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Copines-1, -2, -3, -6 and -7 show different calcium-dependent intracellular membrane translocation and targeting

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The copines are a family of C2- and von Willebrand factor A-domain-containing proteins that have been proposed to respond to increases in intracellular calcium by translocating to the plasma membrane. The copines have been reported to interact with a range of cell signalling and cytoskeletal proteins, which may therefore be targeted to the membrane following increases in cellular calcium. However, neither the function of the copines, nor their actual movement to the plasma membrane, has been fully established in mammalian cells. Here, we show that copines-1, -2, -3, -6 and -7 respond differently to a methacholine-evoked intracellular increase in calcium in human embryonic kidney cell line-293 cells, and that their membrane association requires different levels of intracellular calcium. We demonstrate that two of these copines associate with different intracellular vesicles following calcium entry into cells, and identify a novel conserved amino acid sequence that is required for their membrane translocation in living cells. Our data show that the von Willebrand factor A-domain of the copines modulates their calcium sensitivity and intracellular targeting. Together, these findings suggest a different set of roles for the members of this protein family in mediating calcium-dependent processes in mammalian cells.

Structured digital abstract
- MINT-8049236: *Copine-6* (uniprotkb:Q9Z140) and *transferrin* (uniprotkb:P02787) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)
- MINT-8049176: *CD2* (uniprotkb:P06729) and *Copine-2* (uniprotkb:P59108) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)

Introduction

The copines are a family of proteins that share a common structure, with two N-terminal C2-domains and a C-terminal von Willebrand factor A (vWA)-domain. The former has similarity with the C2-domains found in protein kinase C, phospholipase C, synaptotagmin and rabphilin, which are known to be responsible for calcium-dependent phospholipid binding [1,2]. The vWA-domain has a distant similarity to the vWA-domain of certain integrins, which can bind other proteins, usually in a Ca^{2+}-, Mg^{2+}- or Mn^{2+}-dependent

Abbreviations
2-APB, 2-aminoethyldiphenyl borate; C2A6, chimaera of the C2C2-domains of copine-2 and the vWA-domain of copine-6; C2A6*, chimaera of the C2C2-domains of copine-2 and the vWA-domain of copine-6 with the copine-6 linker; C6A2, chimaera of the C2C2-domains of copine-6 and the vWA-domain of copine-2; COS-7, CV-1 cells stably transformed with the large SV40 T antigen; EGFP, enhanced green fluorescent protein; EYFP, enhanced yellow fluorescent protein; HEK-293, human embryonic kidney cell line-293; vWA, von Willebrand factor type A domain.

manner. The copine vWA-domain has the residues required for metal binding and, in the case of copine-1, has been demonstrated to bind Mn^{2+} [3–5]. The copines were first described in *Paramecium tetraurelia* [4] and, subsequently, in *Caenorhabditis elegans*, *Arabidopsis* and *Dictyostelium* [6–10]. Mutations in genes coding for the copines cause dwarfing, cell death phenotypes and alterations in the expression of the disease resistance gene *SNCI* in *Arabidopsis*, as well as defects in differentiation and vacuolation in *Dictyostelium* [9–14]. Copine expression has been found in many mammalian tissues, including brain, heart, lung, liver and kidney [5]. Screening of human tissues for human copines-1–6 has shown that copines-1, -2 and -3 are ubiquitous, whereas copine-4 has a more restricted distribution in brain, heart and prostate gland, and copine-6 is brain specific [15]. Interestingly, the levels of copine-6 have been shown to increase after the induction of kindling or long-term potentiation in the rat hippocampus [16,17].

The precise role of copines in cells remains unclear, although there is evidence that the copines may be involved in the regulation of plasma membrane protein, or lipid, content. Thus, in *C. elegans*, a copine has been implicated in the insertion, or removal, of a transient receptor potential channel [7], and the synaptic targeting of the levamisole receptor was reduced following RNAi-mediated knockdown of a copine [18]. Another example of such potential regulation is the involvement of OS-9, a copine-6-interacting protein and the product of a gene frequently amplified in osteosarcoma [6,19], in the trafficking of the membrane protease meprin and as a transient receptor potential channel [20,21].

The domain structure of the copines has led to the suggestion that they can target proteins to the plasma membrane in response to an intracellular increase in calcium, with the C2-domains acting as the calcium sensor and directing the copine to the plasma membrane. The vWA-domain is thought to bind the copine's target protein(s) [8]. Potential target proteins for human copines-1, -2 and -4 include transcription factors, cytoskeletal-associated proteins, phosphorylation regulators, proteins associated with protein ubiquitinylation [22] and members of the calcium-binding protein family, the neuronal calcium-binding proteins [23]. It should be noted, however, that, although there is evidence for calcium-dependent interaction of human copine-6 with OS-9, this interaction appears to be with the C2-domain and not the vWA-domain [19].

If the copines do act to target specific proteins to the cell membranes in response to increases in intracellular calcium, they should show calcium-dependent membrane binding. *In vitro* studies using phospholipid vesicles have shown that some copines, or their C2-domains, can exhibit calcium-dependent phospholipid binding [4,5,11,16]. However, *in vivo* evidence for such behaviour is limited, with a single report in *Dictyostelium* showing transient membrane binding of enhanced green fluorescent protein (EGFP)-tagged copine A in response to starvation and subsequent expression of cAMP receptors [11].

We have therefore characterized the calcium responses of copines-1, -2, -3, -6 and -7 with respect to their calcium-dependent intracellular movement, when expressed in human embryonic kidney cell line-293 (HEK-293) cells. Our results show that, in these cells, after ionomycin treatment, all of the copines exhibit calcium concentration-dependent translocation to the plasma membrane, and copines-1, -2, -3 and -7 also translocate to the nucleus. However, only copine-2 and copine-7 respond to a methacholine-induced intracellular increase in calcium. We also show that the C2-domains alone are not sufficient to cause the translocation of the proteins to the plasma membrane, and that their membrane association requires a conserved 22-amino-acid sequence that immediately follows the last C2-domain. In addition, we demonstrate that the vWA-domains of these proteins modulate both their calcium responses and intracellular targeting. The C2- and vWA-domains therefore have distinct and crucial roles in the translocation and targeting of the copines. Together, these findings suggest that the copines may have other roles in addition to targeting proteins to cell membranes.

Results

Expression of copines in mammalian cells

In order to examine the behaviour of copines in cultured HEK-293 and COS-7 (CV-1 cells stably transformed with the large SV40 T antigen) cells, a number of N-terminal antigen-tagged (myc- or HA-), as well as N- and C-terminal EGFP- or enhanced yellow fluorescent protein (EYFP)-tagged, variants of full-length copines, their domains and cross-domain fusions were made (illustrated in Fig. 1). Western blot analysis of lysates from cells expressing the myc- and EGFP- or EYFP-tagged copines showed robust expression of the recombinant proteins in HEK-293 cells (Fig. 2A) and COS-7 cells (not shown). Immunocytochemical analysis of the expressed copines displayed a diffuse cytoplasmic distribution (Fig. 2B). However, in HEK-293 cells, copines-1, -2, -3, and -7, but not copine-6, also exhibited nuclear staining (Fig. 2B). Similar patterns of intracellular localization were seen with the myc- and EYFP-tagged constructs, and none of the copines had

