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Receptor crosstalk protein, calcyon, regulates affinity state of dopamine D1 receptors

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Abstract

The recently cloned protein, calcyon, potentiates crosstalk between G_s-coupled dopamine D1 receptors and heterologous $G_{q/11}$-coupled receptors allowing dopamine D1 receptors to stimulate intracellular Ca^{2+} release, in addition to cAMP production. This crosstalk also requires the participating $G_{q/11}$-coupled receptors to be primed by their agonists. We examined the ability of calcyon and priming to regulate the affinity of dopamine D1 receptors for its ligands. Receptor binding assays were performed on HEK293 cell membrane preparations expressing dopamine D1 receptors either alone or in combination with calcyon. Co-expression of dopamine D1 receptor and calcyon affected neither the affinity of this receptor for antagonists nor the affinity of agonist binding to this receptor high and low-affinity states. However, the presence of calcyon dramatically decreased the proportion of the high-affinity dopamine D1 receptor agonist binding sites. This decrease was reversed by carbachol, which primes the receptor crosstalk by stimulating endogenous $G_{q/11}$-coupled muscarinic receptors. Our findings suggest that calcyon regulates the ability of dopamine D1 receptors to achieve the high-affinity state for agonists, in a manner that depends on priming of receptor crosstalk.

Keywords: G protein; Agonist; Antagonist; Affinity; Receptor crosstalk; Receptor binding assay

1. Introduction

G protein-coupled receptors, such as dopamine receptors, regulate second-messenger systems by interacting with heterotrimec G proteins (Hamm, 1998). The coupling of G proteins to these receptors has also been shown to elevate their affinity for agonists (Perez et al., 1996) by increasing the lifetime of receptor–agonist complex (Kent et al., 1980; Birnbaumer et al., 1990). Consequently, chemical compounds interfering with G protein–receptor coupling [such as guanosine 5′-triphosphate (GTP) and its analogs] usually decrease the proportion of the high-affinity agonist binding sites (Heidenrich et al., 1980; Schulz et al., 1985; Hess et al., 1986). This suggests that G proteins not only play a key role in relaying signals from receptors to intracellular cascades but also are involved in a feedback regulation of the affinity state of G protein-coupled receptors for agonists.

Over the past several years, evidence has accumulated supporting the idea that G protein coupled receptors can interact with other intracellular partners (Hall et al., 1999). One of these proteins, calcyon, was shown to confer on dopamine D1 receptor the ability to stimulate intracelllular Ca^{2+} release by potentiating a crosstalk between this G_s-coupled receptors and heterologous $G_{q/11}$-coupled receptors (Lezcano et al., 2000). This crosstalk also requires the priming of cells with agonists to participating $G_{q/11}$-coupled receptors (Lezcano et al., 2000). Priming in combination with application of dopamine D1 receptors agonists has been shown to significantly strengthen the interaction of calcyon and dopamine D1 receptors (Lezcano et al., 2000).

The present study examines the ability of calcyon and crosstalk priming to regulate the affinity of dopamine D1 receptor for its ligands. It demonstrates that, along with G proteins, calcyon is also involved in regulation of the

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affinity state of dopamine D1 receptors for agonists, and that this regulatory activity of calcyon is modifiable by priming of receptor crosstalk.

2. Materials and methods

2.1. Cell cultures

A human embryonic kidney 293 (HEK293) cell line stably transfected with human dopamine $D1_A$ receptor complimentary deoxyribonucleic acid (cDNA), and henceforth designated as D1 HEK293 cells, was maintained in Dulbecco's modified Eagle's medium containing 2 mM glutamine, 10% fetal calf serum, and 250 μg/ml Geneticin Selective Antibiotic (Gibco BRL, Rockville, MD). D1-HEK293 cells were transfected using Effectene (Qiagen, Valencia, CA) with pCI expression vectors (Promega, Madison, WI), or pCI containing the full-length calcyon cDNA (Lezcano et al., 2000). After 16–24 h, cells were washed with phosphate-buffered saline (pH 7.4), dounce homogenized, nuclei removed by centrifugation at 3400 rpm for 5 min, and crude membrane fractions isolated by centrifugation at 16,000 rpm at 20 min at 4 °C. Pellets were resuspended in 10 mM Tris–HCI (pH 7.4), briefly sonicated, and protein concentrations determined by Bicinchaninic Acid Assay Kit (BCA Assay Kit; Pierce, Rockford, IL). Expression of calcyon was verified by Western blot with affinity-purified calcyon antibodies (Lezcano et al., 2000).

