Elevated Intracellular Calcium Triggers Recruitment of the Receptor Cross-talk Accessory Protein Calcyon to the Plasma Membrane

Mohammad Kutub Ali and Clare Bergson

Calcyon is a called a “cross-talk accessory protein” because the mechanism by which it enables the typically Gq-linked D1 dopamine receptor to stimulate intracellular calcium release depends on a priming step involving heterologous Gq-linked G-protein-coupled receptor activation. The details of how priming facilitates the D1R calcium response have yet to be precisely elucidated. The present work shows that calcyon is constitutively localized both in vesicular and plasma membrane compartments within HEK293 cells. In addition, surface biotinylation and immunofluorescence assays revealed that priming stimulates a 2-fold increase in the levels of calcyon expressed on the cell surface and that subsequent D1R activation produces further accumulation of the protein in the plasma membrane. The effects of priming and D1R agonists were blocked by nocomazol implicating microtubules in the delivery of calcyon-containing vesicles to the cell surface. Accumulation of calcyon in the plasma membrane correlated well with increased intracellular calcium levels as thapsigargin mimicked, and 2-aminoethoxydiphenylborane abrogated, the effects of priming. KN-62, an inhibitor of calcium/calmodulin-dependent protein kinase II (CaMKII) also blocked the effects of priming and D1R agonists. Furthermore, expression of constitutively active forms of the kinase bypassed the requirement for priming indicating that CaMKII is a key effector in the Ca2+ and microtubule-dependent delivery of calcyon to the cell surface.

The D1-like class of dopamine (DA) receptors (D1R and D5R subtypes) belong to the superfamily of seven transmembrane domain, G-protein-coupled receptors (GPCRs), which regulate various biological functions, including movement, endocrine function, and memory processes by G-protein-coupled intracellular signaling pathways (1). In addition to the heterotrimeric G-proteins, signaling via D1Rs appears to be regulated by direct association with other proteins called dopamine receptor interacting proteins (DRIPs). Currently identified DRIPs for the D1R include adenosine (2) and NMDA (3) receptors, as well as a group of non-receptor proteins like calcyon (4), Drp78 (5), and neurofilament-M (6). Both Drp78 and neurofilament-M regulate the levels of D1Rs expressed on the cell surface, but by different mechanisms. For example, neurofilament-M hinders agonist-promoted internalization of D1Rs, whereas Drp78 regulates the processing of newly synthesized D1Rs in the endoplasmic reticulum (ER).

Calcyon is a predicted single transmembrane protein that is widely distributed in brain (7) and is abundant in D1R-positive neurons and dendrites in the prefrontal cortex and hippocampus (4). Interaction with calcyon has been shown to enhance the signaling capabilities of D1Rs. Specifically, calcyon enables typically Gq-linked D1Rs to elicit robust intracellular calcium ([Ca2+]i) release by a mechanism that requires that heterologous Gq-linked GPCRs have first been activated in a step called priming (4). Recent evidence from cells in primary culture revealed that endogenous D1 and D5Rs can stimulate [Ca2+]i release in hippocampal and cortical neurons via a priming-dependent mechanism similar is that described for heterologous cells (8). However, details as to the role of Gq-linked GPCR activation in priming the D1R Ca2+ response in either native or heterologous systems are incomplete. Similarly, although the role of calcyon in potentiating D1R-stimulated [Ca2+]i release is well documented, the mechanism by which this is accomplished has yet to be fully elucidated.

Several recent studies suggest that the specificity and sensitivity of IP3-mediated [Ca2+]i release is influenced by the spatial proximity of receptors and IP3 signaling “microdomains” (9–11). Although agonists appear to stabilize the D1R-calcyon protein complex (4), it is not known whether the spatial arrangement of calcyon and D1Rs is an important aspect of D1R-stimulated [Ca2+]i release. The present study was conducted to better understand how the subcellular distribution of calcyon is regulated during priming and D1R activation. We find that calcyon is constitutively incorporated into the plasma membrane. Levels of cell surface-localized calcyon, however, are significantly increased by heterologous Gq-linked GPCR activation (priming) and subsequent D1/D5 agonist stimulation. Furthermore, intact microtubules and CaMKII activity were required for the agonist-evoked recruitment of calcyon to the plasma membrane. These results provide support for the idea that agonist-dependent trafficking of calcyon-containing vesicles to the plasma membrane may be important for potentiating D1R-stimulated [Ca2+]i release.
Calcium Triggers Calcyon Surface Expression

Human embryonic kidney (HEK293) cells stably expressing D1 (D1 HEK293 cells) were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS) and 250 μg/ml G418, whereas HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% FBS (4). Cells were transfected when 50% confluent with the plasmid constructs as specified and processed 18–24 h later. Plasmids used in this study include pCI vector expressing the full-length human calcyon cDNA (pCI-calcyon); pCMV-Tag2C vector expressing FLAG epitope-tagged human calcyon (pFLAG-calcyon); and pCI-ΔN1 (pFLAG-ΔN1) cDNAs. For some experiments, D1 HEK293 cells were co-transfected with FLAG-calcyon and a plasmid encoding one of two constitutively active forms of CaM kinase II (CaMKII) designated pRSV-CaMKIICα1-296 and pRSV-CaMKIIT286D (12, 13). Agonists were applied in HBS containing 150 mM NaCl, 10 mM HEPES, 10 mM glucose, 2.5 mM KCl, 4 mM CaCl₂, and 2 mM MgCl₂, pH 7.4, or HEPES-MEM containing 25 mM HEPES, pH 7.4, in MEM. Carbachol, and thapsigargin were obtained from Tocris. Dolylmaleimide I, bisindolylmaleimide V, and jasplikinolide were purified from Cytochip (Roche Applied Science).