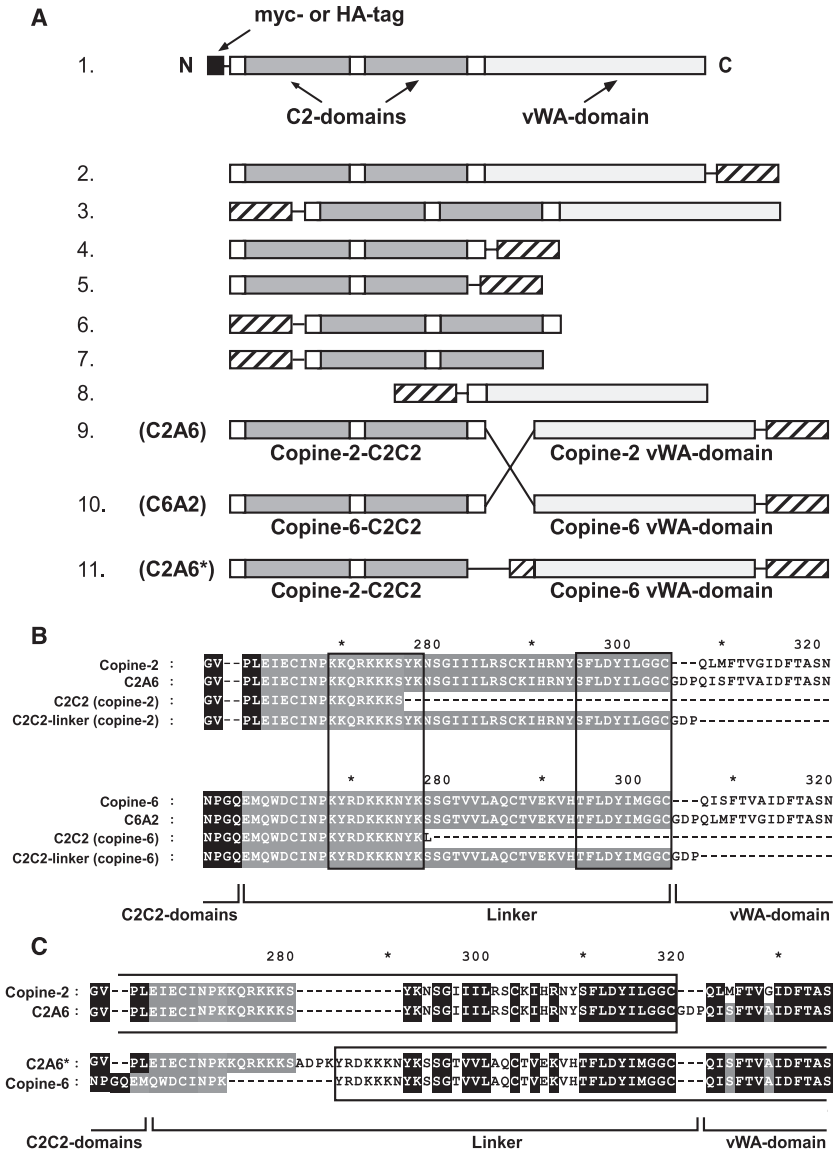

Fig. 1. Cloned fluorescent protein-tagged copines and their domain chimaeras. (A) Schematic diagrams of the domain structure of copines, with the position of the tag in myc- or HA-tagged copines indicated (1), and the fluorescent-protein tagged full-length copines-2, -3 and -6 prepared for this study (2,3). In addition to truncated versions of copines-2 and -6 containing only specific domains (4–8), domain swaps of copines-2 and -6 (9–11) were also constructed as copine-2 C2-domain chimaeras with the copine-6 vWA-domain connected through the copine-2 (9) or copine-6 (11) linker. (B) Alignment of the linker (grey background) between the end of the C2C2-domains (black background) and the beginning of the vWA-domains for copines-2, -6 and their derivatives, with the conserved sequences boxed. (C) Alignment of the linker area of copines-2 and -6 against the corresponding sequences of C2A6 and C2A6* constructs.

significant effects on cell morphology after 24–48 h of expression (see also Fig. S1).

Copines show different plasma membrane translocation responses to increases in intracellular calcium and require extracellular calcium to show maximal responses

To examine the responses of the different copines to changes in intracellular calcium, HEK-293 cells were transiently transfected with individual copines and treated with ionomycin, an ionophore from *Streptomyces conglobatus*, which increases intracellular calcium by making both endoplasmic reticulum and plasma membranes of the cell permeable to Ca^{2+}. In preliminary experiments, myc-tagged copine-2 was found to translocate to the periphery of the cell within 90 s of ionomycin treatment (5 μM; Fig. 3A), where it colocalized with the plasma membrane protein CD2. In addition, an increase in the nuclear immunoreactivity of myc–copine-2 was observed. Thus, ionomycin treatment of the cells caused the translocation of myc–copine-2 from the cytoplasm to both the plasma membrane and nucleus.

To quantify the translocation of the copines, we made use of the different copine–EYFP constructs and monitored the change in the amount of copine in a region of interest following ionomycin treatment (as shown in Fig. 3E, G). Copines-2, -3 and -6 all translocated to the membrane in response to increases in

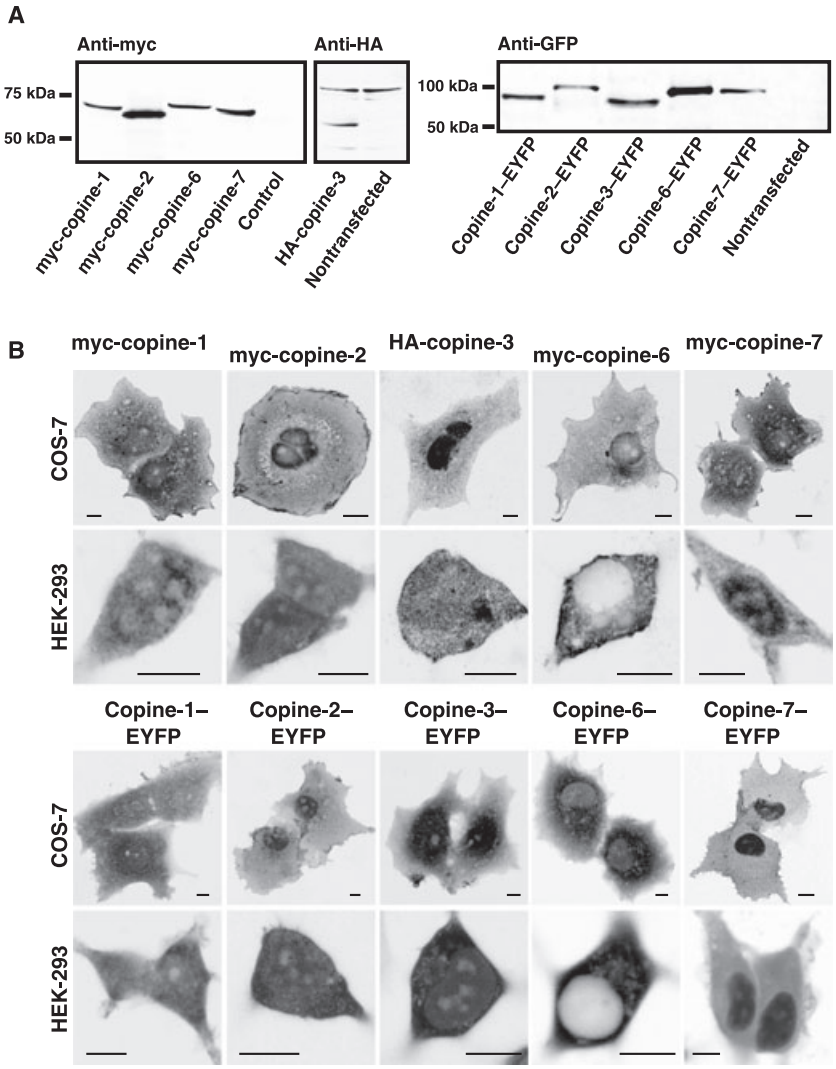

Fig. 2. Expression of recombinant copines-1, -3, -6 and -7 in cultured mammalian cells. (A) Western blots of myc-/HA- and EYFP-tagged full-length copines in cultured HEK-293 cells. The top bands in the anti-HA panel represent nonspecific bands that were present in nontransfected cells. (B) Expression patterns of myc-/HA- and EYFP-tagged full-length copines in cultured COS-7 and HEK-293 cells. Apart from the weak nuclear staining of anti-HA IgG, the antibodies showed no nonspecific binding in cells (see also Fig. S1). Scale bars, 10 μm.

intracellular calcium; however, they did so at different rates (Fig. 3B), with the movement of copine-2 being the most rapid, followed by copine-6 and then copine-3. To determine whether extracellular calcium is necessary for the translocation of the copines, the experiments were repeated in calcium-free medium. Under these conditions, ionomycin caused a small increase in intracellular calcium (Fig. 3C), but did not lead to the translocation of copine-2 or copine-6 (Fig. 3D). The addition of 2 mM calcium to the ionomycin-treated cells in calcium-free medium, however, caused a large increase in intracellular calcium (Fig. 3C) and the rapid translocation of copine-2 and copine-6 to the membrane (Fig. 3D). Copine-1 and copine-7 showed similar ionomycin responses, as did N-terminally tagged EYFP–copine-2 (Fig. S2A). Thus, the ionomycin-induced translocation of the copines was dependent on the presence of extracellular calcium.

We next characterized copine-2 and copine-6 in greater detail with respect to their responses to an increase in intracellular calcium. Treatment of HEK-293 cells with thapsigargin caused a marked increase in intracellular calcium because of its release from intracellular stores, as well as the influx of extracellular calcium through calcium channels. Calcium added to cells treated for 2–3 min with thapsigargin in calcium-free medium produced a dramatic increase in calcium caused by its entry through store-operated calcium channels. This calcium influx can be blocked by the addition of 2-aminoethyldiphenyl borate (2-APB) or 2 μM Gd^{3+} (Fig. 4A). In calcium-free medium, treatment of cells, transfected with either copine-2 or

