

# G protein–coupled receptor/arrestin3 modulation of the endocytic machinery

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**N**onvisual arrestins (arr) modulate G protein–coupled receptor (GPCR) desensitization and internalization and bind to both clathrin (CL) and AP-2 components of the endocytic coated pit (CP). This raises the possibility that endocytosis of some GPCRs may be a consequence of arr-induced de novo CP formation. To directly test this hypothesis, we examined the behavior of green fluorescent protein (GFP)-arr3 in live cells expressing  $\beta_2$ -adrenergic receptors and fluorescent CL. After agonist stimulation, the diffuse GFP-arr3 signal rapidly became punctate and colocalized virtually completely with preexisting CP spots, demonstrating that activated complexes accumulate in previously formed CPs rather than nucleating new CP formation. After arr3 recruitment, CP appeared larger: electron microscopy analysis revealed an increase in both

CP number and in the occurrence of clustered CPs. Mutant arr3 proteins with impaired binding to CL or AP-2 displayed reduced recruitment to CPs, but were still capable of inducing CP clustering. In contrast, though constitutively present in CPs, the COOH-terminal moiety of arr3, which contains CP binding sites but lacks receptor binding, did not induce CP clustering. Together, these results indicate that recruitment of functional arr3–GPCR complexes to CP is necessary to induce clustering. Latrunculin B or 16°C blocked CP rearrangements without affecting arr3 recruitment to CP. These results and earlier studies suggest that discrete CP zones exist on cell surfaces, each capable of supporting adjacent CPs, and that the cortical actin membrane skeleton is intimately involved with both the maintenance of existing CPs and the generation of new structures.

## Introduction

During clathrin (CL)\* coated pit (CP)–mediated endocytosis, coats form on the cytosolic side of the plasma membrane through the assembly of CL triskelia by the tetrameric assembly protein (AP-2). A potential link between the endocytic pathway and extracellular stimuli is suggested by the ability of several coat proteins to bind phosphoinositides produced by receptor signaling (Norris et al., 1995; Ye et al., 1995; Gaidarov et al., 1996; Gaidarov and Keen, 1999) and to undergo reversible phosphorylation (Wilde and Brodsky, 1996; Slepnev et al., 1998; Chen et al., 1999; Chu et al., 1999) in response to receptor stimulation.

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\*Abbreviations used in this paper: AChR, acetyl cholinergic receptor; AR, adrenergic receptor; arr, arrestins; CBC, carbachol; CL, clathrin; CP, coated pit; CPZ, CP zone; GFP, green fluorescent protein; GPCR, G protein–coupled receptor; ISO, isoproterenol.

Key words: clathrin; AP-2; endocytosis; actin; membrane skeleton

Previous work from our laboratory showed that CPs preferentially form at discrete locations on the cell membrane and exhibit restricted lateral mobility (Gaidarov et al., 1999b), suggesting that the membrane cytoskeleton may function to anchor the coat assembly machinery. Furthermore, the number and location of these CP formation sites was not affected by increased levels of receptors on the plasma membrane (Santini and Keen, 1996; Santini et al., 1998; Warren et al., 1998). Nevertheless, in some cells, CP formation can be rapidly induced in response to certain stimulants (e.g., EGF, NGF, carbachol) (Connolly et al., 1981, 1984; Geisow et al., 1985; Wilde et al., 1999; Beattie et al., 2000), further suggesting a functional relationship between signaling receptors and the endocytic machinery, perhaps at the level of actin-anchored CP formation sites.

Many GPCRs have been shown to undergo CL-mediated endocytosis after stimulation, and arrestins (arr) ( $\beta$ arr and arr3, also called  $\beta$ arr1 and  $\beta$ arr2, respectively) (Attramadal et al., 1992), through their binding to both CL (Krupnick et al., 1997a) and AP-2 (Laporte et al., 1999), are pivotal in directing and concentrating activated GPCR into CPs for internalization (Goodman et al., 1996; Zhang et al., 1997). Arr are also thought to play a broader role as scaffolding pro-

teins in the formation of signaling platforms under stimulated GPCR (for a reviews see Ferguson, 2001; Miller and Lefkowitz, 2001; Pierce et al., 2001).

Here we have investigated responses of the endocytic machinery to GPCR activation. We show that the activated GPCR-arr3 complexes do not recruit coat components to form new CPs, but rather undergo endocytosis as a consequence of their rapid accumulation in preexisting CPs. However, cells responded to agonist stimulation by increasing the number and clustering of their CPs. Remarkably, rearrangement of the cortical actin membrane skeleton appears to be required to support formation of these clustered CPs. Our results thus provide evidence for the existence of actin-dependent geographically distinct assembly domains on the cell surface which we term "coated pit zones" (CPZ).

## Results

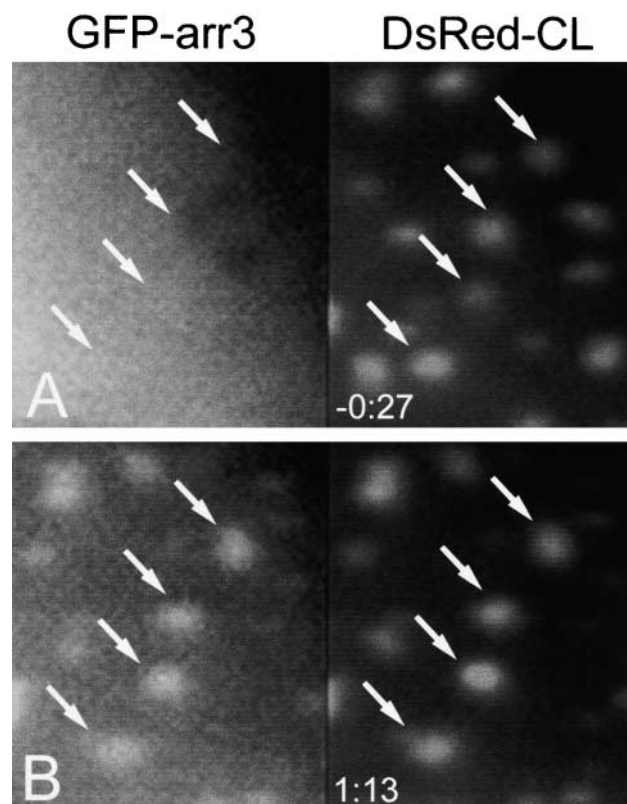
### Arr3 is recruited to preexisting CPs upon agonist activation

To directly determine whether activated GPCRs induce formation of new CPs or accumulate in preexisting structures, we visualized the behavior of both CPs and arr in living cells following agonist stimulation. HEK293 cells expressing DsRed-CL were cotransfected with green fluorescent protein (GFP)-arr3 and Flag-tagged  $\beta_2$ -adrenergic receptor (AR). In the absence of agonist, GFP-arr3 was predominantly diffuse (Fig. 1 A, and frame 1 of Video 1). In contrast, the DsRed-CL signal, as reported previously (Gaidarov et al., 1999b; Engqvist-Goldstein et al., 2001), was present in punctate endocytic CPs at the plasma membrane (Fig. 1) and in the perinuclear localization characteristic of the TGN (Keen et al., 1981).

Within seconds after isoproterenol (ISO) addition there was a striking concentration of arr3 signal in punctate spots which colocalized with DsRed-CL signal (Video 1, frames 11–15). With time, a gradual decrease in the level of diffuse arr3 signal also became apparent (compare first with last frame of Video 1), consistent with recruitment of arr from a cytosolic pool to the receptor at the plasma membrane. Comparison of the emerging GFP-arr3 spots with the punctate DsRed-CL signal marking CPs revealed that virtually all of the arr3 puncta appeared at loci of preexisting CPs (Fig. 1, and frames 11–30 of Video 1). For example, of 130 arr3 spots detectable 40 s after ISO addition (frame #20), 128 (98.5%) appeared at preexisting CP sites (i.e., CPs present in frame # 7). In subsequent frames both signals can be seen to disappear together, consistent with internalization of the arr- $\beta_2$ -AR complex by CP-mediated endocytosis (Video 1). Similar results were observed in transfected COS1 cells (unpublished data). These results provide direct evidence that the endocytic sequence initiated by activation of  $\beta_2$ -AR results in recruitment of arr3-receptor complexes to preexisting CPs. Video 1 is available at <http://www.jcb.org/cgi/content/full/jcb.200110132/DC1>.