Cell lysates of the cleavable and non-cleavable biotinylated samples were sonicated for 5 s, and then centrifuged at 14,000 rpm for 30 min at 4 °C. Supernatants were collected, and protein concentrations were determined. Samples were then diluted with lysis buffer to give equivalent protein concentrations in all samples. Streptavidin slurry (100 μl) was added to samples containing 500 μg of protein in 150 μl and digested for 2 h at 4 °C. Streptavidin-bound biotinylated proteins were washed twice with 300 μl of mild lysis solution followed by two washes with 300 μl of 1.5 M guanidine HCl, and two additional washes with 300 μl of mild lysis solution (150 μl). Proteins were eluted with 300 μl SDS–PAGE sample buffer. Alternatively, samples containing 1200 μg of protein were incubated at 4 °C overnight with 10 μl of calcyon antisera (4) and then digested for 2 h at 4 °C before addition of 30 μl of protein A/G-agarose. The protein complexes were washed three times with mild lysis solution (300 μl) and eluted in SDS–PAGE sample buffer. Eluted proteins were subjected to SDS-PAGE and electroblotted to polyvinylidene difluoride membranes, and processed as described above. For proteins immunoprecipitated with the calcyon antisera, blocked membranes were incubated directly with HRP-conjugated streptavidin (Bio-Rad) (1:2000) for 1 h, and biotinylated proteins were visualized by ECL after washing three times with PBS. Non-cleavable Biotin Studies—D1 HEK293 cells were grown on coverslips, transfected as described above, and processed for immuno-chemistry 20–24 h later. For nonpermeabilized conditions, cells were washed three times with MEM media containing 25 mM HEPES, pH 7.4 (25 mM HEPES-MEM), then blocked with 25 mM HEPES-MEM containing 20% normal goat serum (25 mM HEPES-MEM, 20% NGS) at 4 °C for 20 min. Cells were incubated with mouse anti-FLAG M2 antibody (Sigma) (1:1000) diluted in blocking solution for 1 h at 4 °C and washed three times each for 20 min with ice cold 25 mM HEPES-MEM. The cells were then fixed with 2% paraformaldehyde for 20 min, washed three times with PBS, and blocked again for another 5 min, prior to incubation with Alexa 488-conjugated anti-mouse antibody (1:1000) for 20 min. Nonpermeabilized cells were washed three times with PBS prior to fixation for 20 min with 2% paraformaldehyde at room temperature and then washed three times with PBS. After incubation in blocking solution (50 mM Tris, pH 7.4, containing 3% nonfat dry milk, 50 mM CaCl₂, 5% NGS, and 0.1% Triton X-100) for 15 min, cells were incubated with primary antibodies (FLAG M2 Sigma), golgin-97 (Molecular Probes) or the endoplasmic reticulum (ER) vital dye, DiIC₁₆ (Molecular Probes) diluted according to the manufacturer’s suggestion in blocking solution for 45 min at room temperature. After four washes with PBS each for 20 min, the cells were blocked again for 5 min and then incubated with the appropriate secondary antibody in blocking solution for 30 min. The non- permeabilized and permeabilized samples were then fixed for six times with PBS each for 20 min, followed by three additional washes with PBS. Coverslips were mounted in Prolong Antifade (Molecular Probes). Antibody labeling was viewed using a Zeiss Axiovert LMS510 META confocal microscope and documented using LSM510 software. Luminescence Assays—D1293 and HEK293 cell lines were plated on 48-well plates containing 4% transwell inserts coated with 0.5 mg/ml cell-impermeant, non-cleavable sulfo-NHS-Biotin (Pierce) in PBS for 30 min at 4 °C. Unreared biotin was quenched and removed by three washes of ice-cold PBS supplemented with 10 mM glycine. The cells were then washed with ice-cold PBS, harvested in 1.5 ml of ice-cold PBS with a rubber policeman, and collected by centrifugation at 1500 rpm for 5 min. Cell pellets were dissolved in a minimum volume of mild lysis solution (Cytosignal) containing protease inhibitors (Roche Applied Science).

Labeling of Cell Surface Proteins with N-Hydroxysulfosuccinimide Esters of Biotin

Non-cleavable Biotin Studies—20–24 h after transfection, HEK293, or D1 HEK293 cells were washed twice with PBS to pre-warn at 37 °C and exposed to agonist(s) for times as specified under Results. “Cells” were immediately transferred to ice after agonist treatment and washed twice with 10 ml of PBS. Cells were then incubated with 0.5 mg/ml cell-impermeant, non-cleavable sulfo-NHS-Biotin (Pierce) in PBS for 30 min at 4 °C. Unreacked biotin was quenched and removed by three washes of ice-cold PBS supplemented with 10 mM glycine. The cells were then washed with ice-cold PBS, harvested in 1.5 ml of ice-cold PBS with a rubber policeman, and collected by centrifugation at 1500 rpm for 5 min. Cell pellets were dissolved in a minimum volume of mild lysis solution (Cytosignal) containing protease inhibitors (Roche Applied Science).
FIG. 1. Sucrose density gradients indicate that calcyon is distributed in vesicle- and plasma membrane-containing fractions of pCI-calcyon-transfected D1 HEK293 cells. 50 µg of protein was loaded per lane of the SDS containing 7.5% (IP<sub>3</sub> receptor), 15% (calcyon) polyacrylamide gels, and immunoblotted. Blots were probed with antibodies against the Na<sup>+</sup>/K<sup>+</sup> ATPase, α<sub>1</sub> subunit, D1R, type III IP<sub>3</sub> receptor, or calcyon. Similar results were obtained in two independent experiments. Protein bands were detected using the appropriate HRP-conjugated secondary antibodies followed by ECL. The positions of molecular weight markers are shown to the left of the blots.