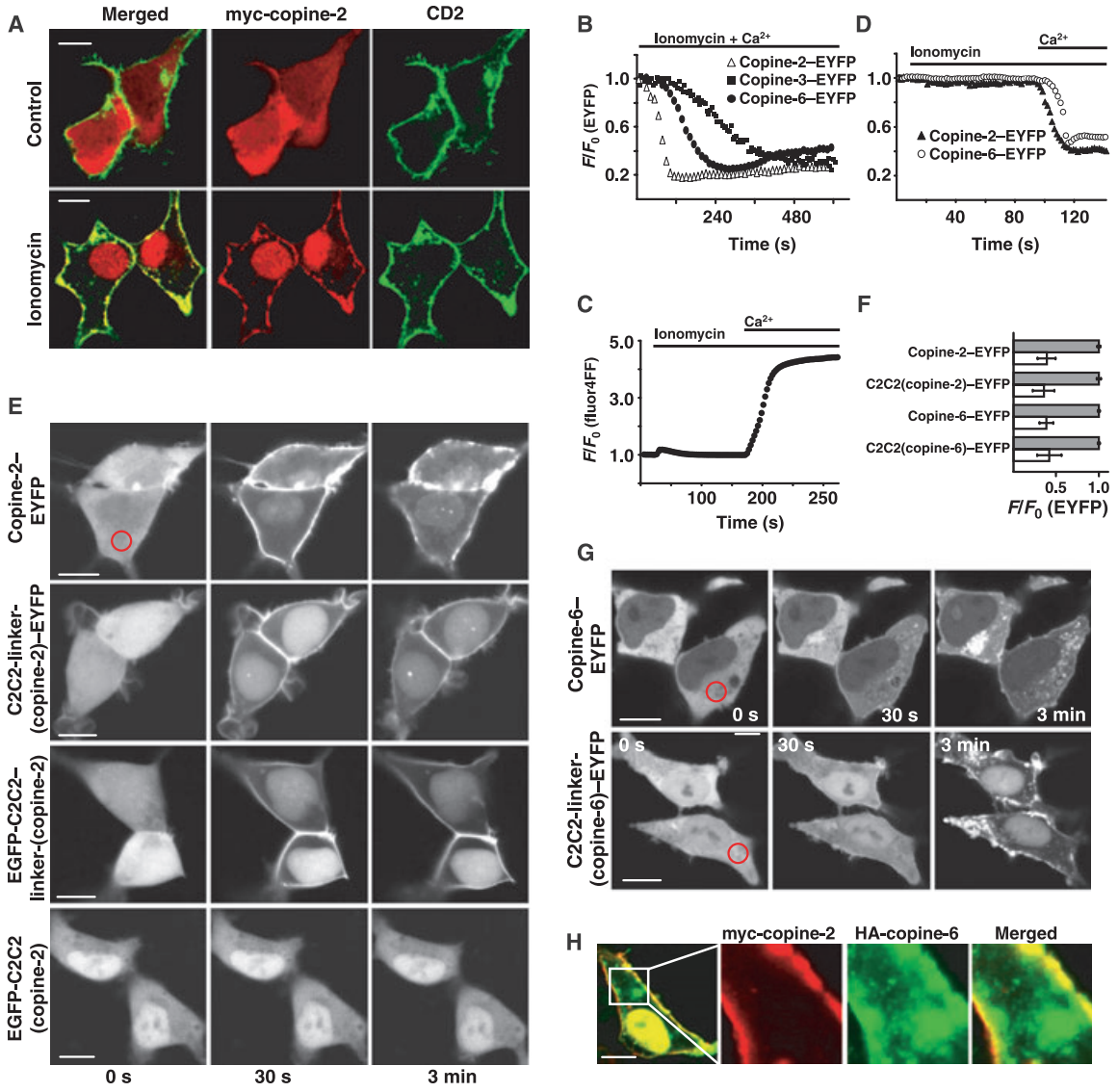

Fig. 3. Ionomycin treatment of HEK-293 cells causes translocation of the copines to the plasma membrane. HEK-293 cells were transfected with the different copines and treated with ionomycin in medium containing 1.8 mM $CaCl_2$. Cells were either fixed with paraformaldehyde, permeabilized and immunostained for the copines (A, H), or the localization of EYFP-tagged copines was visualized by confocal microscopy of live cells (E, G). (A) HEK-293 cells expressing the lymphocyte membrane protein CD2 and myc-tagged copine-2 were treated with ionomycin and immunostained for both proteins. Copine-2 (red) showed rapid movement to the plasma membrane where it colocalized with CD2 (green). (B) Fluorescence levels of cytosolic EYFP-tagged copines-2, -3 and -6 were monitored in HEK-293 cells (30–40 cells) expressing the copines, using circular regions of interest as illustrated in (E) and (G). (C) The effect of ionomycin on EYFP fluorescence in these areas over time, in Ca^{2+}-containing medium, was calculated, and the results were plotted. (D) The effect of ionomycin on cytoplasmic calcium levels in HEK-293 cells (30–40 cells) in calcium-free medium was visualized using the fluorescent calcium indicator Fluo-4FF. In the absence of extracellular calcium, ionomycin had no effect on the cytoplasmic fluorescence of EYFP-tagged copines-2 and -6. (E) Confocal images of the ionomycin responses of copine-2–EYFP and its C2C2-domain constructs in HEK-293 cells. (G) Typical responses of copine-6–EYFP and its C2C2–EYFP construct to ionomycin treatment. The average ionomycin responses of EYFP-tagged copines-2 and -6 and their C2C2–EYFP constructs are summarized in (F) (30–50 cells), where the grey bars are the responses in calcium-free medium and the open bars are those in medium containing calcium. For all the constructs, the response in medium containing Ca^{2+} was significantly greater than that in calcium-free medium ($P > 0.001$, *U*-test). All the quantitative data are expressed as F/F_0, and the data represent the means from at least 10 cells per experiment. HEK-293 cells were cotransfected with myc-tagged copine-2 and HA-tagged copine-6 and treated with ionomycin for 3 min. The cells were fixed, permeabilized and stained for the two different epitopes. The results show that copine-2 is not associated with copine-6 when the latter is internalized (H). Scale bars represent 10 μm.

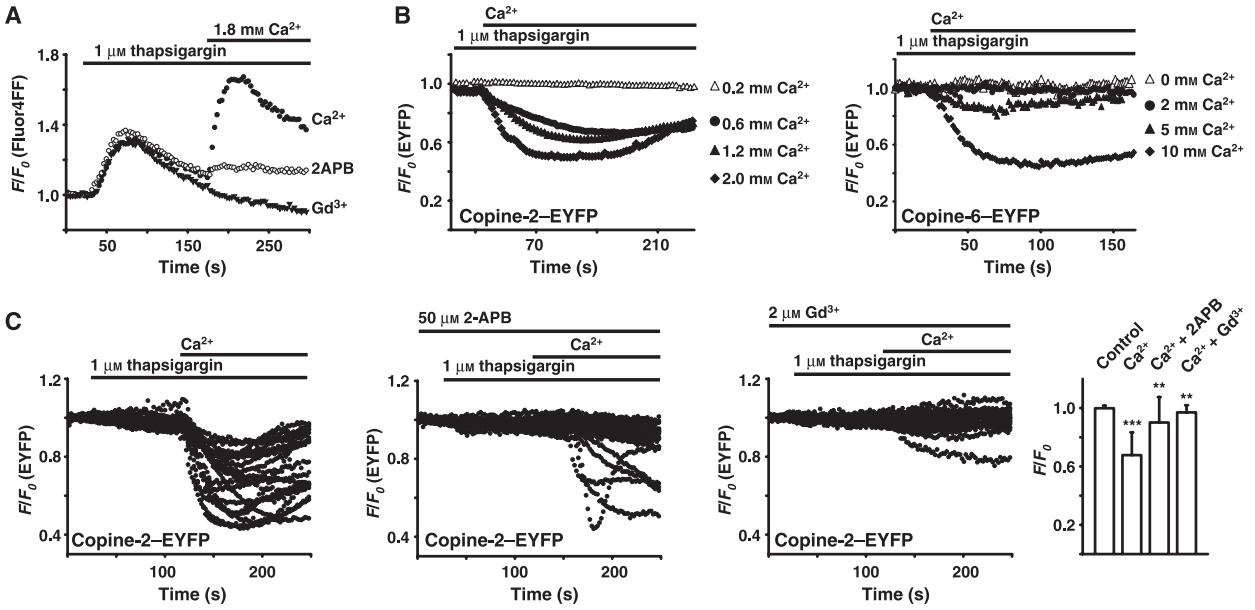

Fig. 4. Calcium-dependent intracellular translocation of the copines depends on the opening of store-operated calcium channels. (A) Fluo-4FF fluorescence in the cell cytoplasm was used to visualize the changes in calcium levels in HEK-293 cells in response to thapsigargin treatment. Measurements were made first in calcium-free medium, and then in medium to which calcium was restored. Changes in the intracellular calcium levels were recorded over time. The effects of 2-ABP or Gd^{3+} ions on the entry of calcium to the cells were also examined. The traces shown represent the average results from 250–300 cells. (B) Changes in the localization of copine-2–EYFP and copine-6–EYFP were imaged using confocal microscopy of live cells. The localization of both copines was affected by thapsigargin treatment, but only when the levels of extracellular calcium were increased. Here, each plot represents the average (~ 50 cells) reduction in cytoplasmic copine–EYFP at the indicated time points. (C) The inhibitory effects of 2-APB and Gd^{3+} on the copine-2–EYFP responses to extracellular calcium in cells with Ca^{2+} stores depleted by thapsigargin are shown. Each plot shows the decrease in cytosolic copine as a fraction of the original fluorescence for an individual cell, and the results are from approximately 20–25 cells per coverslip (three coverslips each). The chart in (C) shows the decrease in cytosolic copine-2 fluorescence as an average of the data from multiple experiments, approximately 150–200 cells in total ($P < 0.001$*** for Ca^{2+} and $P \gg 0.05$** for 2-APB or Gd^{3+}, *U*-test).

copine-6, with thapsigargin, did not stimulate their movement to the membrane, despite the increase in intracellular calcium as a result of release from intracellular stores. However, the addition of calcium to the medium of treated cells caused a rapid shift in both copines to the membrane, although copine-6 required significantly greater extracellular calcium concentrations to initiate membrane translocation (Fig. 4B). In calcium-containing medium, the copine responses were also dependent on the opening of the store-operated calcium channels, as the inhibitors 2-APB and 2 μM Gd^{3+} reduced both the number of cells responding and the extent of their response, as shown for copine-2 (Fig. 4C).