2.2. Receptor binding

Saturation assays for dopamine D1 receptor were performed with 12 concentrations (0.1–24 nM) of antagonist radioligand, [^{3}H]7-cloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride ([^{3}H]SCH23390; Amersham, Piscataway, NJ) and with 12 concentrations (0.7–7000 nM) of agonist radioligand, [^{3}H]*N*-propylnorapomorphine (New England Nuclear, Boston, MA). While [^{3}H]*N*-propylnorapomorphine has similar affinities for dopamine D1 and D2 receptors (Creese et al., 1979; Titeler and Seeman, 1979), it can be used as a selective dopamine D1 receptor ligand in our D1-HEK293 cell preparations lacking dopamine D2 receptors.

Displacement of 2 nM [^{3}H]SCH23390 was carried out by series of 13 concentrations (0.1 nM − 1.0 mM) of dopamine, and dopamine D1 receptor agonists, 1-phenyl-2,3,4,5-tertahydro-(1*H*) − 3-benzazepine-7,8-diol hydrochloride (SKF38393), 6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepinehydrobromide (SKF82958), and (−)-apomorphine (RBI, Natick, MA).

All assays were performed using membrane preparations from D1-HEK293 cells transfected either with pC1 or with pCI-calcyon plasmid DNA. The saturation assays were performed either without or with 100 μM carbachol (RBI) being added to the incubation mixture to prime the crosstalk between $G_{q/11}$-coupled muscarinic M1 receptor endogenously expressed by the HEK293 cells and dopamine D1 receptor (Wang et al., 1997; Lezcano et al., 2000). Displacements of [^{3}H]SCH23390 by SKF38393 from membranes of both cell types were conducted in the presence and of 0, 10, 50, 100 or 400 μM carbachol. Displacement assays with SKF38393 in the presence and absence of 100 μM carbachol were also performed with or without the addition of 0.3 mM GTP (Sigma, St. Louis, MO). As mentioned earlier, GTP is known to prevent G proteins from supporting the high-affinity state of neurotransmitter receptors, and is detectable as a reduction in the proportion of the high-affinity binding sites for agonists (Heidenrich et al., 1980; Schulz et al., 1985; Hess et al., 1986). Therefore, a decrease in the percentage of the high-affinity binding sites for SKF38393 in the presence of GTP would demonstrate that the affinity state of dopamine D1 receptor is regulated by G proteins. Displacements of [^{3}H]SCH23390 by other agonists were performed without addition of either carbachol or GTP.

To obtain the total binding, membrane preparations containing 5 μg of protein per test tube (determined using a BCA Assay Kit; Pierce) were incubated at room temperature for 1 h with the appropriate concentrations of [^{3}H]SCH23390 or [^{3}H]*N*-propylnorapomorphine in 50 mM Tris–HCI buffer (pH 7.4) containing 120 mM NaCl, 5 mM KCI, 2 mM $CaC1_2$, 1 mM $MgC1_2$. In the case of [^{3}H]SCH23390, 1μm mianserin (RBI) was also added to block possible [^{3}H]SCH23390 binding to serotonin receptor sites). When appropriate, dopamine D1 receptor agonists, carbachol, and/or GTP were added to incubation buffer. Nonspecific binding was determined by addition of either 1 μM *cis*-flupentixol (for [^{3}H]SCH23390) or SCH23390 (for [^{3}H]*N*-propylnorapomorphine), both purchased from RBI. Incubations were terminated by rapid filtration and three washes with 5 ml of ice-cold 50 mM Tris–HCI buffer (pH 7.4) through Whatman GF/B filters using an M-242 cell harvester (Brandel, Gaithersburgh, MD). The radioactivity trapped in the filters was measured in a Beckman LS5801 liquid scintillation counter (Beckman, Fullerton, CA). For every saturation assay, binding at each radiologand concentration was performed in three separate test tubes for determination of the total binding and in three test tube for determination of the nonspecific binding. For every displacement assay, binding at each concentration of agonist was also conducted in three separate test tubes. In addition, all saturation and displacement assay were done in triplicate. The results of binding were analyzed by a nonlinear curve-fitting computer program (EDBA/LIGAND, Elsevier-Biosoft, Cambridge, UK) and reported as mean ± S.E.M. The statistical analysis included two-way analysis of variance (ANOVA) with calcyon expression and carbachol priming as variables. This