with PBS, the cells were incubated with HRP-conjugated anti-mouse antibodies for 1 h at 37 °C and then washed six times with PBST, each for 5 min. After the final wash, the volume was adjusted to 2.0 ml with PBST, and 100 µl of ECL solution was added. The samples were incubated for 10 min at room temperature to allow light emission rates to plateau. The luminescence was then determined using a TD-20/20 luminometer (Turner Systems) (16, 17). The values shown for luminescence intensity represent the average of two to four independent experiments. Protein bands were detected by yeast two-hybrid, pull down, and co-immunoprecipitation experiments with each experiment including three to five replicates per treatment group. Controls included untransfected cells incubated with primary and secondary antibodies, and transfected cells incubated with secondary antibody only. The luminescence of both controls was negligible, whereas the values of the FLAG-calcyon transfected D1 HEK293 cells in HBS averaged ~3000 absolute luminescence units. Luminescence data, reported as the mean ± S.E., were standardized relative to the absolute luminescence values determined for FLAG-calcyon in the D1 HEK293 HBS samples (defined as 100% basal cell surface calcyon) and analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparison post test with 95% confidence intervals using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA).

RESULTS

Subcellular Localization of Calcyon—Sucrose density gradient fractionation was performed to obtain information on the subcellular distribution of calcyon (Fig. 1). Three bands with molecular masses of 24, 28, and 35 kDa were detected in lysates of pCI-calcyon-transfected D1 HEK293 cells immunoblated with calcyon antibodies. Calcyon is a predicted 217-residue single transmembrane-containing protein, therefore, the variety of molecular sizes detected likely correspond to core, immature, and mature calcyon proteins during different stages of biosynthesis. Previous studies suggest that the 28- and 24-kDa bands correspond to N-linked glycosylated calcyon and unmodified core calcyon, respectively (4), whereas the post-translation modifications involved in synthesis of the 35-kDa species have yet to be elucidated. Enrichment of the 28- and 35-kDa forms of calcyon was detected in gradient fractions two and three, whereas only the 28-kDa band could be detected in fractions four and five. The α<sub>1</sub> subunit of the Na<sup>+</sup>/K<sup>+</sup> ATPase, an integral plasma membrane protein, was concentrated in fractions one and two with reduced amounts detectable in fractions three and four. In contrast, type III IP<sub>3</sub> receptors, which are primarily expressed in vesicular stores but have also been detected in the plasma membrane (15), were much more abundant in fractions three to five compared with fractions one and two. Given the differential distribution of IP<sub>3</sub> receptors and Na<sup>+</sup> pumps across gradient fractions, plasma membrane proteins appeared to be enriched in fractions one and two, and vesicular proteins concentrated in fractions three to five. Therefore, the profile of calcyon in the gradient was consistent both with a vesicular and a plasma membrane protein. As expected, D1Rs were also enriched in plasma membrane protein-containing fractions, but more concentrated in fraction two where calcyon also seemed most abundant.

Confocal immunolocalization studies were next conducted to visualize the subcellular distribution of calcyon in transfected HEK293 cells. Labeling with affinity-purified calcyon antibodies produced a punctate staining pattern in the cytoplasm and at the cell periphery reflecting the vesicular and plasma membrane localization of the protein (Fig. 2A). In contrast, detection of FLAG-tagged D1R yielded much stronger labeling of cell boundaries (Fig. 2B). When the confocal sections were merged, colocalization of D1Rs and calcyon was evident in the cytoplasm and at the plasma membrane (Fig. 2C). Furthermore, as expected for a transmembrane protein, calcyon antibody staining overlapped with that of the ER marker, DiIC<sub>16</sub> (Fig. 2E), and the Golgi apparatus marker, golgin-97 (Fig. 2F). The native calcyon protein exhibits an analogous distribution in neurons (4).

Activity-dependent Regulation of Calcyon Cell Surface Expression—To obtain more direct evidence for the plasma membrane localization of calcyon, surface proteins of pCI-calcyon-transfected D1 HEK293 cells were labeled with membrane-impermeant, non-cleavable biotin (sulfo-NHS-biotin). Surface biotinylation was used as a strategy to specifically label proteins expressed on the cell surface and to distinguish whether calcyon was an integral plasma membrane protein or merely enriched in plasma membrane fractions due to association with D1Rs. Immunoblotting with calcyon antibodies revealed three bands of molecular mass 24, 28, and 35 kDa in both the streptavidin-bound fractions and lysates (Fig. 3A). In contrast, antibodies against two intracellular proteins, ERK42 (Fig. 3B) and rab11 (data not shown), detected bands only in “lysatelanes” and confirmed that the biotinylation conditions did not permeabilize the cells. These results further support the idea that some fraction of calcyon is inserted in the plasma membrane.