In order to examine the response of the copines to a more physiological stimulus, we took advantage of the expression of the muscarinic acetylcholine receptor in HEK-293 cells [24]. Selective muscarinic agonists, such as acetyl-β-methylcholine (methacholine), can activate these receptors and induce extracellular calcium influx, as well as its intracellular release, in HEK-293 cells. We observed that only copine-2 and copine-7 showed robust responses to treatment of the cells with 10 μM methacholine (63 ± 6.5% and 78.4 ± 8.7% of the transfected cells, respectively) (see both Figs 5 and 6). Copines-1, -3 and -6 showed little response to methacholine treatment, with fewer cells responding and a reduced extent of translocation. For example, only 2.4 ± 1.1% of copine-3-transfected cells weakly responded to methacholine treatment (Fig. 6B, top right). Unlike the ionomycin or thapsigargin responses, the responses of copine-2 and copine-7 to methacholine were transient because of the transient increase in intracellular calcium induced by methacholine, as shown in Fig. 6A. The methacholine-induced translocation of all of the copines, and the copine constructs, was blocked by cotreatment with the muscarinic receptor antagonist atropine (data not shown).

In order to confirm that the intracellular increase in calcium caused by methacholine was sufficient to translocate copine-2 or its C2-domain construct to the membrane, cells expressing these proteins were treated

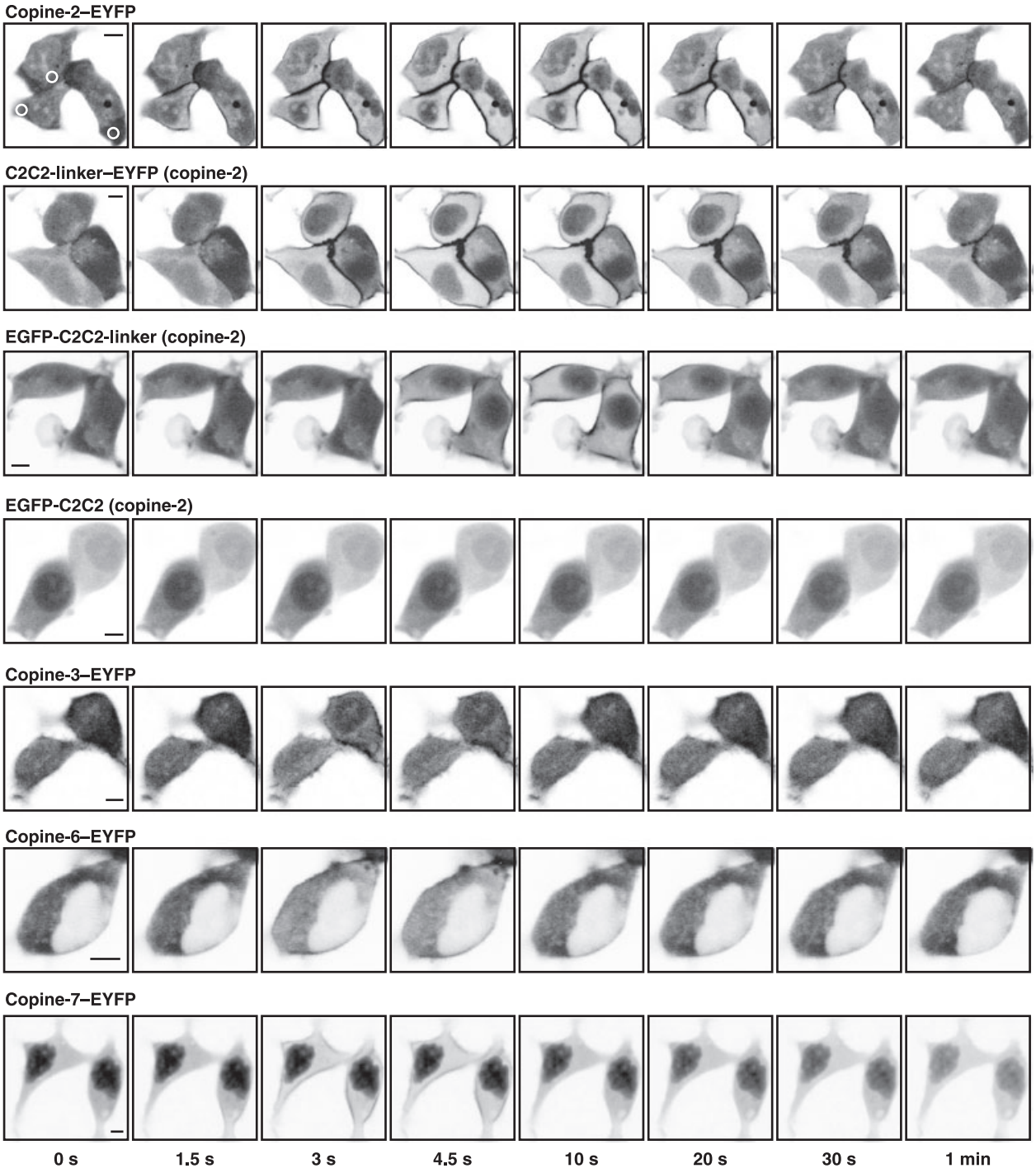

Fig. 5. Methacholine-induced translocation of different copines and C2C2-constructs to the plasma membrane in response to transient elevation of Ca^{2+} in cultured HEK-293 cells. HEK-293 cells were transfected with copine-3–EYFP, -6–EYFP, -7–EYFP and copine-2–EYFP/EGFP and its different C2C2-constructs as indicated above each panel. Methacholine was added to the medium containing extracellular calcium, at time point 0 s, and the changes in cytoplasmic fluorescence were imaged at the indicated time points. Scale bar corresponds to 5 μm.

with methacholine in the presence or absence of extracellular calcium, and in the presence of calcium and 2-APB. The treatment of HEK-293 cells with methacholine caused a robust transient increase in intracellular calcium that could be reduced either by removing extracellular calcium or by blocking the