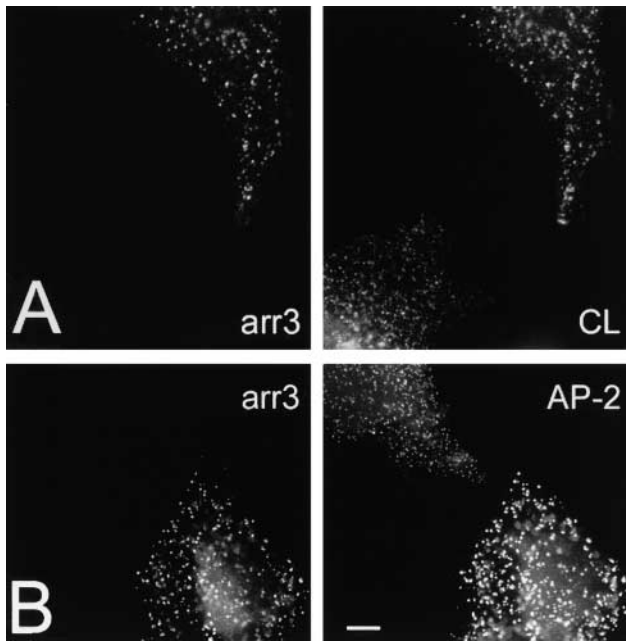
### CP changes are induced by $\beta_2$ -AR activation

In agonist-stimulated live cells we also observed significant changes in the appearance and distribution of plasma membrane CPs (unpublished data), but monitoring live



**Figure 1. Agonist activation induces recruitment of arr3 to preexisting CPs.** HEK293 cells coexpressing GFP-arr3, DsRed-CL, and Flag-tagged  $\beta_2$ -AR were viewed as described in Materials and Methods. A is frame 1 of Video 1 and shows the distribution of DsRed-CL and GFP-arr3 27 s before addition of ISO (10  $\mu$ M) to the cells. B is frame 20 of Video 1, acquired 73 s after stimulation and shows the redistribution of GFP-arr3 to preexisting CPs (arrows). Video 1 is available at <http://www.jcb.org/cgi/content/full/jcb.200110132/DC1>.

cells over extended periods presents technical difficulties. Accordingly, we used immunostaining to more easily investigate these changes in COS1 cells transiently expressing  $\beta_2$ -AR and arr3. As expected from the results described above and earlier work (Goodman et al., 1996; Krupnick et al., 1997b), after ISO stimulation for 5 min arr3 became colocalized with CL (Fig. 2 A). Under these conditions we noted that CPs adopted an altered distribution, with the CL puncta on the plasma membrane appearing both noticeably larger and, to some extent, fewer in number compared with those of nonexpressing cells in the same field. Because AP-2 is a structural component of plasma membrane CPs but, unlike CL, is not present in the TGN (Robinson, 1987), we used AP-2 labeling as a more direct indicator of endocytic CP changes upon agonist treatment. Following ISO stimulation of arr3-expressing cells, AP-2-labeled CPs underwent redistribution similar to that observed with CL (Fig. 2 B). Interestingly, we noticed that the AP-2 spots had become not only larger but also brighter after agonist stimulation, amounting to a 4–8-fold increase in comparison to neighboring nonexpressing cells. We also found that arr2 ( $\beta$ arr1), although capable of translocating to CPs and of mediating the internalization of activated  $\beta_2$ -AR upon expression in COS1 cells (Goodman



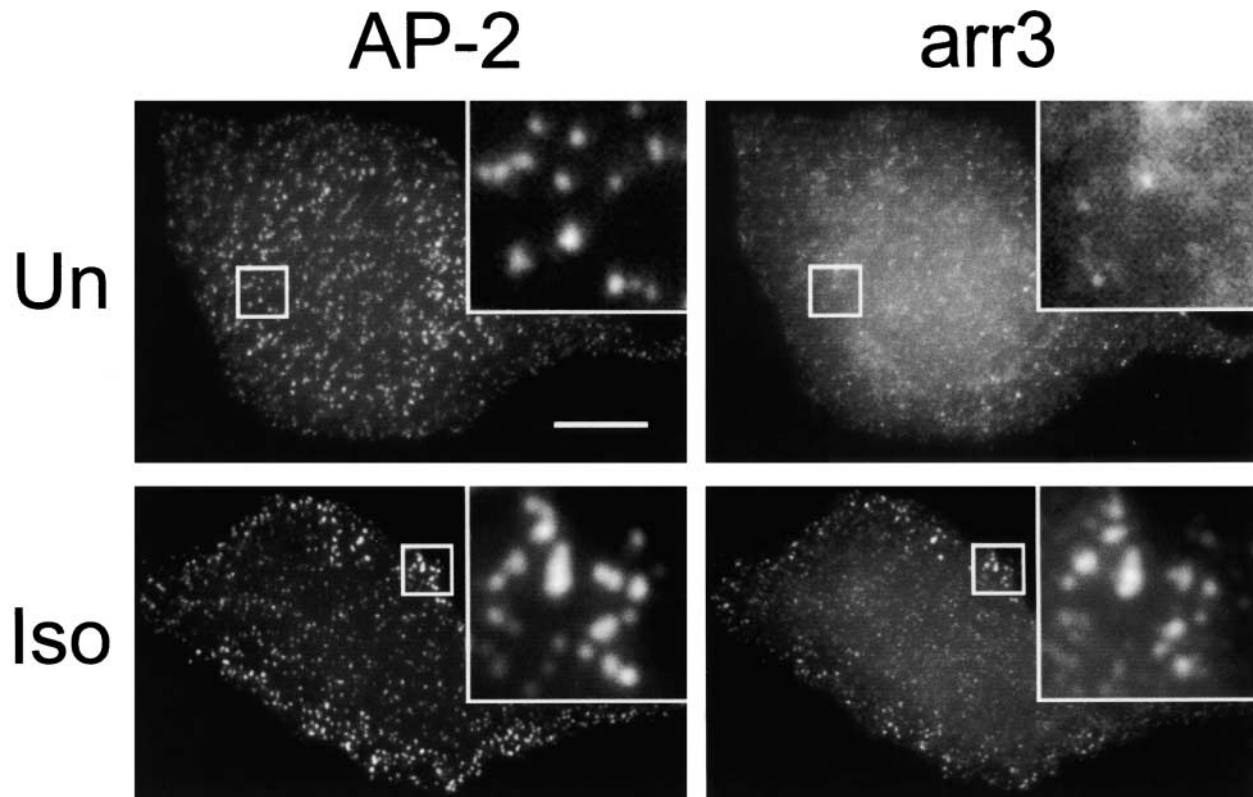
**Figure 2. Changes in CP appearance upon  $\beta_2$ -AR activation.** Following treatment with ISO, COS1 cells coexpressing Flag- $\beta_2$ -AR and arr3 show recruitment of arr3 to CPs, increase in CP size, and increased CP AP-2 signal in comparison to arr3-nonexpressing cells. (A) COS1 cells transiently expressing Flag- $\beta_2$ -AR and arr3 were stimulated with ISO (10  $\mu$ M, 5 min), fixed, and stained for CL and arr3. (B) COS1 cells transiently expressing Flag- $\beta_2$ -AR and arr3 were stained for AP-2 and arr3 after stimulation with ISO (10  $\mu$ M, 5 min). Bar, 10  $\mu$ m.

et al., 1996), was much less effective in inducing changes in CP distribution (unpublished data).

To investigate whether  $\beta_2$ -AR is able to induce alterations in CP properties in the absence of exogenous arr3 expression, we used RBL( $\beta_2$ ) cells, an RBL-2H3 subline that stably expresses Flag-tagged  $\beta_2$ -ARs (Santini et al., 2000). RBL cells express detectable levels of endogenous arr, making them an ideal system to study cellular responses to GPCR stimulation. Additionally, we have shown recently that treatment of these cells with ISO induces the selective recruitment of endogenous arr3 to CPs (Santini et al., 2000). Following ISO stimulation of RBL( $\beta_2$ ), examination of CP localization by immunostaining with either CL (unpublished data) or AP-2 antibodies (Fig. 3) revealed arr3 accumulation in larger CPs with increased AP-2 signal, similar to that observed in COS1 cells.