Because calcyon and D1Rs were shown to physically interact by yeast two-hybrid, pull down, and co-immunoprecipitation assays (4), an alternative explanation for the above results is that the calcyon detected in the streptavidin eluates may have co-precipitated with biotinylated D1Rs and may not itself be biotinylated. To address this possibility, cell surface biotinylated proteins were immunoprecipitated with calcyon antiserum, and immunoblots were probed with horseradish peroxidase (HRP)-conjugated streptavidin. This approach confirmed the cell surface localization of calcyon because bands migrating at ~28 kDa could be detected in both pCI-calcyon-transfected
HEK293 and D1 HEK293 cells (Fig. 3C). In contrast, no bands were detected in untransfected HEK293 and D1 HEK293 cells. Because equivalent levels of calcyon are expressed in HEK293 and D1 HEK293 cells (data not shown), the greater abundance of biotinylated calcyon detected following immunoprecipitation from D1 HEK293 compared with HEK293 cells indicated that, to some extent, D1Rs enhance expression of calcyon on the cell surface.

We next examined whether the expression of calcyon on the cell surface may be dynamically regulated by priming and D1R agonists. In these experiments, transfected cells were pre-treated with the Gq-linked muscarinic receptor agonist carbachol for 5 min, and subsequently treated with a D1/D5 receptor agonist SKF81297 for 10 min before biotinylation. This combination of agonists produced a striking increase in the intensity of the 28- and 35-kDa calcyon immunoreactive bands detected in the streptavidin-bound material compared with similarly processed samples from untreated cells incubated in HBS for an equivalent period of time (Fig. 3C). Priming with the Gq-linked purinergic receptor agonist ATP for 5 min followed by treatment with DA for 10 min also produced a visible increase in levels of streptavidin-precipitated calcyon (Fig. 3D). In contrast, treatment with DA or ATP alone resulted in either a slight decrease or increase in the 35-kDa calcyon band, respectively, but both agonists had little effect on the intensity of the 28-kDa band. Collectively, these results confirm that some fraction of calcyon is constitutively localized in the plasma membrane and strongly indicate that levels of surface localized calcyon are modulated by priming and D1R stimulation.

The effect of agonist stimulation on levels of calcyon localized in the plasma membrane was examined in a time-course experiment. Calcyon-transfected D1 HEK293 cells were exposed to SKF 81297 alone for 2, 5, or 15 min or to the D1 agonist for similar times after pretreatment with the priming agent carbachol for 5 min. Immunoblotting of streptavidin-bound fractions with calcyon antibodies revealed a visible increase in the 35-kDa band after 2 min of treatment with SKF81297 following priming with carbachol (Fig. 3E). Although the intensity of the 35-kDa band increased with time of carbachol and SKF treatment compared with samples from cells incubated in HBS for 35 min, more variation was detected in the strength of the 28-kDa band. In contrast, treatment of SKF81297 alone also resulted in a temporary increase in the intensity of the calcyon immunoreactive bands, but the magnitude was less and not sustained compared with the combined treatment with carbachol and SKF81297.

Surface D1Rs are known to internalize following agonist stimulation (18). We therefore examined whether priming and/or D1R stimulation also results in internalization of plasma membrane-localized calcyon. In these experiments, surface proteins were labeled with a cleavable, cell-impermeant biotin compound (sulfo-NHS-S-S-biotin), and agonists were

---

**Fig. 2.** Calcyon co-localizes in the plasma membrane, ER, and Golgi apparatus by confocal immunofluorescence analysis. Distribution of calcyon (A, D, and G) and FLAG tagged D1R (B), ER vital dye (E), or golgin-97 (H) in (A–C) HEK293 cells co-transfected with pCI-calcyon and pFLAG-D1R, or in (D–I) D1 HEK293 cells transfected with pCI-calcyon. Merged images (C, F, and I) show an overlap in the distribution of calcyon with D1Rs (C), ER (F), and Golgi (I) indicating localization of calcyon in various subcellular compartments, including the plasma membrane, ER and Golgi apparatus, respectively. Calcyon proteins were detected with affinity-purified rabbit calcyon antibody; FLAG-D1Rs with anti-FLAG M2 monoclonal antibody; ER with DiIC16 and Golgi with golgin-97. Primary antibodies were visualized with either Alexa 568 or Alexa 488 anti-mouse and anti-rabbit antibodies. Bar = 10 μm.
Calcium Triggers Calcyon Surface Expression

**Fig. 3.** Cell surface levels of calcyon are regulated by priming and D1R agonists. A and B, immunoblots of lysates and streptavidin-agarose-bound material obtained from non-cleavable sulfo-NHS-biotinylation of pCI-calcyon-transfected D1 HEK293 cells. Blots were probed with calcyon (A) and ERK42 (B) antibodies. C, streptavidin-HRP detection of biotinylated proteins in pCI-calcyon transfected or untransfected HEK293 and D1 HEK293 cells immunoprecipitated with calcyon antiserum. Prior to labeling with non-cleavable sulfo-NHS-biotin, transfected D1 HEK293 cells were treated with carbachol (CCL) or DA for 10 min, or incubated in HBS for 15 min. D, calcyon antibody-immunoreactive bands in streptavidin-agarose-bound material precipitated from pCI-calcyon transfected D1 HEK293 cells. Surface proteins were biotinylated after treatment with either ATP (50 μM) for 15 min, DA (10 μM) for 10 min, or ATP for 5 min followed by DA for 10 min. E, increased calcyon surface biotinylation with agonist stimulation. Samples were treated with CCL for 5 min before addition of SKF for the times indicated. For comparison, cells were incubated in HBS for 35 min before exposure to sulfo-NHS-biotin and precipitation with streptavidin HRP. Similar results were obtained in at least two independent repetitions of each of the experiments shown.