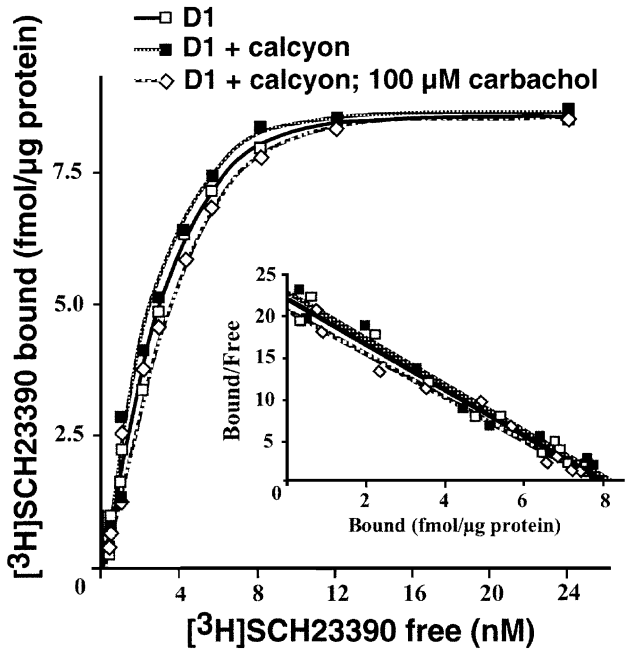

Fig. 1. Specific saturation binding of a dopamine D1 receptor-specific antagonist radioligand, [^{3}H]SCH23390, to membrane preparations from cells expressing D1 receptor alone or together with calcyon. In the latter membranes, the bindings were performed in the absence and in the presence of 100 μM of a priming agent, carbachol. The insert represents the corresponding Scatchard transformation of the data. Each point represents a mean value from three separate assays. Note saturability of the binding in all three assays' conditions. Also, note a significant similarity of the saturation curves at all three conditions. In all assays, the data best fitted by a single linear regression line suggesting a single affinity site. The slopes of the regression lines are also very similar indicating the lack of appreciable differences in the K_d values (calcyon-free membranes, $K_d = 0.36 \pm 0.07$ nM, $B_{max} = 8.1 \pm 2.6$ fmol/μg/prot; calcyon-containing membranes in the absence of carbachol, $K_d = 0.32 \pm 0.17$ nM, $B_{max} = 8.4 \pm 1.5$ fmol/μg/prot; calcyon-containing membranes in the presence of carbachol, $K_d = 0.41 \pm 0.09$ nM; $B_{max} = 7.8 \pm 3.4$ fmol/μg/prot; these K_d or B_{max} values are statistically indistinguishable, $P > 0.05$).

was followed by Tukey's post-hoc between-group comparison. The differences were considered significant when $P < 0.05$.