### m1 muscarinic receptor activation induces clustering of CPs

To probe whether the ability to alter CP patterns was shared by other GPCRs, we examined RBL-2H3 cells (RBL[m1]) stably expressing m1 acetylcholinergic receptors (m1AChR). Signaling events following agonist activation of the m1AChR in RBL-2H3 cells have been studied extensively (Jones et al., 1991; Hirasawa et al., 1995; Dippel et al., 1996) and include arr3 recruitment to CPs and m1AChR internalization (Santini et al., 2000). As observed after stimulation of  $\beta_2$ -AR in COS1 cells, stimula-



**Figure 3. Changes in plasma membrane CPs in RBL cells following  $\beta_2$ -AR stimulation.** RBL( $\beta_2$ ) cells stably expressing Flag- $\beta_2$ -AR show formation of larger CPs with increased AP-2 signal as well as recruitment of endogenous arr3 (arr3) to CPs after stimulation with ISO (ISO) (10  $\mu$ M, 5 min). Insets show fourfold magnified views of the boxed region of the cells. Bar, 10  $\mu$ m.

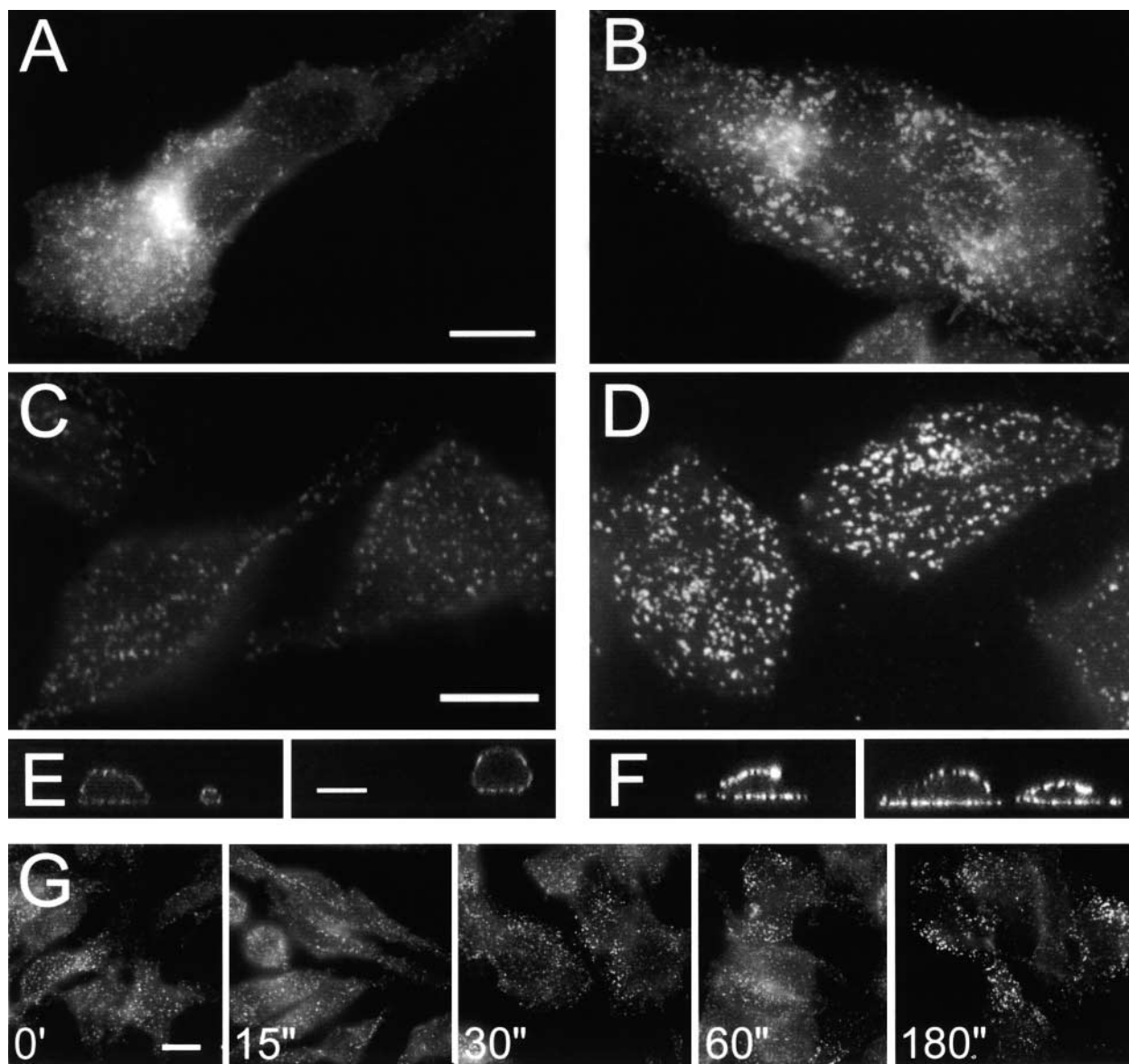


Figure 4. **m1AChR stimulation induces changes in plasma membrane CPs.** Stimulation of resting RBL(m1) cells (A) with CBC (1 mM, 3 min) induces the formation of larger CPs (B) as revealed by immunostaining for CL. Confocal microscopy images of untreated (C) or CBC-treated cells (D) immunostained for AP-2 also reveals the appearance of larger CPs with greatly increased AP-2 signal. These CPs are appropriately restricted to the plasma membrane (x-z sections; E and F). The confocal images were acquired and reproduced using identical gain settings and the same minimum and maximum display values for each unstimulated/stimulated pair. (G) Lower magnification views of changes in AP-2-labeled CPs over a 3 min period following stimulation. Bars: 10  $\mu$ m.

tion of RBL(m1) cells with the agonist carbachol (CBC) resulted again in CL puncta on the plasma membrane becoming noticeably larger than those observed in unstimulated cells (Fig. 4, compare A to B).

#### Digital image analysis of immunofluorescence CP spots

The apparent size of spots in fluorescence microscopy, especially for objects such as CPs that are at or below the diffraction limit, is a function of both the object size and relative brightness. Thus, a trivial explanation for the larger apparent size of CPs revealed by CL staining in stimulated cells could be that the individual spots are simply brighter. However, measurement of mean CL pixel intensities of spots in images from agonist-treated and untreated RBL(m1) cells collected

under identical instrument settings revealed no significant change in spot brightness (Table I). Rather, almost a doubling in the apparent CP spot size and a drop in the apparent density of CPs was observed (Table I). These results suggest that the physical size and/or distribution of CPs on the cell was indeed altered by agonist activation of m1AChRs.

When RBL(m1) cells were stained for AP-2 $\alpha$  the puncta in agonist-stimulated cells appeared larger (Fig. 4, compare C to D), consistent with the changes in the appearance of CL (Fig. 4, A and B). Furthermore, the AP-2 signal in these spots was also brighter compared with CP spots in unstimulated cells, though less pronounced than the increase observed in COS1 cells in response to  $\beta_2$ -AR stimulation. To assess whether these larger and brighter spots indeed re-



Table I. Changes in CP properties upon agonist stimulation characterized by light microscopy

	Basal	Stimulated
Mean brightness <sup>a</sup>	334.37 ± 5.22 ( <i>n</i> = 15)	376.85 ± 8.1 ( <i>n</i> = 24)
Spot density <sup>b</sup>	18.9 ± 1.18 ( <i>n</i> = 15)	12.8 ± 0.9 ( <i>n</i> = 24)
Average spot size <sup>c</sup>	9.07 ± 0.76 ( <i>n</i> = 284)	18.46 ± 1.89 ( <i>n</i> = 306)

<sup>a</sup>Average integrated pixel intensity ± SE in a 5.4 × 5.4 μm box. See Materials and methods for details.

<sup>b</sup>Average number of spots ± SE in a 5.4 × 5.4 μm box. See Materials and methods for details.

<sup>c</sup>Average size of a spot ± SE in square pixels. See Materials and methods for details. *n*, number of spots.

flected CPs at the cell surface rather than aberrant internal structures, we used confocal *x-z* sectioning of AP-2-immunostained cells. The images (Fig. 4, E and F) confirmed that the CPs were indeed restricted to the cell periphery as expected. In RBL(m1) cells marked changes in CP properties occurred between 1–3 min (Fig. 4 G). In the continued presence of agonist the altered CP profiles persisted, subsiding gradually after 10–15 min.

All of the properties noted above following GPCR stimulation were consistently observed when multiple antibodies against CL (mAb X22 or polyclonal R5, COM, 27004) or AP-2 (mAb AP.6 or polyclonal #31) were employed. In addition, varied fixation conditions, compatible with specific antibodies, all gave similar results.