Applied, and residual surface-bound biotin was stripped prior to lysis. D1Rs were detected in streptavidin-bound material precipitated from samples treated with SKF81297 or DA reflecting the ability of the D1 agonists to stimulate receptor internalization (18). The receptors also internalized under conditions that lead to [Ca^{2+}], release as strong D1R-immunoreactive bands were detected in samples from cells primed with either carbachol or ATP and subsequently stimulated with D1R agonist (Fig. 4A). In contrast, much weaker D1R bands were detected in the samples from cells treated with priming agent or HBS only. Similar results were obtained for calcyon as immunoreactive bands were detected in the streptavidin-bound material precipitated from samples stimulated with D1 agonist, or priming agent plus D1 agonist, but not from samples treated with priming agent alone. However, the levels of calcyon detected seemed quite reduced compared with those seen in the non-cleavable biotinylation studies. Cleavable and non-cleavable biotinylated transfected cell samples were therefore treated and immunoprecipitated with calcyon antiserum in parallel to more directly compare the relative levels of surface localized and internalized calcyon. As shown in Fig. 4B, streptavidin-HRP bands migrating at 24, 28, and 35 kDa could be detected in the non-cleavable biotinylated pCI-calcyon-transfected HEK293 and D1 HEK293 samples. Equivalent bands were not detectable in lanes loaded with samples from cells biotinylated with the cleavable compound. This difference would suggest that the percentage of presumably biotinylated internalized calcyon was relatively low compared with the total amount of surface calcyon.

Insertion of the FLAG epitope at the N terminus of the calcyon open reading frame allowed visualization of tagged calcyon at the surface of non-permeabilized cells (Fig. 5A). In addition, there was a marked increase in immunofluorescent labeling of cells transfected with FLAG-calcyon after priming and exposure to D1R agonist (Fig. 5, B and C) presumably reflecting agonist-evoked trafficking of calcyon to the plasma membrane. We used luminescence assays (16, 17) to quantify the effects of agonist treatment and to examine mechanisms regulating the agonist-dependent trafficking of calcyon to the plasma membrane. Higher levels of FLAG-calcyon were detected on the surface of transfected D1 HEK293 cells compared with HEK293 cells, and a significant increase in luminescence was detected in both cell types relative to untreated controls following a 5-min exposure to carbachol (\(p < 0.001\) for both D1 HEK293 and HEK293 cells) (Fig. 5D). Subsequent treatment of D1 HEK293 cells with SKF81297 for 10 min also significantly augmented surface FLAG-calcyon levels compared with the carbachol-only-treated cells (\(p < 0.001\)). A parallel effect of D1 agonist was not detected in the transfected HEK293 cells (Fig. 5D) further indicating some involvement of D1Rs in calcyon surface expression. Combined treatment with ATP and DA produced a similar synergistic increase in surface FLAG-calcyon levels in D1 HEK293 cells indicating that both priming and D1 agonist treatment play a role in regulating calcyon surface levels (Fig. 5E).

A time-course study suggested that the effect of priming and D1 agonist treatment produces a relatively stable build up of calcyon at the cell surface (Fig. 5F). Transfected cells were exposed to either carbachol, or SKF81297 alone, or to a 5-min carbachol pretreatment followed by SKF81297 for varying times. Carbachol-evoked increases in surface calcyon reached maximum levels within 5 min and gradually returned to baseline levels by 20 min. In contrast, as seen in the non-cleavable biotinylation studies (Fig. 3), the combined treatment of carba-
agonist-stimulated recruitment of calcyon to the cell surface in jasplakinolide-treated compared with control cells (p < 0.001) (Fig. 6A). In addition, nocodazole pretreatment reduced basal levels of surface FLAG calcyon indicating that intact microtubules are important both for the constitutive and agonist-regulated trafficking of calcyon to the plasma membrane (Fig. 6A).

\[ \text{Ca}^{2+}/\text{H}11021 \]

**CaM Kinase Activation, and Recruitment of Calcyon to the Plasma Membrane**—Because [Ca\(^{2+}\)]\(_i\) is involved in vesicle trafficking in both secretory as well as non-secretory cells (21, 22), we asked whether [Ca\(^{2+}\)]\(_i\) release plays a role in the ability of the priming agonists to increase trafficking of FLAG calcyon to the cell surface. We explored this possibility using 2-APB, an inhibitor of IP\(_3\) receptors, and thapsigargin, an inhibitor of sarcoendoplasmic reticulum Ca\(^{2+}\) ATPases. Although basal levels of surface calcyon were unaffected by treatment with 2-APB, the ability of both carbachol and ATP to promote accumulation of the FLAG epitope on the cell surface was completely blocked (Fig. 6B). This result suggested that IP\(_3\)-mediated release of Ca\(^{2+}\) from intracellular stores is an essential step in the mechanism by which priming agents trigger trafficking of calcyon to the cell surface.

Thapsigargin mimicked the effects of priming. That is, levels of surface calcyon in thapsigargin-treated cells were not significantly different from control cells treated with the priming agents, ATP or carbachol (p > 0.05) (Fig. 6B). Also, in contrast to control cells, priming did not stimulate recruitment of significantly higher levels of calcyon to the cells surface of the thapsigargin treated cells (p > 0.05). However, priming followed by D1 receptor agonist significantly increased levels of surface calcyon compared with thapsigargin-only-treated cells (p < 0.001). Taken together the thapsigargin and 2-APB studies indicate that elevated [Ca\(^{2+}\)]\(_i\) is crucial for triggering accumulation of calcyon at the plasma membrane. However, these data also suggest that independent, D1R agonist-activated processes can further stimulate trafficking of calcyon to the cell surface.