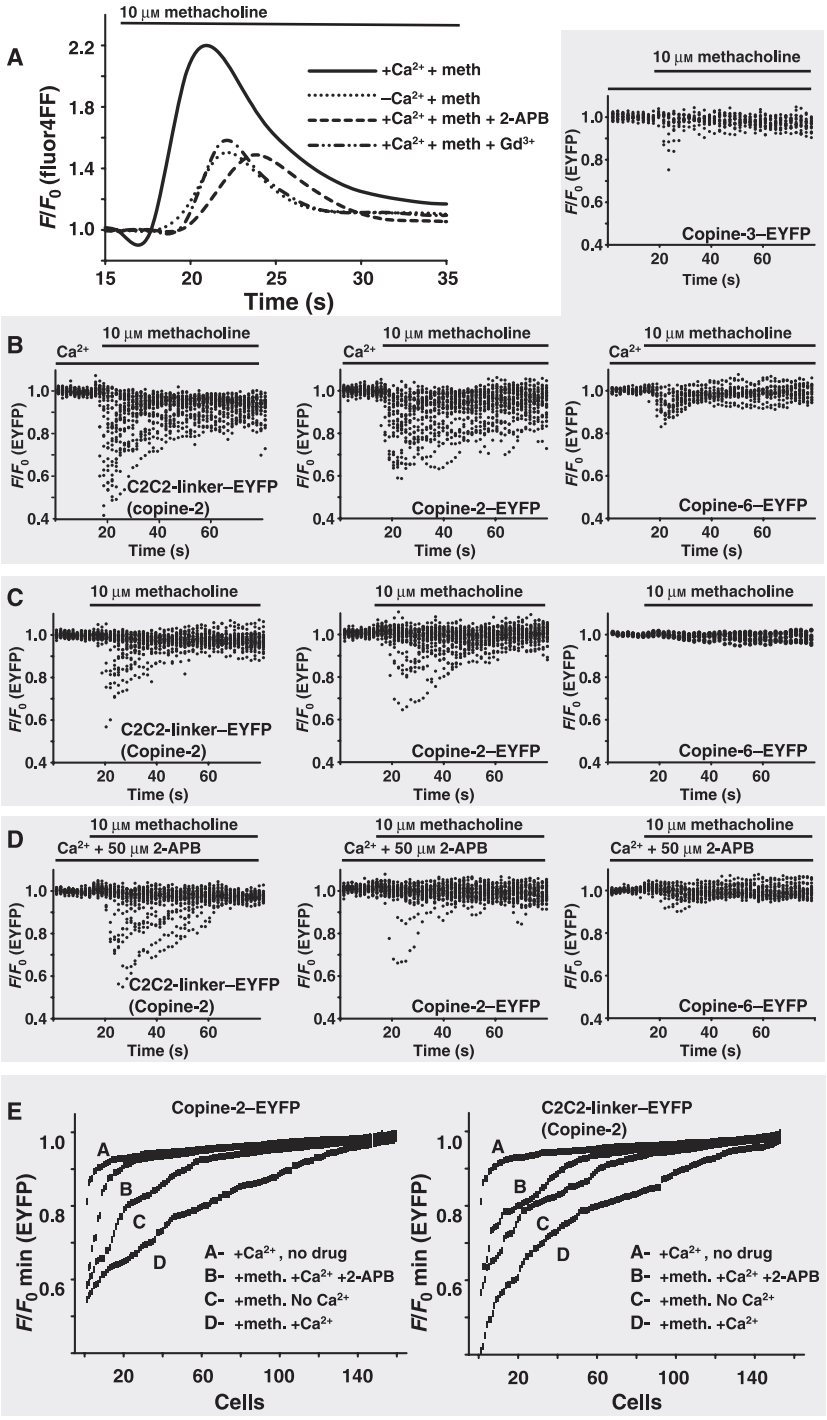

Fig. 6. Full methacholine-induced copine responses depend on extracellular calcium. (A) Intracellular levels of calcium in HEK-293 cells were increased by 10 μM methacholine treatment and the calcium levels were monitored using Fluo-4FF. The stimulatory effect of methacholine was prevented by either cotreating the cells with 2-APB or Gd^{3+}, or by incubating the cells in calcium-free medium. The peak value for the intracellular rise in calcium was significantly lower when the cells were exposed to 2-APB relative to 2 μM Gd^{3+} ($P \gg 0.05$, $n_{1-2} > 300$, *U*-test). (B) Methacholine application affects the level of cytoplasmic fluorescence of copine-2–EYFP, -3–EYFP and -6–EYFP, as well as the C2C2–EYFP domains of copine-2, to varying degrees, in the presence of extracellular calcium. The effect of methacholine is attenuated for copine-2–EYFP and copine-6–EYFP in the absence of extracellular Ca^{2+} (C) and in the presence of 2-APB (D). The experiments show the traces from 20–25 cells per coverslip (three coverslips in total). Copine-3–EYFP did not respond to methacholine in the absence of Ca^{2+} or the presence of 2-APB (data not shown). (E) The cumulative representation of three to five independent experiments (~160–250 cells in total) of copine-2–EYFP and its C2C2-EYFP domain response to methacholine. The graphs show sorted minimal cytoplasmic fluorescence (i.e. the maximum response to methacholine) for each individual cell. Full-length copine-2–EYFP and its C2C2-EYFP domain responded similarly to methacholine in the presence ($P = 0.59$; $n_1 = 254$, $n_2 = 187$; *U*-test) or absence ($P = 0.31$; $n_1 = 240$, $n_2 = 173$; *U*-test) of extracellular Ca^{2+}, suggesting that the vWA-domain is not important for the translocation of copine-2–EYFP to the plasma membrane.

store-operated channels and IP3 receptors with 2-APB (Fig. 6A). In the absence of extracellular calcium or in the presence of both calcium and 2-APB, the weak responses of copines-3 and -6 to methacholine were completely inhibited (Fig. 6B–D). In contrast with thapsigargin treatment, in calcium-free medium, the responses of copine-2 and its C2C2-linker construct to methacholine were not ablated. The response was reduced significantly, however, with fewer cells responding to treatment and, in the cells that did respond, the extent of translocation being attenuated (Fig. 6C), suggesting that a maximal response required

the influx of extracellular calcium. However, further investigation revealed that store-operated channels were also involved, as methacholine-induced translocation of copine-2 was reduced by 2-APB treatment. Moreover, this reduction was even greater than the reduction produced by the elimination of extracellular calcium ($P < 0.001$; $n_1 = 188$, $n_2 = 161$; *U*-test; Fig. 6D, E), reflecting the inhibition of release of calcium from intracellular stores by 2-APB [25]. In contrast, the C2C2-linker domains (copine-2) gave similar responses to methacholine whether in calcium-free medium or in the presence of calcium plus 2-APB ($P = 0.074$; $n_1 = 160$, $n_2 = 177$; *U*-test; Fig. 6D, E). Thus, in the full-length protein, the presence of the vWA-domain may modulate the intracellular translocation of the copines by reducing the sensitivity of the C2-domains to calcium. Together, these results show that the copines have different sensitivities to increases in intracellular calcium, and that they require extracellular calcium to exhibit their maximal translocation responses.

The copine C2-domains and linker region are crucial for ionomycin-induced membrane translocation

The predicted domain structure of the copines suggests that the C2-domains might be responsible for the calcium-mediated membrane association of copines [4,8,16]. In order to test this hypothesis, the C2-domains of copine-2 alone were fused with EYFP at both the N- and C-termini and in the presence and absence of the linker region between the last C2-domain and the start of the vWA-domain (see schematic diagrams 4–7 in Fig. 1A). The response of these EYFP-tagged domains to ionomycin treatment was compared with that of full-length copine-2–EYFP. The results showed clearly that all of the constructs containing the linker region behaved similarly to copine-2–EYFP. However, if the linker region was removed, the protein did not associate with the plasma membrane (Figs 3E and S2B), indicating the importance of the linker region in mediating this interaction. Similar results were obtained with copine-6 (Figs 3G and S2B). Quantitative analysis of several experiments showed that, for copines-2 and -6, the C2-domain constructs behaved similarly to the full-length copine–EYFP following ionomycin treatment (Fig. 3F). In addition, the C2-domain constructs of copine-2 containing the copine-2 linker region, tagged at the N-terminus with EGFP or at the C-terminus with EYFP, responded robustly to methacholine, whereas if the linker region was removed no response was observed (see both Figs 5 and 6). In contrast, the EYFP–vWA-domains of the copines showed no response to ionomycin, despite the presence of the linker region (data not shown).

Taken together, the investigation of the behaviour of the different truncations and domain swap constructs showed that the C2-domains are essential for calcium-mediated membrane binding, but that the binding requires the presence of the linker region, proximal to the vWA-domain (see Fig. S3).

Copine-6 associates with clathrin-coated vesicles in a calcium-dependent manner which is regulated by both the C2- and vWA-domains

During the course of these experiments, we noted that ionomycin treatment of copine-6 (but not copine-2)-expressing cells appeared to show copine-6-containing vesicles in cytoplasm after 3 min of exposure to ionomycin (Fig. 3G, H). Indeed, when myc-tagged copine-2 and HA-tagged copine-6 were co-expressed in the same cells, and the cells were exposed to ionomycin, only HA-tagged copine-6 was found in intracellular vesicles (Fig. 3H). A fusion construct of C2-domains of copine-2 (including the linker of copine-2) and the vWA-domain of copine-6 behaved similarly (Fig. 3G), whereas the C2-domains of copine-2 alone exhibited a pattern identical to full-length copine-2 (Fig. 3E). Thus, the association of copine-6 with vesicles appears to require the vWA-domain of copine-6.

In order to investigate this further, HEK-293 cells expressing either HA- or EYFP-tagged copines-2, -3 or -6 were stimulated for 3–5 min with ionomycin, and immunostained using markers for clathrin-mediated endocytosis (transferrin), caveolar endocytosis (caveolin) or a late endosome marker (mannose-6-phosphate receptor). Neither caveolin nor mannose-6-phosphate receptor staining colocalized with any of the copines (data not shown). However, the copine-6-containing vesicles (Fig. 7A1), but not copines-2 or -3 (Fig. 7A2, A3), colocalized with Alexa-Fluor-568-conjugated transferrin. To visualize the effect of ionomycin on the formation of copine-6-containing vesicles, we imaged live cells, transfected with either copine-6–EYFP or copine-2–EYFP and incubated with fluorescent transferrin. In untreated cells, transferrin was associated with internalized clathrin-coated vesicles and was partially diffused throughout the cell cytoplasm (Fig. 7A1, A2, top row). Ionomycin treatment of the cells caused fast translocation of both copines to the plasma membrane, with copine-6, but not copine-2, bound to the internalized clathrin-coated vesicles containing transferrin. Ionomycin therefore did not cause