### Ultrastructural analysis of agonist-induced changes in CP profiles

To learn more about agonist-induced changes in CP appearance, we examined control and treated RBL(m1) cells by electron microscopy. As can be seen from the examples presented in Fig. 5, both untreated (A) and CBC-treated cells (B–I) show similar characteristic, bristle-coated indentations of the cell surface typical of CPs. These images confirmed that the altered appearance of CPs at the light microscopy level after agonist treatment did not reflect gross malformation of coat structures, e.g., accumulation of empty lattices or CL aggregates that have been seen under nonphysiological conditions (Merisko et al., 1986; Sandvig et al., 1988; Heuser and Anderson, 1989).

However, quantitative analysis of these images did reveal several significant changes in CP distribution (Table II). First, stimulation of RBL(m1) cells with CBC for 3 min produced a 50% increase in the number of CPs ( $P = 0.001$ ). Second, a twofold increase in the proportion of shallow

and flat CPs at the expense of deep CPs was observed after stimulation. Most striking in the treated samples was a noticeable increase in the frequency with which CPs appeared to occur in close proximity to other CPs, i.e., the CPs appeared to be clustered, with the rim of one pit almost touching that of another (Fig. 5, C and E), suggesting the existence of CPZs. In the extreme example shown in Fig. 5 H, an extended region of coated membrane is visible, comprised of six or seven contiguous but discrete coated structures. In other instances, adjacent indented CPs appear to share the same opening (Fig. 5, I and G). To quantitatively analyze this phenomenon, we characterized CPs that were within 0.3 μm of each other as being ‘clustered’, i.e., essentially not resolvable by light microscopy. By this conservative criterion, the occurrence of clustered CPs increased 2.5-fold ( $P < 0.001$ ) in cells that had been stimulated for 3 min (Table II). However, the number of CPs in a given cluster was not changed by CBC treatment, and was between two and three on average (control =  $2.27 \pm 0.55$  SD [*n* = 31 clusters] and CBC =  $2.29 \pm 0.56$  SD [*n* = 57]). Thus, the dramatic appearance of larger CPs observed at the light microscope level (Figs. 2 and 4 and Table I) following agonist activation likely reflects multiple factors: the increase in number and clustering of CPs detected ultrastructurally, the intensified signal associated with these structures, and the limited resolution of the fluorescence light microscope.

### Mobilization of arr3 to CPs and onset of CP clustering are separable events

The ultrastructural results we obtained prompted us to consider in more detail the relationship between translocation of arr to CPs and the onset of clustering. In unstimulated RBL(m1) cells some punctate arr3 signal could be detected, but virtually none was coincident with CPs; in fact, the two

Table II. Ultrastructural changes in CP properties upon agonist stimulation

	Coated structures <sup>a</sup>	Shallow pits <sup>b</sup>	Deep pits <sup>c</sup>	Clusters <sup>a,d</sup>	Coated structures in clusters <sup>d</sup>
	per μm	%	%	per μm	%
Control	0.2 ± 0.02 ( <i>n</i> = 36) <sup>e</sup>	18.4	81.6	0.02 ± 0.003 ( <i>n</i> = 36) <sup>f</sup>	19.3
CBC	0.3 ± 0.03 ( <i>n</i> = 26) <sup>e</sup>	38.7	61.3	0.05 ± 0.009 ( <i>n</i> = 26) <sup>f</sup>	35.5

A total of 1.81 mm (Control) and 1.26 mm (CBC) of plasma membrane were analyzed. Coated structures were classified as described in Materials and methods.

<sup>a</sup>Data are means ± SEM.

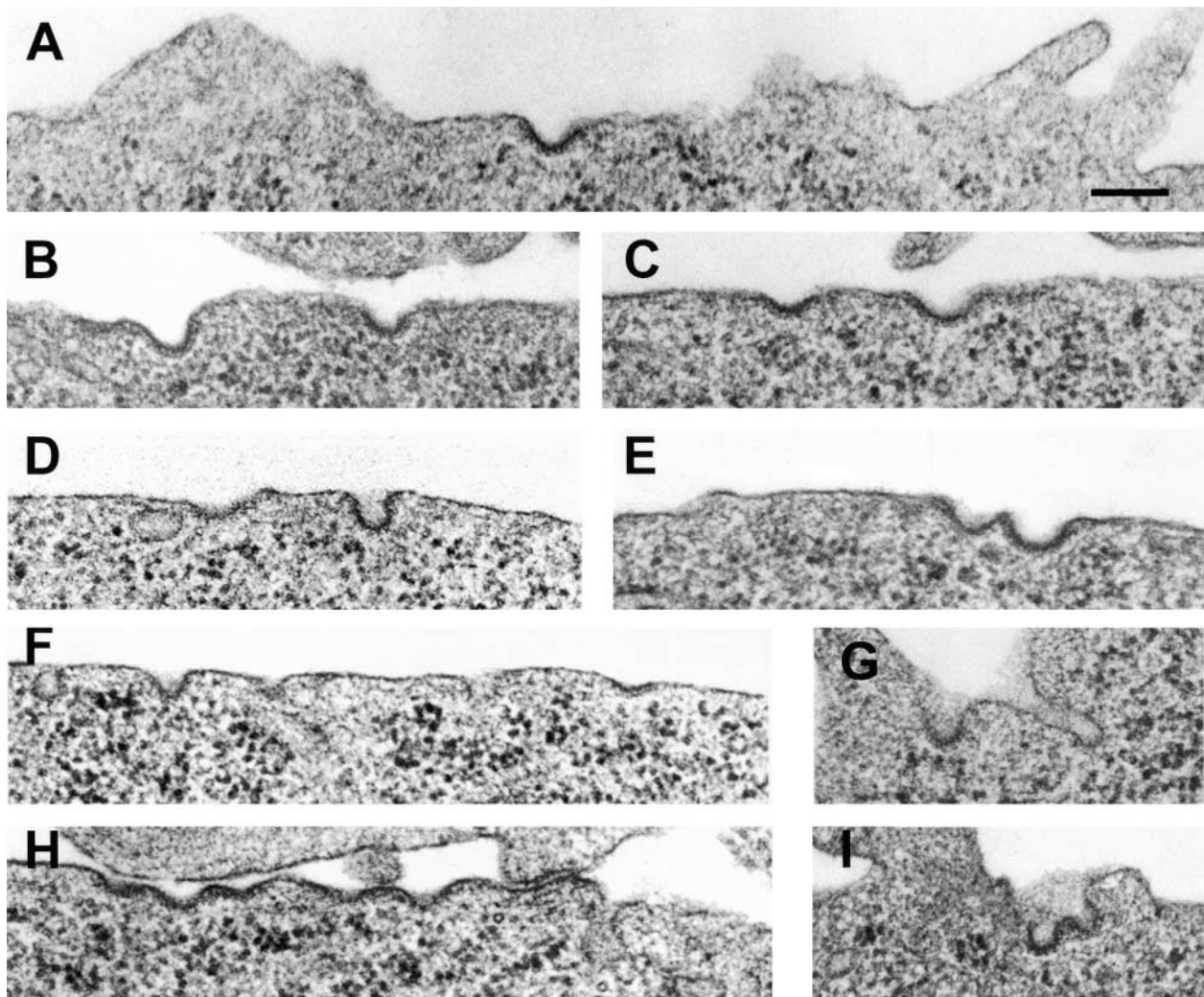
<sup>b</sup>‘Shallow pits’ include flat coated regions of plasma membrane; 3.3% and 7.3% of the total in control and CBC-treated cells, respectively.

<sup>c</sup>‘Deep pits’ include deeply invaginated-coated membranes and putative-coated vesicles (see Materials and methods). These ‘coated vesicles’ accounted for 33 and 23.1% of the total in control and CBC-treated cells, respectively.

<sup>d</sup>Clusters are defined as coated structures within 0.3 μm of each other. Clusters contained  $2.27 \pm 0.55$  SD (*n* = 31 clusters) and  $2.29 \pm 0.56$  SD (*n* = 57) coated structures in control and CBC-treated cells, respectively.

<sup>e</sup>The differences are statistically significant ( $P < 0.001$ ).

<sup>f</sup>The differences are statistically significant ( $P = 0.001$ ).