**The Role of CaM Kinase Activation in Calcyon Transport to Plasma Membranes**—Several studies have implicated Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) in Ca\(^{2+}\)-regulated fusion of vesicles with the plasma membrane during exocytosis (23, 24). Consistent with a role for CaMKII in trafficking calcyon to the cell surface, pretreatment of transfected D1 HEK293 cells with the CaMK inhibitor KN-62 completely blocked the ability of carbachol and SKF81297 to stimulate an increase in levels of surface FLAG-calcyon. In contrast, pretreatment with bisindolylmaleimide I (Bis I), an inhibitor of the Ca\(^{2+}\) and phospholipid-sensitive protein kinase C (PKC), but not the inactive enantiomer Bis V, exerted a comparatively modest inhibitory effect on the ability of priming and D1 agonists to increase surface levels of calcyon. Collectively, these studies with inhibitors of Ca\(^{2+}\)-regulated kinases suggested that CaMK activation is important for agonist-regulated insertion of calcyon in the plasma membrane.

Because KN-62 inhibits CaMK types I and IV as well as II, we examined whether expression of either of two different constitutively active forms of CaMKII could regulate the surface expression of calcyon (12, 13). Although the point mutant CaMKII\(_{T286D}\) seemed more effective, coexpression of FLAG calcyon with either CaMKII\(_{T286D}\) or CaMKII\(_{C2890}\), a constitutively active form resulting from truncation of the kinase, produced significant increases in levels of surface calcyon compared with untreated controls (p < 0.01 and < 0.001, respectively) (Fig. 6D). In addition, levels of surface calcyon in either the CaMKII\(_{C2890}\) or the CaMKII\(_{T286D}\) co-transfected cells treated with priming agent and D1R agonist were not significantly different from similarly treated control cells co-trans-
fected with empty vector (p/H11022 \leq 0.05) (Fig. 6D). Nevertheless, carbachol treatment of both the CaMKII T286D (p/H11021 \leq 0.05) - and CaMKIIca1–290 (p/H11021 \leq 0.01)-transfected cells produced a small, but significant increase in the levels of surface calcyon compared with untreated counterparts. In light of the ability of KN-62 to reduce basal and to block agonist-stimulated accumulation of calcyon at the cell surface, these studies confirm that CaMKII plays a prominent role but that CaMKII-independent factors also influence the levels of calcyon recruited to the cell surface by agonists.

**DISCUSSION**

It is increasingly evident that subcellular location is a critical determinant of specificity and efficiency in GPCR signaling. For example, the ability of the Gq-linked mGluR1 and bradykinin receptors to potently activate intracellular [Ca^{2+}]_i, release requires the formation of microdomains in the plasma membrane where receptors, effectors, and associated scaffolding and accessory signaling proteins are organized in close proximity (9–11). Here, we explored the dynamics of the plasma membrane distribution of calcyon to gain insight into the mechanism whereby this signaling accessory protein potentiates the ability of D1R to stimulate [Ca^{2+}]_i release. A striking increase in recruitment of FLAG-calcyon to the cell surface could be detected in blocked cells after stimulation with carbachol (CCL) for 5 min followed by the D1R agonist, SKF81297 (SKF) for 10 min. Bar = 10 μm. A, effect of carbachol (CCL) and SKF81297 (SKF) treatment on the levels of surface FLAG epitope in transfected HEK293 and D1 HEK293 cells determined by luminescence assay. B, ATP and DA treatments regulate increase surface FLAG-calcyon levels in transfected D1 HEK293 cells. F, time-course comparison of the effects CCL and/or SKF81297 treatments on the expression of FLAG-calcyon at the surface of D1 HEK293 cells. The bars and error bars show the means ± S.E. of two to three independent experiments performed in triplicate. The luminescence values of FLAG-calcyon surface expression detected in D1 HEK293 HBS control samples defined basal levels. The asterisks indicate significantly different from basal levels, or indicate significant differences between the samples highlighted by the brackets (* and ** denote p \leq 0.01, and <0.001, respectively). p values were determined by one-way ANOVA followed by Tukey-Kramer post test.