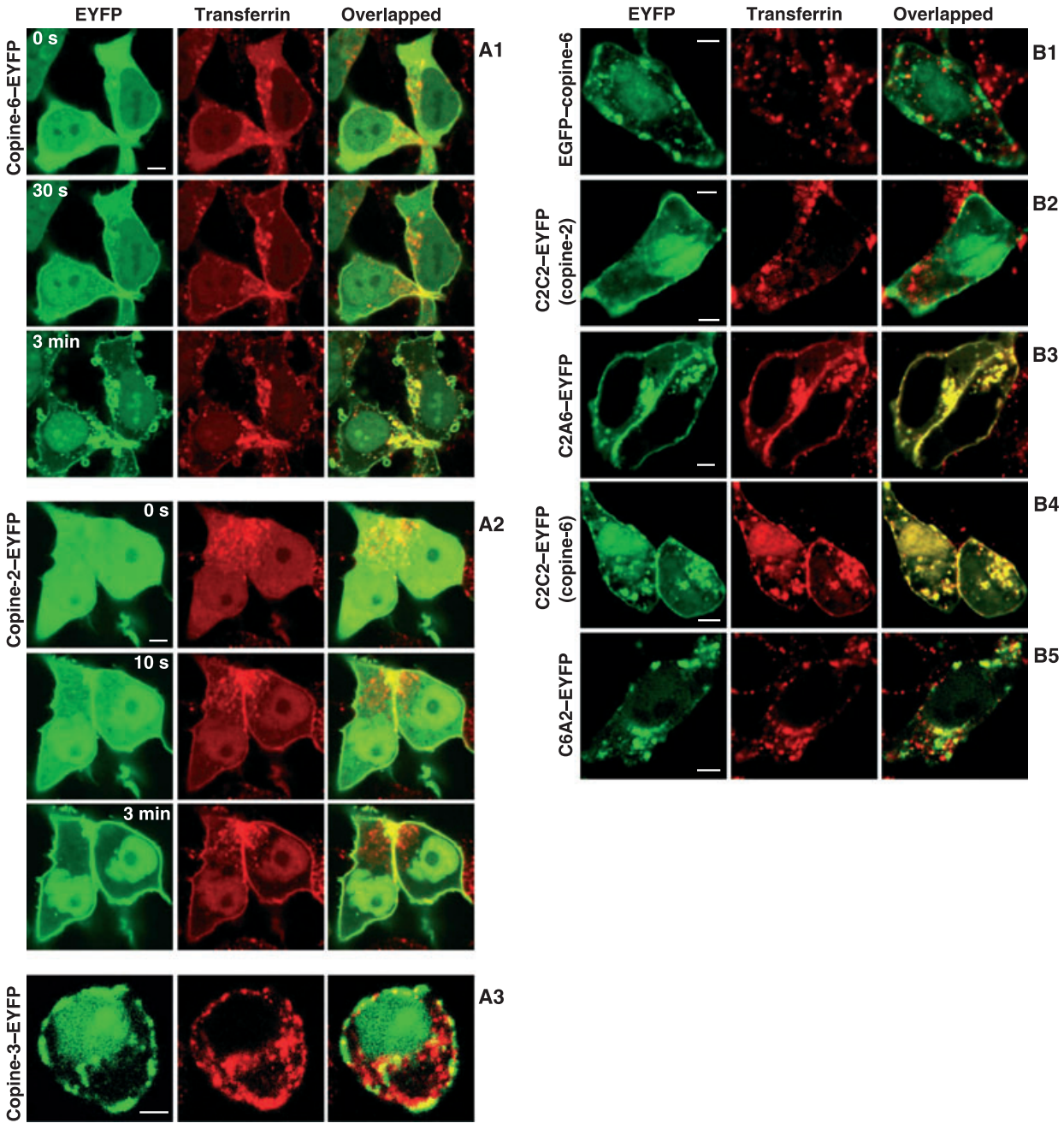

Fig. 7. Copine-6 associates with clathrin-coated vesicles following increases in intracellular calcium. HEK-293 cells expressing copines were pre-incubated with Alexa546-conjugated transferrin, washed and treated with ionomycin for 5 min in the presence of 1.8 mM extracellular Ca^{2+}. Green corresponds to EYFP or EGFP fluorescence, red to transferrin fluorescence. Live HEK-293 cells expressing copine-6–EYFP (A1) or copine-2–EYFP (A2) were imaged before, 10 s and 3 min after ionomycin application. Alternatively, cells were fixed after ionomycin treatment and immunofluorescence was used to visualize copine-3–EYFP (A3). Similar experiments were performed with the cells fixed after 5 min using N-terminally tagged copine-6 (EGFP–copine-6) (B1), the C2C2–EYFP domains of copine-2 (B2) and copine-6 (B4) or the domain recombination constructs C2A6–EYFP (B3) and C6A2–EYFP (B5). Scale bars, 5 μm.

the internalization of copine-6, but rather stimulated its association with clathrin-coated membranes of internalized early endosomes and with the plasma membrane (Fig. 7A1, A2). To determine which domains of the copines contribute to the endosomal association of copine-6, different chimaeric copine constructs were examined following ionomycin treatment of cells labelled with transferrin. Myc-tagged copine-6

and copine-6–EYFP, but not the N-terminally tagged EGFP–copine-6, colocalized with clathrin-mediated internalized vesicles following ionomycin treatment (Fig. 7B1, B2, B3). The domain swap C2A6–EYFP construct and the C2C2–copine-6 derivative also bound to the transferrin-containing vesicles (Fig. 7B3, B4), unlike the C6A2–EYFP construct (Fig. 7B5). In contrast, neither copine-2 nor its C2-domain–EYFP construct bound to the transferrin-containing vesicles (Fig. 7A2, B2). Thus, the N-terminal copine-6 C2-domains appear to contribute to endosomal vesicle binding, but are not sufficient to confer this property to the copine-2 vWA-domain. Thus, the copine-6 vWA-domain seems to carry an endosomal targeting sequence which can confer endosomal binding to the C2-domains of copine-2.

Discussion

In the present study, we have shown for the first time that, in mammalian cell lines, copines-1, -2, -3, -6 and -7 can move to the plasma membrane following increases in intracellular Ca^{2+} triggered by ionomycin treatment of cells in medium containing 1.8 mM calcium. Structural considerations [4,8] and *in vitro* binding studies [5] had suggested that the copines should associate with cell membranes in a calcium-dependent manner. To date, this hypothesis had only been demonstrated in *Dictyostelium*, where a small percentage (1–4%) of cells showed copine-A translocated to the plasma membrane after starvation [11]. Our finding that copine-3 can translocate to the plasma membrane in response to ionomycin treatment has been confirmed in a recent study on the role of copine-3 in cell migration [26]. In that study, and in the present study, ionomycin also caused nuclear translocation of copine-3, as well as copines-1, -2, and -7. In contrast, copine-6 did not show nuclear translocation after exposure of cells to ionomycin, but did bind to intracellular vesicles, identified as early endosomes, as well as the plasma membrane. The copines showed different rates of calcium-induced membrane translocation, with copine-2 and copine-7 moving most rapidly, followed by copines-1, -6 and -3.

In calcium-free medium, neither ionomycin nor thapsigargin caused movement of the copines to the plasma membrane, despite both agents causing increases in intracellular calcium. The addition of 2 mM calcium to the medium of cells treated with either agent caused rapid movement of copine-2 to the cell membrane. However, after thapsigargin, but not ionomycin, treatment, copine-6 required the addition of higher concentrations of extracellular calcium (5 mM) than did copine-2 to trigger its movement. This may reflect the fact that ionomycin forms calcium-permeable pores in the cell membrane, permitting a rapid increase in intracellular calcium from the extracellular medium [27,28]. In contrast, calcium enters the thapsigargin-treated cells through store-operated calcium channels, opened by the release of calcium from intracellular stores by the drug [29–31], and this release can be blocked by 2-APB and Gd^{3+} (Fig. 4), as has been reported previously for HEK-293 cells [32,33]. Therefore, the rate of calcium entry into the thapsigargin-treated cells may be slower than that in ionomycin-treated cells, and more dependent on the calcium concentration difference across the membrane. Thus, the higher extracellular concentrations of calcium needed to mobilize copine-6 following thapsigargin treatment suggest that it has a lower affinity for calcium than does copine-2. A higher affinity for calcium of copine-2 may also explain its more rapid rate of membrane translocation in response to ionomycin treatment and its stronger response to methacholine stimulation, compared with the responses of copine-6. Such differences in calcium sensitivity have been observed in other families of C2-domain-containing proteins, such as, for example, DOC2A and DOC2B [34], and even between different C2-domains from within a protein family (e.g. the protein kinase C family [35]).

Following the activation of the endogenous muscarinic receptor in HEK-293 cells by methacholine, the copines again exhibited different levels of membrane translocation, with copine-2 and copine-7 showing a rapid and robust movement to the cell membrane, with weaker responses from copine-6 and copine-3. In HEK-293 cells, the full response of the proteins to methacholine was dependent on calcium entry through store-operated calcium channels. Thus, regardless of the stimulus used to increase intracellular calcium, the copines showed different rates of membrane association and different sensitivities to the levels of intracellular calcium. Nevertheless, in order to exhibit a full response to either ionomycin or methacholine, the copines require calcium entry from the extracellular medium.