**Figure 5. Ultrastructural analysis reveals increased plasma membrane CP clustering in CBC-stimulated RBL(m1) cells.** Compared with unstimulated cells (A), agonist stimulation (1 mM, 3 min) produced an increase in the occurrence of nearby or adjacent CPs (B–I). These clusters, which often comprised multiple distinct membrane-coated regions, included pits at different stages of invagination (B–F, and H). See text for details. Bar, 0.2  $\mu$ m.

signals are remarkably separate (Fig. 6, A–C). Within 30 s after addition of agonist, some localization of arr3 to CPs is detectable, though no significant clustering of pits is apparent (Fig. 6, D–F). After 1 min of stimulation (Fig. 6, G–I), large CPs could be detected with some frequency. Interestingly, at early times arr3 was often seen to localize to only one of two adjacent AP-2 spots (Fig. 6, G, H, J, and K, arrows): at 30 s, 66% of clusters have arr3 in only one of the two adjacent CPs ( $n = 46$ ). This is suggestive of the presence of arr3 in only one of a nascent cluster of two or more adjacent CPs (Fig. 6, see merged panels and arrows). This phenomenon was less pronounced at longer times after agonist stimulation.

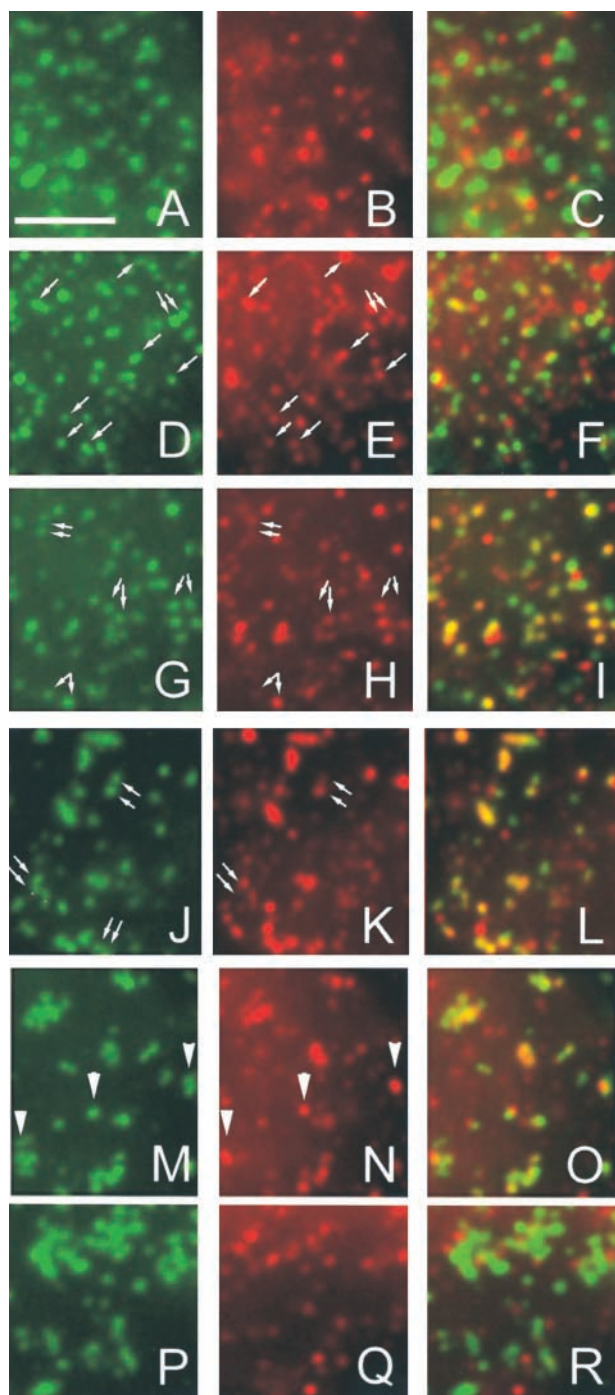
Generally similar results were seen in COS1 cells coexpressing  $\beta_2$ -AR and arr3, though the changes occurred more rapidly. Here, stimulation with ISO induced readily detectable arr recruitment to CPs in most cells as early as 15 s (Fig. 7). At this point, only a small fraction of cells displayed detectable changes in CP distribution, though these transfor-

mations followed rapidly. It was also possible to appreciate that arr3 recruitment preceded the agonist-induced increase in AP-2 brightness in these cells (Fig. 7, compare 15" to 30"), though the latter could not be resolved from changes in CP patterns (unpublished data).

We were further able to distinguish between arr recruitment to CPs and CP changes by stimulating COS1 cells at reduced temperature. Cells transiently expressing Flag- $\beta_2$ -AR and arr3 were stimulated with ISO at 16 $^\circ$ , and arr3 and AP-2 immunostaining were compared with that of cells stimulated at 37 $^\circ$ C. As expected (Cao et al., 1998), agonist was able to mobilize arr3 to CPs readily in both sets of cells. However, cells kept at 16 $^\circ$ C for up to 45 min failed to show changes in the pattern of CPs (unpublished data). Similar results were obtained upon CBC stimulation of RBL(m1) cells at reduced temperature (unpublished data).

Next we evaluated the effect of either agonist removal or antagonist administration upon CP changes. Addition to CBC-stimulated RBL(m1) cells of the m1AChR antagonists





**Figure 6. Endogenous arr3 is often concentrated in one CP within a cluster and CP clusters, but not arr3, persist after antagonist treatment.** RBL(m1) cells were stimulated with CBC (1 mM) for 0 (A–C), 0.5 (D–F), 1 (G–I), 3 (J–L), or 6 min (M–O). At zero time (A–C), endogenous arr3 (red) was not coincident with CPs, marked by AP-2 (green). Some coincidence of the two signals is evident within 30 s of CBC addition (D–F, arrows). Between 1–3 min after agonist treatment, arr3 is often localized within one of two adjacent CPs (G–L, arrows), less resolvable at later times (M–O). Note that the degree of CP clustering does not correlate closely with intensity of arr3 signal (M–O, arrowheads). P–R show cells treated for 3 min with CBC (1 mM) followed by the antagonist atropine (10  $\mu$ M) for 3 min. Under these conditions, arr3 has been virtually completely removed from the CPs but the clustered CP phenotype remains unchanged. Bar, 3  $\mu$ m.

atropine or pirenzepine (Fig. 6, P–R, and unpublished data), which immediately block agonist-induced tyrosine phosphorylation (unpublished data) and histamine release (Jones et al., 1991), caused essentially immediate disappearance of arr3. However, in both cases, the clustered CP persisted for at least several minutes (Fig. 6 P). Similar results were observed in ISO-stimulated COS1 cells expressing arr3 and  $\beta_2$ -AR upon agonist washout or upon addition of the antagonist alprenolol (unpublished data). Collectively, these results indicate that arr3 recruitment to CPs precedes, and is distinct from, reorganization of CP patterns on the surface of the cell. However, once established, maintenance of the clustered CP phenotype does not require the continued presence of arr3.

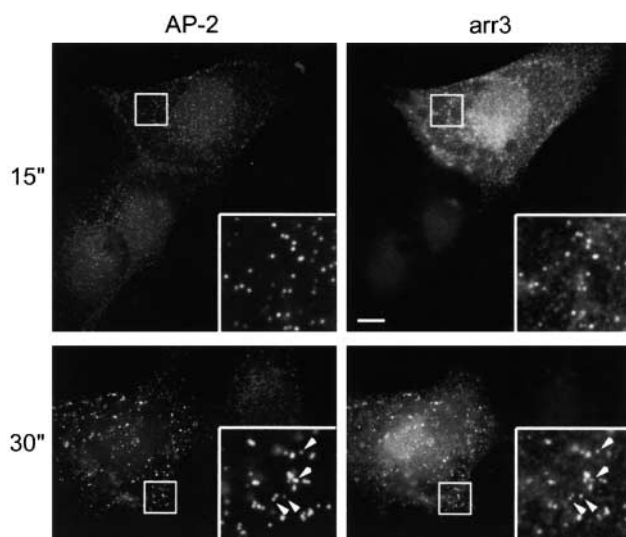
### Arr3 determinants sufficient for CP localization do not support CP reorganization

Alteration of specific residues in arr3 have been described that result in substantial loss of CL (Krupnick et al., 1997a) or AP-2 (Laporte et al., 2000) binding. To investigate the contribution of these sites to the ability of arr3 to induce changes in CP distribution, we cotransfected COS1 cells with Flag- $\beta_2$ -AR and with a mutant construct coding for arr3 with either impaired CL (LIF/A) or AP-2 (R396A) binding. Upon agonist treatment, recruitment of either mutant arr to CPs was substantially reduced in comparison to the wild-type protein (Fig. 8, WT), as was expected from previous work (Krupnick et al., 1997a; Laporte et al., 2000). However, close inspection did reveal clear examples of colocalization of each of the mutant arr with AP-2 in CPs. Importantly, when detected in CPs, both mutants were able to induce clustered CP formation and to increase CP-associated AP-2 (Fig. 8, compare arr-expressing with nonexpressing cells). These results indicate that neither mutation fully abrogates the arr3–CP interaction in the context of the intact cell. Nevertheless, despite impaired CL or AP-2 binding, if successfully recruited to CPs these proteins apparently retain the ability to initiate changes in CP patterns on the cell surface.