**Calcium Triggers Calcyon Surface Expression**

**Fig. 5. Priming and D1 DA receptor stimulation synergistically increase levels of surface calcyon.** A–C, confocal immunofluorescent (upper panels) and merged bright field and immunofluorescent (lower panels) visualization of plasma membrane localized calcyon in FLAG-calcyon transfected D1 HEK293 cells. Fluorescent images (A–C) were scanned with the argon laser, with the settings for the pinhole, detector gain, amplifier offset, and amplifier gain fixed to 90, 756, −0.1, and 1.4, respectively. A, the FLAG epitope was detected under non-permeabilized conditions using FLAG-M2 and Alexa 488-conjugated anti-mouse antibodies. No staining was detectable either in untransfected D1 HEK293 cells or in transfected cells if the FLAG antibody was omitted (data not shown). B and C, basal levels of surface calcyon were blocked by incubating non-permeabilized cells initially labeled with FLAG-M2 antibodies with unconjugated anti-mouse antibodies. After blocking, cells were incubated in HBS (B) or agonist (C) prior to visualization of surface-localized FLAG-calcyon with the FLAG-M2 and Alexa 488 antibodies (A). A striking increase in recruitment of FLAG-calcyon to the cell surface could be detected in blocked cells after stimulation with carbachol (CCL) for 5 min followed by the D1R agonist, SKF81297 (SKF) for 10 min. Bar = 10 μm. B, effect of carbachol (CCL) and SKF81297 (SKF) treatment on the levels of surface FLAG epitope in transfected HEK293 and D1 HEK293 cells determined by luminescence assay. E, ATP and DA treatments regulate increase surface FLAG-calcyon levels in transfected D1 HEK293 cells. F, time-course comparison of the effects CCL and/or SKF81297 treatments on the expression of FLAG-calcyon at the surface of D1 HEK293 cells. The bars and error bars show the means ± S.E. of two to three independent experiments performed in triplicate. The luminescence values of FLAG-calcyon surface expression detected in D1 HEK293 HBS control samples defined basal levels. The asterisks indicate significantly different from basal levels, or indicate significant differences between the samples highlighted by the brackets (* and ** denote p \leq 0.01, and <0.001, respectively). p values were determined by one-way ANOVA followed by Tukey-Kramer post test.
HEK293 cells (p < 0.001). These results suggest that both constitutive, D1R-independent as well as D1R-dependent mechanisms regulate the localization of calcyon in the plasma membrane. Because calcyon physically associates with D1Rs, and the receptors are predominantly localized in the plasma membrane, it seems likely that protein/protein interactions play a part in the D1R-dependent mechanism. Furthermore, previous studies showed that formation of the calcyon-D1R complex is enhanced under conditions that lead to [Ca\textsuperscript{2+}]\textsubscript{i} release (4). Because elevated [Ca\textsuperscript{2+}]\textsubscript{i}, was also recently shown to increase surface expression of D1Rs in neostriatal neurons (26), it seems likely that agonist-regulated accumulation in the plasma membrane plays a role in the formation of the D1R-calcyon complex.

The vast majority of D1Rs undergo dynamin-dependent endocytosis following agonist stimulation (18). Using cleavable biotin to examine endocytosis, we found that in contrast to D1Rs much less surface calcyon internalized following D1 agonist treatment. An explanation for this difference is the possibility that non-biotinylated, vesicular calcyon co-precipitated with internalized biotinylated D1Rs. The ability to detect internalized biotinylated D1Rs following treatment with D1 agonist alone or in combination with priming agents is consistent with this interpretation. It is also possible that the calcyon-D1R complex dissociates upon D1 agonist stimulation such that D1Rs internalize but calcyon remains localized in the plasma membrane. Consistent with this idea, priming combined with D1 agonist treatment produced a sustained accumulation of calcyon at the cell surface. Furthermore, the inability to detect biotinylated calcyon by immunoprecipitation of the cleavable biotinylated samples may reflect the possibility that internalized calcyon is rapidly degraded, or perhaps that only a minimal fraction of surface calcyon actually undergoes endocytosis. Future studies will need to establish whether
Calcium and D1Rs undergo different postendocytic sorting. Specifically, because little is known about functional domains in calcyon or those of two closely related proteins P19 (NEEP21) and P21, it will be of interest to determine whether the calcyon localizes to early endosomes in neurons similar to P19 (27).

Numerous factors, including [Ca$^{2+}$], CaMKII, and cytoskeletal proteins regulate the post-Golgi sorting of transmembrane proteins during trafficking of vesicles to the plasma membrane (20). Increased [Ca$^{2+}$] can promote the fusion of vesicles with the plasma membrane in secretory cells such as neurons and endocrine cells (19, 28–30) as well as non-secretory types of cells like HEK293 cells (21). We observed that trafficking of calcyon to the plasma membranes of HEK293 and D1 HEK293 cells correlated well with elevated [Ca$^{2+}$]. Indeed, application of carbachol or ATP, agonists of G$_i$-linked GPCRs that promote calcium-triggered vesicular transport of calcyon to the plasma membrane (8). In contrast, KN-62, an inhibitor of CaMK I, II, and IV, increased the evoked levels of surface calcyon in vector-transfected cells (27). It will be of interest to determine whether other agonists stimulated trafficking of vesicular calcyon to plasma membranes depends on CaMKII activity and availability.

In neurons, calcyon is localized in dendrites and spines (4) where both CaMKII and microtubules have been implicated in a vesicular trafficking process called Ca$^{2+}$-evoked dendritic exocytosis (CEDE) (23, 35) due to the ability of nocodazole and KN-62 to block CEDE. More recent studies suggest that either overexpression of constitutively active CaMKII, high frequency stimulation, or opening of NMDA receptor Ca$^{2+}$ channels increases the delivery of AMPA glutamate receptor to dendritic spines (36, 37). Given the parallels between the mechanisms regulating CEDE and AMPA receptor surface expression with those reported here for calcyon, it will be important in future studies to determine whether the plasma membrane localization of calcyon in dendrites and spines is influenced by neuronal activity in an analogous manner.

Acknowledgments—We gratefully acknowledge the generous assistance of our colleagues: L. Redmond, Ph.D., Medical College of Georgia, for the constitutively active CaMKII plasmide, R. Hall, Ph.D., Emory University, for the hippocampal assay protocol. We also thank R. Leveson, Ph.D. (Penn State College of Medicine) and L. Redmond for their comments on the manuscript and Shaun Opperman, B.S., for expert technical assistance.