3. Results

3.1. Saturation binding of [^{3}H]SCH23390

The dopamine D1 receptor antagonist, [^{3}H]SCH23390, exhibited saturable specific binding to membranes of cells expressing these receptors either with or without calcyon (Fig. 1). In both cases, the saturation data were best fitted by a model of binding to a single homogenous population of binding sites (Fig. 1). The affinity (represented by K_d) and the total number of binding sites (represented by B_{max}) for [^{3}H]SCH23390 were similar in membranes of cells lacking calcyon and membranes of cells containing calcyon (Fig. 1 legend). Further, the K_d and B_{max} values for [^{3}H]SCH23390 were not significantly influenced by the presence or absence of carbachol in the binding assays (Fig. 1 legend).

3.2. Displacement assays

The displacements of [^{3}H]SCH23390 binding by all dopaminergic agonists tested, including SKF38393, SKF82958, (−)-apomorphine, and dopamine, were best modeled by a heterogenous, two-site fit. Calcyon expression did not significantly affect the affinity (represented by K_i) of all these agonists to either high- or low-affinity states of D1 receptor (Table 1). However, expression of calcyon greatly reduced the proportion of the high-affinity dopamine D1 receptor binding sites. In the absence of calcyon, the percentage of high-affinity sites among dopamine D1-specific binding sites was close to 50%. The presence of calcyon reduced the percentage of these sites to less than 10% (Table 1).

3.3. Displacement assays in the presence of carbachol

The effect of carbachol were analyzed in the displacement assays of [^{3}H]SCH23390 by SKF38393. Carbachol increased in the percentage of high-affinity SKF38393 binding sites in the dopamine D1 receptor plus calcyon

Table 1
Dissociation constants for high- (K_iH) and low- (K_iL) affinity binding sites and the percentages of high-affinity sites among the total number of the specific binding sites (% RH) for a series of dopamine D1 receptor agonists

Agonists	Dopamine D1 receptor			Dopamine D1 receptor + calcyon		
	K_iH (nM)	K_iL (nM)	% RH	K_iH (nM)	K_iL (nM)	% RH
SKF38393	4.23 ± 0.24	118.17 ± 5.31	51 ± 4	5.42 ± 0.37	124.16 ± 6.22	3 ± 2 *
SKF82958	1.96 ± 0.30	59.79 ± 2.12	67 ± 3	2.00 ± 0.23	55.56 ± 3.31	7 ± 4 *
(−)-Apomorphine	7.82 ± 0.31	830.62 ± 26.97	47 ± 5	8.44 ± 0.69	860 ± 34.90	4 ± 3 *
Dopamine	23.25 ± 1.43	3300.51 ± 219.07	45 ± 3	25.41 ± 2.06	3223.27 ± 318.23	5 ± 3 *

These data were generated from the analysis of the ability of these agonists to displace the binding of [^{3}H]SCH23390, from the membrane preparations of cells expressing dopamine D1 receptor alone or in combination with calcyon.
Each value represents a mean ± S.E.M. of $n = 3$.
Note that calcyon does not affect appreciably either K_iH or K_iL values for any of the agonists tested. The percentage of high-affinity sites for all agonists, however, is substantially decreased by co-expression of dopamine D1 receptor with calcyon.
* Values obtained from calcyon-containing membrane preparations that are statistically different from those obtained from calcyon-free membranes.