Previous work has shown that constructs coding for the COOH-terminal half of arr lack high affinity interaction with receptors but retain CL and AP-2 binding determinants and are constitutively localized in CPs in the absence of agonist (Krupnick et al., 1997b). We used such a construct (C-ter, see Materials and methods) to attempt to distinguish between arr-coat binding and CP clustering. Upon transient expression in COS1 cells, C-ter was prominently localized to CPs in the absence (unpublished data) as well as the presence of agonist (Fig. 8). However, no changes in CP distribution or AP-2 brightness were evident in comparison to control cells. Together, these results indicate that stable occupancy of arr binding sites on the CP lattice is necessary but not sufficient for initiating events leading to reorganization of CPs on the cell surface. Rather, the formation of a complex between arr3 and agonist-activated receptors is likely to be a prerequisite to the changes observed.

### Latrunculin B blocks CP reorganization in stimulated cells

The observation that the presence of the CP binding region of arr3 in CPs is insufficient to induce CP reorganization indicated that other components were also required. To probe whether signaling initiated by GPCR activation was involved,



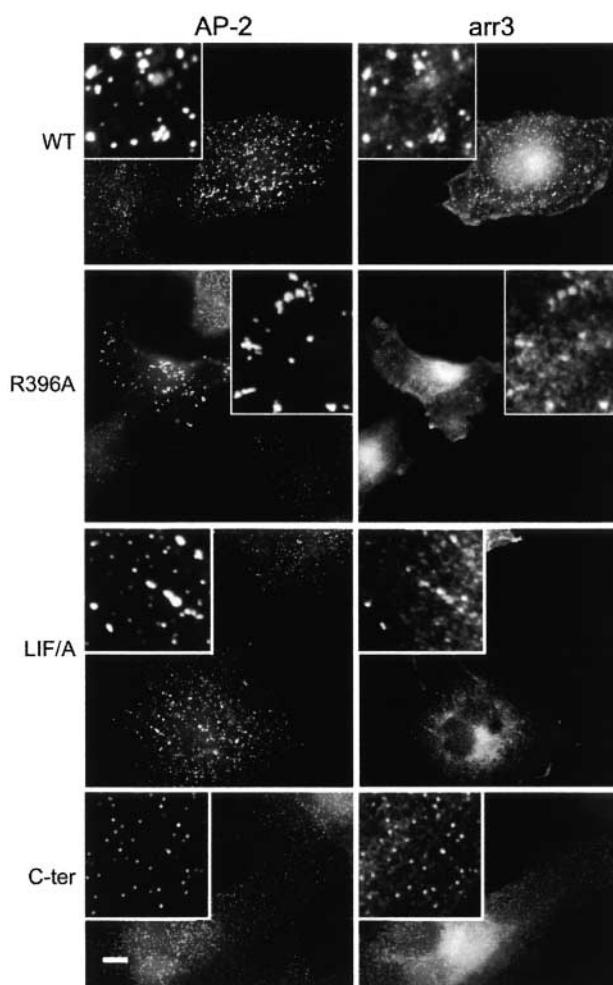
**Figure 7. Changes in CP morphology and distribution follow arr3 recruitment to CPs.** Recruitment of arr3 to CPs (AP-2) can be visualized after only 15 s (15") of ISO (10  $\mu$ M) stimulation in COS1 cells transiently expressing  $\beta_2$ -AR and arr3. At this time, the appearance of CPs in the arr3-expressing cells is indistinguishable from that in nonexpressing counterparts. Subsequent to arr3 recruitment (30"), increases in CP size and in AP-2 signal become apparent. Note that there is little proportionality between the levels of arr3 and the AP-2 spot size and brightness (see arrowheads in insets). Insets are fourfold magnified view of the cell area boxed. Bar, 10  $\mu$ m.

RBL(m1) cells were pretreated with AIF $_4^-$  (30  $\mu$ M, 3 min), cholera (1  $\mu$ g/ml, 4 h), or pertussis (0.1  $\mu$ g/ml, 4 h) toxins to target the heterotrimeric G proteins  $G_{\alpha s}$  and  $G_{\alpha i}$ . These treatments did not detectably impair the agonist-induced CP response (unpublished data), indicating that signaling through these G proteins is not involved in the reorganization of CPs upon agonist stimulation. In addition, RBL(m1) cells were pretreated with EGF receptor kinase targeted (AG1478; 10  $\mu$ M, 10 min) or broad-spectrum protein kinase inhibitors (quercetin; 10  $\mu$ M, 10 min), as well as the src family kinase inhibitor PP1 (10  $\mu$ M, 15 min) (Hirasawa et al., 1995; Wilde et al., 1999). None of these treatments blocked agonist-activated changes in CP distribution on the cell surface, or induced their occurrence in unstimulated cells (unpublished data).

Finally, we also tested a number of agents that affect cell ultrastructure. Pretreatment with either colchicine (10  $\mu$ g/ml, 30 min) or cytochalasin D (2  $\mu$ M, 20 min) did not induce changes in CP distribution nor inhibit the effects of  $\beta_2$ -AR or m1AChR stimulation (unpublished data). However, brief incubation with the G-actin sequestering agent latrunculin B (0.6  $\mu$ g/ml, 3 min) was sufficient to completely block both the CBC-induced changes in CP clustering and increased AP-2 recruitment in RBL(m1) cells (Fig. 9, and compare with Figs. 3 and 6). Under these conditions, significant drug-induced actin rearrangement was also observed (unpublished data). Interestingly, recruitment of arr3 to CPs was not detectably affected by latrunculin B treatment (Fig. 9, +).

## Discussion

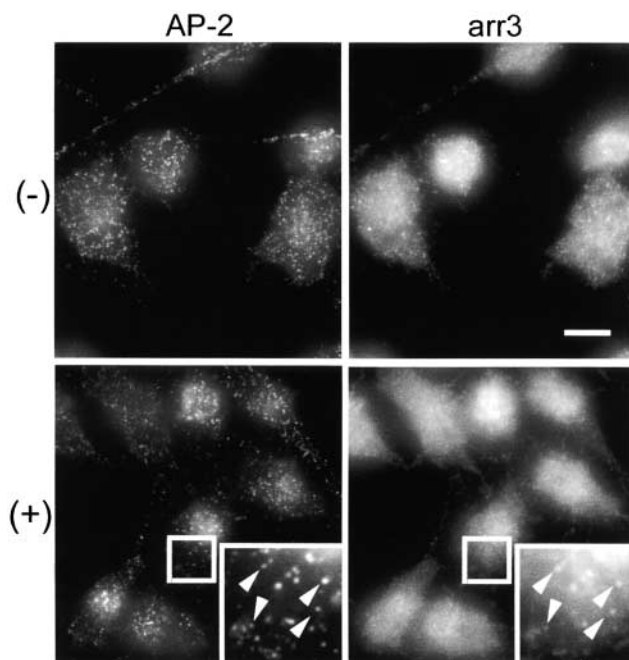
Nonvisual arrestins play critical roles in the internalization of GPCRs in vivo. Because they bind both CL and



**Figure 8. Clustering of CPs requires recruitment of functional arr3.** COS1 cells expressing Flag- $\beta_2$ -AR and either wild-type arr3 (WT), arr3 with impaired AP-2- (R396A) or CL-binding (LIF/A), or arr3 284–409 (C-ter) were stimulated with ISO (10  $\mu$ M, 5 min). Although the extent to which either of the mutant full-length arr3 proteins was recruited to CPs (AP-2, left) was greatly diminished, each was capable of inducing formation of the larger, clustered CPs. However, the COOH-terminal domain of arr3, which colocalized with AP-2 in the absence (unpublished data) or presence (C-ter) of agonist, was incapable of inducing this change in CP appearance. Insets show fourfold magnification of the boxed area of the cell. Bar, 10  $\mu$ m.