References
1. Missale, C., Nash, S. R., Robinson, S. W., Jaber, M., and Caron, M. G. (1998) Physiol. Rev. 78, 189–225
2. Gines, S., Hillion, J., Torvinen, M., Le Crom, S., Casado, V., Canela, E. I., Rondin, S., Lew, J. Y., Watson, S., Zoli, M., Agnati, L. F., Vernieri, P., Liuisi, C., Forero, S., Fuxe, K., and Franco, R. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 8606–8611
3. Lee, F. J., Xue, S., Lei, P., Vukusic, B., Chen, Y., Wang, Y., Yang, Y., Namkhi, H. B., Yu, X. M., and Liu, F. (2002) Cell 111, 219–230
4. Lezcano, N., Mrzljak, L., Elkoubski, S., Leveson, R., Goldman-Rakic, P., and Bergson, C. (2000) Science 287, 1660–1664
5. Bernek, J. C., Li, M., Bullock, C., and Zhou, Y. Q. (2001) Nat. Cell Biol. 3, 492–498
6. Kim, O. J., Ariano, M. A., Lazzarini, R. A., Levine, M. S., and Sibley, D. R. (2002) J. Neurosci. 22, 5920–5930
7. Zelenin, S., Aperia, A., and Diaz, H. R. (2002) J. Comp. Neurol. 446, 37–45
8. Lezcano, N., and Bergson, C. (2002) J. Neurophysiol. 87, 2167–2175
9. Xiao, B., To, J. C., Petrakis, R. S., Yuan, J. P., Doan, A., Breder, C. D., Ruggiero, A., Lanahan, A. A., Wenthold, R. J., and Worley, P. F. (1998) Neuroscience 24, 707–716
10. Fagni, L., Chavis, P., Ango, F., and Bockaert, J. (2002) Trends Neurosci. 23, 80–88
11. Delineas, P., Wanaverbeek, N., Abogadie, F. C., Mistry, M., and Brown, D. A. (2002) Neuron 34, 209–220
12. Sun, P., Enslen, H., Myung, P. S., and Maurer, R. A. (1994) Genes Dev. 8, 2327–2339
13. Yang, E., and Schulman, H. (1999) J. Biol. Chem. 274, 26199–26208
14. Tewhun, H., Stahlstein, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4550
15. Tanimura, A., Toyojo, Y., and Turner, R. J. (2000) J. Biol. Chem. 275, 27488–27493
16. He, J., Xu, J., Castleberry, A. M., Lau, A. G., and Hall, R. A. (2002) Biochem. Biophys. Res. Commun. 297, 545–572
17. Xu, J., He, J., Castleberry, A. M., Balasubramanian, S., Lau, A. G., and Hall, R. A. (2003) J. Biol. Chem. 278, 10770–10777
18. Vickery, R. G., and von Zastrow, M. (1999) J. Cell Biol. 144, 31–43
19. El-G, O. G., Morris, R. L., Lazzarini, R. A., Levine, M. S., and Sibley, J. M., and Steinhardt, R. A. (1997) J. Cell Biol. 138, 998–1008
20. Kreitzer, G., Schmoranz, J., Low, S. H., Li, X., Gu, Y., Weimbs, T., Simon, M., and Rodrigues-Boulan, E. (2003) Nat. Cell Biol. 5, 126–136
21. Coorsens, J. R., Schmitt, H., and Almers, W. (1999) EMBO J. 18, 3757–3761
22. Burgoyne, R. D., and Morgan, A. (1998) J. Neurosci. 18, 6814–6821
23. Easom, R. A. (1999) Diabetes 48, 675–684
24. Lan, J. Y., Skeberdis, V. A., Jover, T., Zheng, X., Bennett, M. V., and Zunin, P. (2001) J. Neurosci. 21, 9058–9068
25. Scott, L., Kruse, M. S., Forsberg, H., Brismar, H., Greenberg, P., and Aperia, A. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 1661–1664
26. Steinherz, P., Barria, J. C., Glavas, U., Magnas, C., Siciliano, S., and Hirling, H. (2002) J. Cell Biol. 157, 1197–1209
27. Steinhardt, R. A., Bi, G., and Alderton, J. M. (1994) Science 263, 390–393
28. Geerlings, A., Nunez, E., Lopez-Corcuera, B., and Aragon, C. (2001) J. Biol. Chem. 276, 17584–17590
29. Fukuda, M., Kanno, E., Ogata, Y., Saegusa, C., Kim, T., Loh, Y. P., and Yamamoto, A. (2003) J. Biol. Chem. 278, 3220–3226
31. Apodaca, G. (2001) *Traffic* 2, 149–159
32. Doctor, R. B., Dahl, R., Fouassier, L., Kilic, G., and Fitz, J. G. (2002) *Am. J. Physiol. Cell Physiol.* 282, C1042–C1052
33. Wang, X., Butowt, R., Vasko, M. R., and von Bartheld, C. S. (2002) *J. Neurosci.* 22, 931–945
34. Yamamoto, H., Yamauchi, E., Taniguchi, H., Ono, T., and Miyamoto, E. (2002) *Arch. Biochem. Biophys.* 408, 255–262
35. Maletic-Savatic, M., and Malinow, R. (1998) *J. Neurosci.* 18, 6803–6813
36. Shi, S. H., Hayashi, Y., Petralia, R. S., Zaman, S. H., Wenthold, R. J., Svoboda, K., and Malinow, R. (1999) *Science* 284, 1811–1816
37. Hayashi, Y., Shi, S. H., Esteban, J. A., Piccini, A., Ponce, J. C., and Malinow, R. (2000) *Science* 287, 2262–2267