The calcium-dependent association of the copines required the presence of the C2-domains, as these contain the calcium-binding sites [36]. However, interestingly, we found that both copine-2 and copine-6 required a specific region between the second C2-domain and the vWA-domain for membrane association, which we termed the 'linker region' (Figs 1C and S4). The linker region contains two sections that are strongly conserved in all mouse copines. The first is proximal to the C-terminus of the second C2-domain, which contains several positively charged

amino acids, and the second is situated near the start of the vWA-domain. The positively charged sequence is preserved in the C2-domain constructs that did not show calcium-dependent membrane association, and therefore the membrane-binding site must lie in the C-terminal segment of the linker region. Lipid binding of C2-domains of other proteins has been attributed to two regions: the calcium-binding region and a cationic β-groove located in strands β3 and β4 of the protein [35,36]. In the case of the copines, the linker region identified here lies outside the canonical C2-domain. In the only study to date on isolated copine-6 domains, binding of both domains without the linker region to phosphatidylserine vesicles was observed, with the first C2-domain exhibiting calcium-independent vesicle binding [16]. This indicates that the single C2-domains fused to glutathione S-transferase and expressed in *Escherichia coli* can bind to lipids in *in vitro* assays, but our results show clearly that the linker is critical for membrane association *in vivo* in cells, where perhaps it acts to stabilize membrane binding following a transient C2-domain-mediated initial interaction.

The linker appears to be insufficient to promote membrane association, as the vWA-domains containing the linker do not bind to cell membranes. However, the vWA-domains can clearly modulate the responses of the C2-domains to calcium, and possibly play a role in intracellular targeting. The altered methacholine response of the different constructs of copine-2 indicates a role for the vWA-domain in modulating its calcium responsiveness. Thus, replacement of the copine-2 vWA-domain with that of copine-6 ablates the methacholine response, but not the ionomycin response, of the hybrid construct. Similarly, removing the copine-2 vWA-domain renders the C2C2-linker construct of copine-2 more responsive than full-length copine-2 to methacholine challenge in both calcium-free medium and in the presence of 2-APB (see Figs 5 and 6). A contribution of the vWA-domains to the intracellular targeting of the copines is indicated by our finding that the exchange of the vWA-domain of copine-6 with that of copine-2 is sufficient to prevent endosome association of the chimaeric construct C6A2. Furthermore, unlike copine-2, the C2A6–EYFP construct does not show nuclear localization, but does show early endosome binding, following ionomycin treatment of the cells (see Fig. 7). The fact that the majority of the copines, but not copine-6, show nuclear targeting suggests a role for these copines in nuclear processes. The recent demonstration that copine-3 appears to bind several nuclear proteins, including interleukin enhancer-binding protein 2, nucleolin and DNA topoisomerase 1, is consistent with such a role for copine-3 [26], as is the finding that copine-1 is involved in the regulation of NF-kappaB transcriptional responses via endoproteolysis of the p65 protein [37,38].

The physiological function of the majority of the copines is currently unclear. However, a recent report showing that copine-3 interacts with Erb-2, is upregulated in breast and prostate tumours, and promotes tumour migration by recruiting RACK1 to focal adhesion plaques [26] indicates the importance of studying this family of proteins. The data presented here show, for the first time, that the copines will move to the plasma membrane in response to increases in intracellular calcium. However, it is clear that they show different sensitivities to calcium. The finding that copines have distinct properties suggests that each copine may be tailored to respond to specific physiological stimuli: for example, in the present study, copine-3 did not respond to stimulation of the muscarinic receptor, whereas previously it was found to respond to activation of the ErbB2 receptor by heregulin [26]. In addition, our data show that, although the C2-domains of the copines are essential for calcium-dependent membrane binding, they are not sufficient, and we have identified a conserved linker region in the copines, between the C2- and vWA-domains, that is necessary for membrane binding in living cells. We have also shown that the vWA-domains contain targeting information, and can modulate the calcium sensitivity of the proteins. Our data also indicate that many of the copines, but not copine-6, show nuclear localization, either normally (e.g. copines-2 and -7) or following elevation of intracellular calcium (copines 1 and -3). We conclude that the copines are likely to mediate calcium-dependent targeting of proteins to various intracellular locations, including the plasma membrane and the nucleus. The rapid translocation of the copines in response to changes in intracellular calcium suggests that this family of proteins may play an important role in calcium-dependent intracellular signalling.

Materials and methods

DNA constructs

Coding sequences of mouse copines-2 and -6, and human copines-1, -3 and –7, were derived from image clones: IMAGE:3985959, IMAGE:6591063, IMAGE:3502122, IMAGE:5300530 and IMAGE:5727324, respectively (GeneService Ltd.; http://www.geneservice.co.uk). N-terminally HA-tagged copine-3 was amplified by PCR from the IMAGE clone DNA in two steps, where the product of the first step was used in the second round of PCR. Step 1

primers: 5′-CCGTATGACGTCCCAGATTACGCATCGA TGGCTGCCCAGTGTGTCAC-3′ and 5′-GGGGATCCT CACTGCTTCTGTTGTTTCGTGG-3′. In step 2, primer 5′-CCTCTAGACGCCGCCACCATGCCGGATTACGCG TCTTACCCGTATGACGTCCCAGATT-3′ was used with the second primer of step 1. The PCR product was cloned into the *Xba*I and *Bam*HI sites of the pcDNA3.1(–) vector (Invitrogen, Paisley, Renfrewshire, UK). The same scheme was applied to clone HA–copine-6 with primers 5′-CCGTA TGACGTCCCAGATTACGCATCGATGTCGGACCCA GAGATGGGATG-3′ and 5′-GGGGATCCTCATGGG CTAGGGCTGGGAGTC-3′ used in the first round of PCR. N-terminally myc-tagged copine-2 was amplified from its IMAGE clone with the primers 5′-CGAATTCGGATG GCCTACATTCCGGATGG-3′ and 5′-CGCTCGAGTCA GGCAGGCTCTGAGTTGGTG-3′, and cloned between the *Eco*RI and *Xho*I sites of pCMV-myc (Clontech, Mountain View, California, USA). N-terminally myc-tagged copine-6 was made by substitution of the *Spe*I–*Cla*I fragment of HA–copine-6 with the DNA fragment amplified from pcDNA3.1(–) with primers 5′-GTTTCTGATTATTGAC TAGTTATTAATAGTAATCAATTACGGG-3′ and 5′-GT TTCTATCGATGACAAGTCCTCTTCAGAAATGAGCT TTTGCTCCATGGTGGCGGCGTCTAGAG-3′. N-terminally myc-tagged copines-1 and -7 were made by substitution of the *Hind*III–*Cla*I fragment in the myc–copine-6 construct with the respective products of PCR amplification (5′-GTTTCTATCGATGGCCCACTGCGTGACCTTGG-3′, 5′-GTTTCTAAGCTTTTAAGCCTGGGGGGCCTGTGC AG-3′ and 5′-GTTTCTATCGATGAGCGCGGGCTCGG AGCG-3′, 5′-GTTTCTAAGCTTTCACGGTGTGCAGCC TGGGCTG-3′).