AP-2 in vitro, it has been suggested that arr could mediate the assembly of CL coats beneath the activated GPCR–arr complex by directly recruiting coat proteins (Zuker and Ranganathan, 1999; Laporte et al., 2000). Several studies from our laboratory and others have found no direct relationship between CP formation and the overall density of receptors undergoing either constitutive or stimulus-induced internalization (Santini and Keen, 1996; Santini et al., 1998; Warren et al., 1998). However, in some cells CP formation can be rapidly induced in response to activation of some signaling receptors (Connolly et al., 1981, 1984; Geisow et al., 1985; Wilde et al., 1999; Beattie et al., 2000), indicating that physiological mechanisms exist that regulate CP levels. In view of these findings, the potential role of GPCRs and arr in CP formation and dynamics deserved investigation.





**Figure 9. Latrunculin B inhibits agonist-induced CP clustering.** RBL(m1) cells were treated with latrunculin B for 3 min before treatment for 5 min with vehicle (–) or CBC (+). Cells were fixed, permeabilized, and immunostained for AP-2 and arr3. Latrunculin B did not affect CP appearance (AP-2) in unstimulated cells (–) nor did it inhibit agonist-induced arr3 recruitment to CPs (+, insets, arrows). However, it did block CP clustering and the rise in CP-associated AP-2. Insets are fourfold magnified views of the cell area boxed. Bar, 10  $\mu$ m.

Our live cell studies with GFP-arr3 provide direct evidence that activated receptor–arr complexes are targeted to and accumulate in preexisting CPs, which then undergo physiological internalization. These results argue for a highly restricted distribution of CP assembly sites (see below), consistent with previous observations (Estes et al., 1996; Santini and Keen, 1996; Gonzalez-Gaitan et al., 1997; Santini et al., 1998; Gaidarov et al., 1999b). Though the GPCR–arr3 complex does not nucleate CPs, after stimulation of  $\beta_2$ -AR or m1AChR and recruitment of the GPCR–arr3 complex to CPs we observed a change in the number and distribution of plasma membrane CPs. This response was rapid, agonist- and temperature-dependent and required GPCR and arr3 recruitment to CPs. These changes must be distinct from those involved in the membrane ruffling that occurs in response to activation of many receptors (Beck et al., 2001): stimulation of RBL cells via the Fc $\epsilon$ RI receptor (Apgar, 1990) or by PMA (Apgar, 1995) causes membrane ruffling and changes in actin microfilaments (see below), but fails to induce the CP rearrangements elicited by  $\beta_2$ -AR or m1AChR activation reported here (unpublished data).

Agonist stimulation of GPCRs often initiates transactivation of other receptors, including receptor tyrosine kinases (Luttrell et al., 1999). Though tyrosine phosphorylation of CL heavy chain has been suggested to affect CPs (Wilde et al., 1999), no CL heavy chain phosphorylation was detectable upon agonist stimulation (unpublished data). More-

over, inhibitors of the EGFR kinase (AG1478), of Src and Src-related kinases (PP1), and a broad spectrum tyrosine kinase inhibitor (quercetin) all failed to block CBC-stimulated CP changes (unpublished data), though the latter two reagents (Hirasawa et al., 1995; Amoui et al., 1997) did block degranulation and blunt overall phosphotyrosine production following Fc $\epsilon$ RI or m1AChR activation in our hands (unpublished data). Thus, tyrosine kinases do not appear to play a role in these CP changes.

The CP response was elicited by stimulation of  $\beta_2$ -AR and m1AChR, which work through distinct G-proteins ( $G_{\alpha s}$  and  $G_{\alpha q/11}$ , respectively) and effectors (adenylate cyclase and PLC, respectively). In addition, incubation with cholera or pertussis toxins or AIF $^{-4}$  did not affect the CP response mediated by either receptor. Further, treating the cells with forskolin in the presence of cAMP phosphodiesterase inhibitors also was without effect (unpublished data). However, physical recruitment of competent, even if binding impaired, arr3–GPCR complexes to CPs was required for CP increases and clustering (Fig. 8). Together, these results suggest that the CP changes require: (a) formation of a complex between arr3 and activated receptors; (b) the presence of additional factor/s recruited to the arr–receptor signaling complex; and/or (c) downstream signaling by the activated GPCR–arr complex distinct from that mediated by  $G_{\alpha s}$ / $G_{\alpha i}$  trimeric G proteins.

GPCR stimulation resulted in increased AP-2 signal associated with CPs, without a concomitant increase in the CL signal. Interestingly, despite the variation in AP-2/CL proportions the lattice size of CPs upon GPCR stimulation remained unaffected as confirmed by direct ultrastructural analysis (unpublished data). Increases in AP-2 brightness have been noted previously following GPCR stimulation (Laporte et al., 1999), and variation in AP-2/CL ratios are also known to occur (Brown et al., 1999; Keren et al., 2001). Stoichiometric AP-2/arr3 binding (Laporte et al., 1999) is unlikely to explain this phenomenon, as their signals were not generally proportional (Fig. 7, arrowheads). Alternatively, additional AP-2 may have bound directly to unoccupied sites on the CL lattices (Keen et al., 1991) indirectly through interaction with other coat-associated proteins, or to nearby potential assembly sites in response to GPCR activation.

Ultrastructural analysis of stimulated cells revealed both an increase in the number of CPs and in the proportion of CPs occurring in clusters. Together, these changes likely account for the striking apparent increase in CP size observed by light microscopy. Arr3 recruitment to preexisting CPs is the earliest detectable response to GPCR activation. However, there is an intervening period, before frankly larger CPs are observed, in which arr3 is detected in only one of a pair of adjacent CPs (Fig. 6). Based on these images and the ultrastructural data, we propose that initial accumulation of arr3–GPCR complexes in preexisting CPs induces the formation of nearby CPs that do not contain, at least initially, any arr3. These new CPs then rapidly become loaded with arr3–GPCR complexes.

One reagent effective in blocking agonist-induced CP clustering was latrunculin B, which acts by sequestering G-actin monomers (Spector et al., 1983, 1989; Coue et al., 1987).

There is ample evidence to suggest that actin plays a role in endocytosis (Lamaze et al., 1997; Munn, 2001) and several coat and associated proteins bind to or regulate actin assembly (Engqvist-Goldstein et al., 2001; Hussain et al., 2001). Furthermore, several members of the Rho family of small GTPases are known to be involved in both CL endocytosis and in actin rearrangements induced by receptor signaling (Lamaze et al., 1996; Yang et al., 2001).

Interestingly, cytochalasin D, a drug that inhibits actin polymerization by capping the barbed end of existing actin filaments and thereby preventing their elongation (Spector et al., 1989), was ineffective in blocking the GPCR-induced CP changes. These results extend our previous observations in which we found that treating cells with latrunculin but not cytochalasin increased the lateral movement of CPs in the plasma membrane (Gaidarov et al., 1999b). Together, they argue that the CP machinery is intimately connected to a cortical actin network, often resistant to cytochalasin (Cooper, 1987) but susceptible to latrunculin (Cassimeris et al., 1990), and which provides a fence-like restriction to the movement of established CPs (Gaidarov et al., 1999b). Further, actin rearrangements upon agonist stimulation appear to provide conditions necessary for the assembly of new clustered CPs.

Work from our lab and others has established that endocytic CPs occur at defined loci, rather than being randomly distributed on the cell surface. The results presented here suggest that these sites should actually be envisaged as CPZs, capable of supporting formation of adjacent, clustered CPs. Conversely, substantial expanses of cell surface lack this capability at any given time. We propose that each CPZ exists as a discrete, comparatively stable plasma membrane-cytoskeleton domain with micron dimensions, perhaps analogous to raft structures whose properties are being increasingly appreciated (Ikonen, 2001). Within each CPZ, an actin framework may help to physically organize the assembly of signaling complexes and provide a mechanical basis for inter-CPZ movements. The clustered distribution of CPs, in turn, could facilitate functional cross-talk between assemblies of different receptor signaling platforms.

