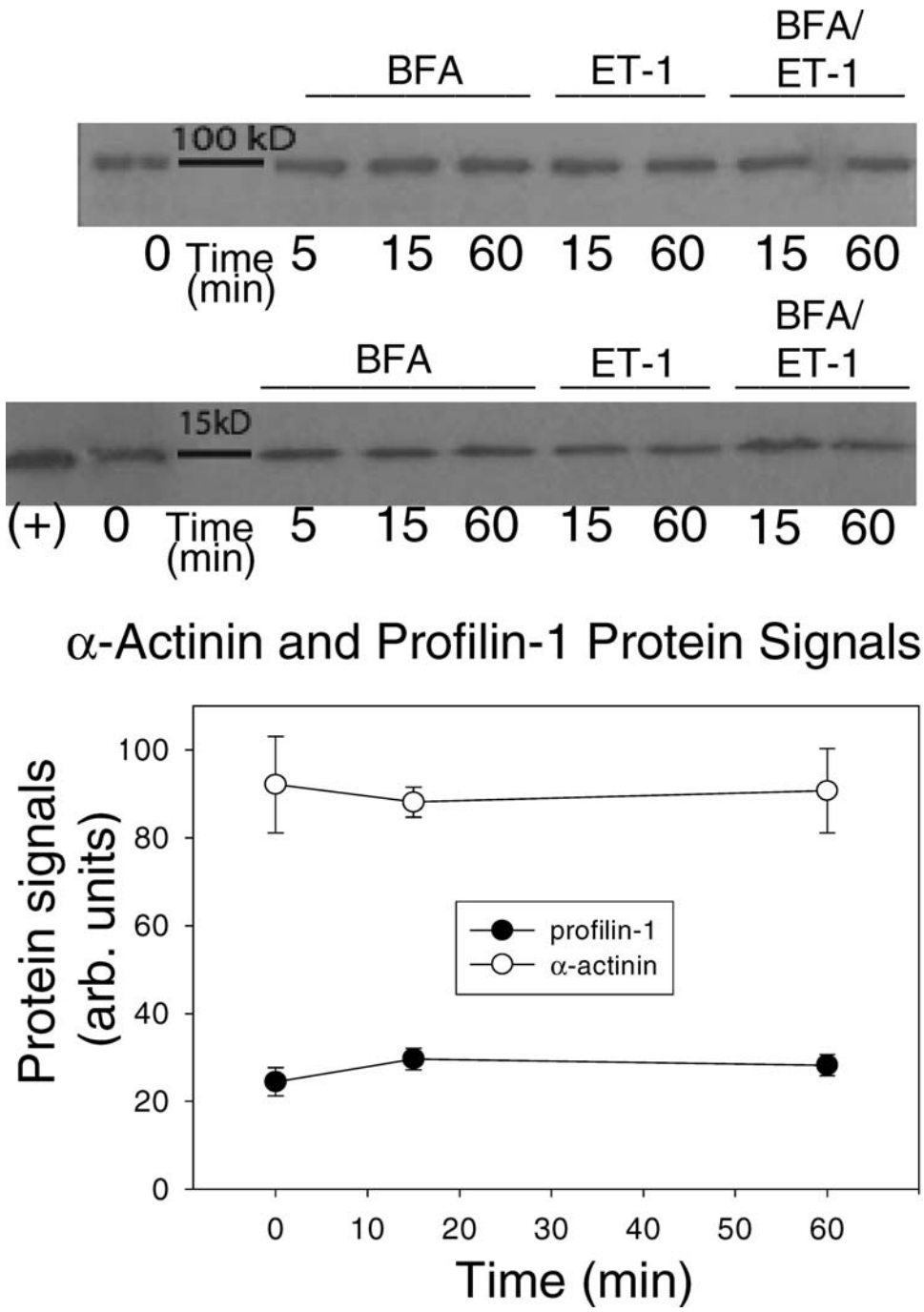

Figure 4. α-Actinin and profilin-1 protein signals. Samples were blotted with an anti–α-actinin antibody or an anti–profilin-1 antibody and resultant band densities were graphed over a 60-min time course. (Upper) Representative blots and (lower) graph summarizing results of three separate experiments. The lane labeled with (+) refers to a profilin-1 marker.

Elucidating the precise mechanism of BFA's action on ET-1 receptor function will require further investigation.

Further evidence for buffering of PIP_2 levels by profilin-1 was obtained using BFA treatment alone. The addition of BFA (in the absence of ET-1) would be expected to prevent PIP_2 binding to ARFs and consequently generate a surplus of PIP_2 in the membrane pool. Such a surplus could then drive PIP_2 onto its cytoskeletal buffer profilin-1, albeit only transiently as observed here.

In conclusion, we have provided evidence that the actin-binding protein profilin-1 is an important PIP_2-binding protein in cardiac muscle tissues. In contrast to the PIP_2 bound to other cardiac cytoskeletal proteins such as α-actinin, gCap39, and capZ, the pool of PIP_2 bound to profilin-1 was depleted by ET-1 stimulation and was enhanced by treatment with BFA. Profilin-1 protein was also localized in this study to cardiac T-tubules or Z-lines where it would be in or near the same membrane

compartment as ET receptors. The data indicate that profilin-1 may participate in the dual roles of regulating availability of PIP_2 for ET signaling and mediating GPCR control of the actin cytoskeleton.

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