To clone EGFP fusion proteins, the PCR amplification products (5′-GTTTCTGAATTCCATGGCCTACATTCCG GATGGG-3′ and 5′-GTTTCTGGATCCTCAGGCAGG CTCTGAGTTGGTG-3′) of the copine-2 IMAGE clone were inserted into the pEGFP-C1 vector (Clontech) using its *Eco*RI and *Bam*HI sites. The EGFP–HA–copine-6 construct was cloned by inserting the *Xba*I–*Bam*HI fragment of HA–copine-6 into the pEGFP-C1 vector. To clone C-terminal EYFP-tagged copines-1, -2, -3, -6 and –7, PCR amplification products of corresponding IMAGE clones were inserted into the pEYFP-N1 (Clontech) vector (copine-1–EYFP: 5′-GTTTCTGAATTCGCCACCATGGCC CACTGCGTGACCTTGG-3′ and 5′-GTTTCTACCGGT CCTGAAGCCTGGGGGGCCTGTGCAG-3′, *Eco*RI–*Age*I; copine-2–EYFP: 5′-CCAGATCTCCATGGCCTAC ATTCCGGATGGG-3′ and 5′-CCCTCGAGGGCAGGCT CTGAGTTGGTG-3′, *Bgl*II–*Xho*I; copine-3–EYFP: 5′-GT TTCTCTCGAGGCCGCCACCATGGCTGCCCAGTGTG TCAC-3′ and 5′-GTTTCTGGATCCGTACTCTGCTTCT GTTGTTTCGTGG-3′, *Xho*I–*Bam*HI; copine-6–EYFP: 5′-GTTTCTGAATTCTAGCCACCATGTCGGACCCAG AGATGGGATG-3′ and 5′-GTTTCTGGATCCG ATGGG CTAGGGCTGGGAGTCATAG-3′, *Eco*RI–*Bam*HI; copine-7–EYFP: 5′-GTTTCTGCTAGCGCCACCATGAGC GCGGGCTCGGAGCG-3′ and 5′-GTTTCTAAGCTTTG ACGGTGTGCAGCCTGGGCTG-3′, *Nhe*I–*Hind*III). The C2C2-domains of copines-2 and -6 were amplified by PCR from the corresponding IMAGE clones and inserted between the *Eco*RI–*Bam*HI sites of the pEYFP-N1 vector. The primers used to produce the C2C2-domains lacking the linker region were 5′-GTTTCTGAATTCTGGCCAC CATGGCCTACATTCCGGATGGG-3′ and 5′-GTTTCT GGATCCGCGCTTTTCTTCTTCCTCTGCTTCTTGG-3′ (C2C2–copine-2), and 5′-GTTTCTGAATTCTGGCCACC ATGTCGGACCCAGAGATGGGATG-3′ and 5′-GTTTC TGGATCCAGCTTGTAATTCTTCTTCTTGTCTCGGTA CTTGG-3′ (C2C2–copine-6). The reverse primers for the C2C2-linker domains were 5′-GTTTCTGGATCCCCGCA GCCTCCCAGAATGTAGTCCAG-3′ (C2C2-linker, copine-2) and 5′-GTTTCTGGATCCCCGCAGCCACCCAT GATATAATCCAGG-3′ (C2C2-linker, copine-6). To produce N-terminally EGFP-tagged C2C2-domains of copine-2 with/without the linker area, products of EGFP–copine-2 PCR amplification with primers 5′-GTTTCTGCAGAGC TGGTTTAGTGA-3′ and 5′-GTTTCTGGATCCCTAGCA GCCTCCCAGAATGTA-3′/5′-GTTTCTGGATCCCTAG CTTTTCTTCTTCCTCTG-3′ were cloned into the pEGFP-C1 vector using the *Age*I and *Bam*HI sites. The vWA-domains of copines-2 and -6 were amplified by PCR and inserted into the *Eco*RI–*Bam*HI sites of the pEGFP-C1 vector using the primers 5′-GTTTCTGAATTCAAAGA AGCAGAGGAAGAAGAAAAGCTACAAG-3′ and 5′-GT TTCTGGATCCTCAGGCAGGCTCTGAGTTGGTG-3′ (copine-2), and 5′-GTTTCTGAATTCAAAGTACCGAG ACAAGAAGAAGAATTACAAGAG-3′ and 5′-GTTTCT GGATCCTCATGGGCTAGGG CTGGGAG-3′ (copine-6). To clone EYFP-tagged chimaera of the C2C2-domains of copine-2 with the vWA-domain of copine-6 (C2A6) with the copine-2 linker, the copine-6 vWA-domain, amplified with primers 5′-GTTTCTGGATCCTCAGATCAGCTTC ACGGTGGCTATC-3′ and 5′-GTTTCTGGATCCGATG GGCTAGGGCTGGGAGTCATAG-3′, was inserted into the *Bam*HI site of the C2C2–copine-2* construct. For the C2A6* chimaera, containing the copine-6 linker between the C2C2- and vWA-domains, the PCR amplification product (5′-GTTTCTGGATCCAAAGTACCGAGACAA GAAGAAGAATTACAAGAG-3′ and 5′-GTTTCTGGAT CCGATGGGCTAGGGCTGGGAG-3′) was inserted into the *Bam*HI site of the C2C2–copine-2 construct. The C6A2 (fusion of the C2C2-domains of copine-6 and the vWA-domain of copine-2) chimaera was obtained by insertion of the PCR amplification product of the copine-6 vWA-domain into the *Bam*HI site of the C2C2-linker (copine-6) construct (primers: 5′-GTTTCTGGATCCTCAGCTCATG TTCACCGTTGGAATAG-3′ and 5′-GTTTCTGGATCCG AGGCAGGCTCTGAGTTGGTGGG-3′). The assignment of the domain boundaries of the different regions of the copines was based on their analysis in the Simple Modular

Architecture Research Tool (SMART: http://smart.embl-heidelberg.de/). The range and structure of the constructs used in this study are illustrated schematically in Fig. 1A.

Cell culture and transfection

HEK-293 cells (ECACC Cat. No. 851120602) and COS-7 cells were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich, Poole, Dorset, UK), supplemented with 10% (v/v) fetal bovine serum (GIBCO, Paisley, Renfrewshire, UK), 2 mM L-glutamine, 50 $U \cdot mL^{-1}$ penicillin and 50 $\mu g \cdot mL^{-1}$ streptomycin (all from Sigma-Aldrich), at 37 °C in 5% CO_2, 100% humidity. Only the third to 15th passages of HEK-293 cells were used for live imaging. For microscopy, cells were plated onto borosilicate glass coverslips coated with poly-D-lysine for HEK-293 cells, and grown for 48 h prior to transfection. Cells were transiently transfected with polyethyleneimine according to the protocol adopted from Durocher *et al.* [39], 24 h prior to fixation or live imaging. The transfection efficiency varied between 30 and 70%.

Cell imaging and microscopy

All live imaging was performed at 25 °C in HBS buffer (10 mM Hepes, 150 mM NaCl, 5 mM KCl, 1.8 mM $MgCl_2$, 5.3 mM D-glucose, pH 7.4), with 1.8 mM $CaCl_2$ added for Ca^{2+} studies.

For calcium imaging, HEK-293 cells were loaded with 1 μM Fluo-4FF (Invitrogen) in HBS for 30 min at room temperature, followed by three rinses in HBS. Coverslips were mounted in a slide-holder chamber in 0.5 mL HBS for imaging. The fluorescent images (512 × 512 or 1024 × 1024 pixels, one scan per frame) were taken with an LSM510 inverted confocal microscope system and a Plan-NEOFLUAR 40×/1.3 oil DIC immersion lens (Carl Zeiss Ltd., Welwyn Garden City, Hertfordshire, UK; excitation 488-nm and emission 530–550-nm bypass filter, or excitation 543-nm and emission 560-nm long-pass filter; optical slice, 0.1–0.3 μm). For live internalization imaging, cells were pre-incubated with transferrin conjugated with ALEXA 568 (50 $\mu g \cdot mL^{-1}$, Invitrogen) for 5 min at room temperature, rinsed three times with HBS and imaged after 5 min ionomycin stimulation. The fluorescence intensity from selected areas in each frame was calculated using Zeiss LSM510 software, and the data were then exported to Microsoft Excel, SIGMAPLOT 10 (Systat Software Inc., Chicago, IL, USA) or SPSS 16 (SPSS Inc., Chicago, IL, USA) for further analysis. The immunofluorescence was expressed as the fluorescence intensity in a defined region of interest divided by that in the same region at the start of the experiment (F/F_0). For live imaging, calcium and all drugs were added to the cell by bath application in HBS buffer. For intracellular protein localization, cells were fixed in 4% (w/v) paraformaldehyde in HBS (pH 7.4) for 5 min at room temperature, washed 2 × 5 min in Tris-saline and, where appropriate, permeabilized with 0.2% (v/v) Triton X-100 for 5 min. Nonspecific binding was blocked by incubating the cells with 1% (w/v) bovine serum albumin for 30 min. The cells were then incubated in blocking solution with primary antibody for 2 h at room temperature, washed in HBS for 3 × 5 min, incubated for 1 h with the appropriate secondary antibody, washed in HBS for 3 × 5 min and mounted for imaging.

Western blotting

Cells were scraped into solubilization mixture [1% (v/v) Triton X-100, HBS buffer, pH 7.4, 10 mM EDTA] containing the recommended concentration of Complete™ protease inhibitor cocktail (Roche, Welwyn Garden City, Hertfordshire, UK), triturated with a pipette and incubated at 4 °C for 1 h. The lysate was cleared by centrifugation, subjected to SDS/PAGE and blotted onto Polyscreen® poly(vinylidene difluoride) membrane (Perkin Elmer, Cambridge, UK). Blots were probed with antibodies as indicated in the figure legends. Detection was by horseradish peroxidase-conjugated secondary antibodies (Promega, Southampton, Hampshire, UK) and Super Signal® West Pico chemiluminescent substrate (Thermo Scientific, Loughborough, Leicestershire, UK).

Other reagents and antibodies

Reagents: ionomycin from *Streptomyces conglobatus*, thapsigargin, acetyl-β-methylcholine chloride, poly-D-lysine hydrobromide, gadolinium(III) chloride hexahydrate and 2-APB (all from Sigma-Aldrich); carbamoylcholine chloride (Fluka, Gillingham, Dorset, UK). Antibodies and conjugates: chicken anti-mannose-6-phosphate receptor (cation-independent) (Chemicon, Temecula, CA, USA, AB3463); mouse anti-γ-adaptin (BD Transduction Labs, Oxford, UK, A36120); rabbit anti-caveolin-1 (BD Transduction Labs, 610406); rhodamine-conjugated dextran (Invitrogen, D-1824); transferrin from human serum Alexa-568 conjugate (Invitrogen, T-23365); rabbit anti-HA (Abcam, Cambridge, UK, 9119-100); monoclonal anti-myc 9E10, goat anti-rabbit Alexa Fluor 488 or 568 IgG (Invitrogen); goat anti-mouse Alexa Fluor 488 or 568 IgG (Invitrogen). All other high-purity grade chemicals were purchased from Sigma-Aldrich, BDH (West Chester, PA, USA) or Fluka.

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Supporting information

The following supplementary material is available:
Fig. S1. Expression of myc-/HA-tagged copines in cultured mammalian cells.
Fig. S2. Effect of sustained Ca^{2+} influx into HEK-293 cells, triggered by application of 5 μM ionomycin, on some EGFP-/EYFP-tagged copines and their C2C2-domains.
Fig. S3. Schematic diagram of the methacholine and ionomycin responses of copine-2 and copine-6.
Fig. S4. Alignment of linker region sequences of copine family members.

This supplementary material can be found in the online version of this article.

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