## Materials and methods

### Reagents

CBC, (–) isoproterenol, atropine, pirenzepine, quercetin, and cytochalasin D were from Sigma-Aldrich; pertussis toxin was from Research Biochemicals, Inc.; cholera toxin was from List Biological Laboratories, Inc. Brefeldin A was from Epicentre Technologies; latrunculin B and PP1 were from Calbiochem. Other chemicals were reagent grade or better.

### Antibodies

mAb anti-CL (X-22 and TD.1) and anti-AP2 (AP.6) were from American Type Culture Collection. Polyclonal anti-CL (COM) and anti-AP-2 (#31) were gifts from Dr. F. Brodsky (University of California San Francisco, CA) and Drs. G. Carpenter and A. Sorokin (Vanderbilt University, TN), respectively. Arr3 was detected using affinity-purified rabbit antisera 182–6 (Gaidarov et al., 1999a). mAb (M2) against the FLAG epitope was from Eastman Kodak Co. mAb antiphosphotyrosine (clone 4G10) was from Upstate Biotechnology UBI. Fluorescein-, rhodamine-lissamine-, or Cy5-conjugated affinity-purified donkey anti-rabbit or anti-mouse polyclonal antibodies were from Jackson ImmunoResearch.

### Cell culture

HEK-293 and COS1 cells were obtained from American Type Culture Collection and were grown as specified (Krupnick et al., 1997b). RBL-2H3 cells stably expressing the human m1 muscarinic receptor (RBL[m1]) (Jones et al.,

1991) were supplied by Dr. M. Beaven (National Institutes of Health, Bethesda, MD). RBL-2H3 cells stably expressing a Flag-tagged human  $\beta_2$ -adrenergic receptor (RBL[ $\beta_2$ ]) were described previously (Santini et al., 2000). Cell lysis, CL immunoprecipitation, and phosphotyrosine detection were performed as described (Santini and Beaven, 1993; Gaidarov et al., 1999b).

### Plasmids and cell transfection

Constructs coding for Flag- $\beta_2$ AR (Goodman et al., 1996) and arr3 in which Leu-373, Ile-374, and Phe-376 were mutated to Ala (arr3-LIF/A) (Krupnick et al., 1997b) have been described, as has DsRed-CL (Engqvist-Goldstein et al., 2001). Arr3-R396A was generated by PCR and confirmed by DNA sequencing. Constructs coding for GFP-arr3 (Mundell et al., 2000), bovine arr3 short form, and the COOH-terminal region of arr3 (residues 284–409) (Orsini and Benovic, 1998) were gifts of Dr. J. Benovic (Thomas Jefferson University, Philadelphia, PA). Transfection was performed as described (Goodman et al., 1996).

### Agonist and pharmacological treatments

Experiments involving RBL cells were performed in a glucose-saline, PIPES-buffered medium (pH 7.2) that contained 0.1% BSA and 1 mM  $\text{Ca}^{2+}$  (Santini and Keen, 1996). Experiments involving COS1 cells were performed in Hepes-supplemented complete DME medium (Gaidarov et al., 1999a). After 10 min equilibration in the experimental buffer, cells were incubated with the drug or vehicle for the specified period of time before addition of 1 mM CBC, 10  $\mu\text{M}$  ISO, or vehicle. Drugs requiring more than 15 min incubation time (e.g., pertussis and cholera toxins) were added to cells in complete medium.

The following drugs were added to RBL(m1) cells before stimulation with CBC (1 mM, 3 min) to probe their effectiveness in inhibiting the agonist effect on CPs: 2  $\mu\text{M}$  cytochalasin D for 20 min; 5  $\mu\text{g}/\text{ml}$  brefeldin A for 40 min; 10  $\mu\text{M}$  quercetin for 10 min (Hirasawa et al., 1995); 30  $\mu\text{M}$   $\text{AlF}_4^-$  (prepared by mixing 10 mM NaF with 10  $\mu\text{M}$   $\text{AlCl}_3$ ) for 3 min; 1  $\mu\text{g}/\text{ml}$  cholera toxin for 4 h (Ali et al., 1990); 0.1  $\mu\text{g}/\text{ml}$  pertussis toxin for 4 h (Ali et al., 1990); 10  $\mu\text{M}$  AG1478 for 10 min; 10  $\mu\text{M}$  PP1 for 15 min. The mAChR antagonists atropine (10  $\mu\text{M}$  final concentration) and pirenzepine (0.1  $\mu\text{M}$  final concentration) were used as specified in the text. Latrunculin B was used at 0.6  $\mu\text{g}/\text{ml}$ . The effect of each of the treatment alone was also tested in unstimulated cells. Drugs were substituted by vehicle alone in control cells.

### Fluorescence microscopy of DsRed-CL and GFP-arr3-expressing cells

DsRed-CL and GFP-arr3-expressing cells grown on coverslip-bottom chambers (LabTek) overnight were transferred to warm Hepes 0.1% BSA-supplemented serum-free F-12 medium (GIBCO-BRL), placed on the microscope stage (at 37°C), and images were obtained as described previously (Gaidarov et al., 1999b). CP dynamics (DsRed signal) and mobilization of arr3 (GFP signal) following addition of ISO were studied on sequences of 100 or 120 images taken using continuous time-lapse recording. An excitation wavelength alternating between 488 and 554 nm was used which produced a 1.7 s interval between successive frames of the same channel.

### Indirect immunofluorescence and confocal microscopy

Indirect immunofluorescence was performed as described previously (Santini and Keen, 1996, 2000). CL was detected using X-22 (30  $\mu\text{g}/\text{ml}$ ), AP-2 with AP.6 (20  $\mu\text{g}/\text{ml}$ ), and arr3 with 182.6 (1:100). F-actin was visualized using Bodipy 503/512-phalloidin (5 U/ml; Molecular Probes) and primary antibodies were detected with appropriate tagged second antibodies (1:100). Images were processed using Adobe Photoshop®.

### Evaluation of plasma membrane CPs

CP density and distribution was estimated in cells immunostained for AP-2 and analyzed by confocal x-z scanning as described previously (Santini and Keen, 1996; Santini et al., 1998). Alternatively, images of cells immunostained for CL or AP-2 were acquired using a widefield system (Santini and Keen, 2000) as specified above. After determining background level, 50 × 50 pixel boxes were drawn in the cell periphery, and IPLab software version 3.2.4 (Scanalytics) was used to count CP spots and quantitate their signal intensity and area.

### Electron microscopy

Control RBL(m1) cells and RBL(m1) cells stimulated with CBC for 3 min were rinsed in 0.1 M cacodylate buffer, pH 7.4, fixed, and processed as described (Smith et al., 1985). Determination of CP number (Table II) was performed on 36 and 26 low magnification (13,500×) microphotographs of control and stimulated cells, respectively. The length of plasma mem-

brane from cell sections on each photograph was measured and coated structures and clusters, defined as coated structures  $\leq 0.3 \mu\text{m}$  apart, were counted. High magnification photographs (70,200 $\times$ ) were used to classify coated structures as: (a) "shallow pits" if their depth is less than 1/2 of their diameter, including flat coated membrane segments; or (b) "deeply invaginated pits" whose depth is  $\geq 1/2$  of their diameter, including putative "coated vesicles" exhibiting no continuity between the structure and the plasma membrane. Data are expressed as means  $\pm$  SEM. The Wilcoxon rank sum/Mann-Whitney test was performed using SYSTAT.

### Online supplemental material

Agonist activation induces recruitment of arr3 to preexisting CPs. HEK293 cells coexpressing GFP-arr3, DsRed-Cl, and Flag-tagged  $\beta_2$ -AR were incubated in 25 mM Hepes-supplemented F-12 medium and viewed on the 37 $^\circ$  stage of a microscope. DsRed and GFP signals were alternately acquired with an interval of 1.7 s between frame initiation. Total acquisition time for the entire sequence was 2.5 min. ISO (10  $\mu\text{M}$ ) was added 27 s after the start of the series (between frames 8 and 9). The video is played back at a speed of 8.33 frames/s. The video is available at <http://www.jcb.org/cgi/content/full/jcb.200110132/DC1>.

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**Note added in proof.** While this work was in press, it was reported that activation in live cells of thyrotropin-releasing hormone receptor-1 induced recruitment of arr3 to spots containing fluorescently tagged eps15, inferred to be preexisting CPs (Scott, M.G.H., A. Benmerah, O. Muntaner, and S. Marullo. 2001. *J. Biol. Chem.* 277:3552–3559). These results are entirely consistent with the findings reported here.

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