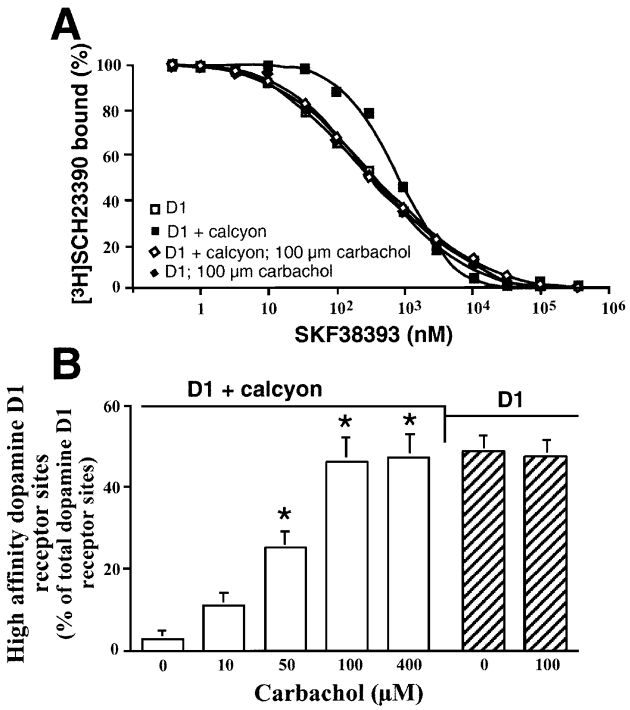

Fig. 2. Effect of the priming agent, carbachol, on [^{3}H]SCH23390 displacement by SKF38393. (**A**) Displacement curves obtained in membranes expressing dopamine D1 receptor alone and together with calcyon. In both cases, the assays were performed in the absence and in the presence of 100 μM carbachol. Each point represents a mean value from three separate assays. In all cases, the analysis of the displacement curves suggests the presence of two affinity sites: 4.23 ± 0.24 and 118.17 ± 5.31 nM for calcyon-free membranes in the absence of carbachol; 5.87 ± 0.55 and 123.28 ± 6.94 nM for calcyon-free membranes in the presence of carbachol; 5.42 ± 037 and 124.16 ± 6.22 nM for calcyon-containing membranes in the absence of carbachol; and 5.01 ± 0.47 and 120.01 ± 7.32 nM for calcyon-containing membranes in the presence of carbachol. There are no statistically significant differences between the K_d values for either high- or low-affinity sites between different membrane preparations ($P > 0.05$). However, the analysis of these displacement curves demonstrates that different membrane preparations may differ in the proportions of the high- and low-affinity sites for dopamine D1 receptor agonists as detained in part B of this figure. (**B**) Histogram representing the percentage of the high-affinity receptor sites detectable in assays of [^{3}H]SCH23390 displacement by SKF38393. Each bar represents a mean $\pm$S.E.M. of $n = 3$. Asterisks mark the statistically significant effects of carbachol ($P < 0.05$). Note that, in the presence of calcyon, carbachol increases the percentage of high-affinity sites in a concentration-dependent manner. However, this effect of carbachol, asymptotes at concentrations above 100 μM, with the percentages of high-affinity sites reaching those seen in membrane preparations lacking calcyon. In calcyon-free membranes, carbachol does not influence the dopamine D1 receptor affinity state for agonists.

containing cells membranes in a concentration-dependent manner (Fig. 2). This effect of carbachol, however, leveled off at concentrations above 100 μM, where the percentage of high-affinity sites were comparable to D1-HEK293 cell membrane preparation lacking calcyon (Fig. 2). Addition of carbachol to D1-HEK293 cell preparations of calcyon-free membranes had no significant effect on the percentage of high-affinity sites for SKF38393 (Fig. 2). Carbachol also had no appreciable effect on K_i values of SKF38393 at either high- or low-affinity sites (Fig. 2 legend).

3.4. Effects of GTP

Addition of 0.3 mM GTP during displacement of [^{3}H]SCH23390 by SKF38393 reduced the percentage of high-affinity sites in the membrane preparations containing dopamine D1 receptors alone by almost one-half (Fig. 3). Similarly, addition of GTP resulted in a nearly 50% decrease in the percentage of the high-affinity binding sites in the dopamine D1 receptor- and calcyon-containing membrane preparations assayed in the presence of carbachol (Fig. 3). Also, when tested in the absence of carbachol the addition of GTP to membrane preparations containing dopamine D1 receptor and calcyon (which already had very low proportion of the high-affinity binding sites for SKF38393), further reduced the percentage of high-affinity sites to below detectable levels (Fig. 3). All these GTP-induced effects were statistically significant. In contrast, GTP did not significantly alter K_i values for either high or low-affinity sites in any of the assays (not shown).

3.5. Saturation binding of [^{3}H]N-propylnorapomorphine

To verify that the effects of the presence of calcyon and priming with carbachol detectable in the displacement assays can also be observed in the saturation assays with dopamine D1 receptor agonists, we conducted such assays using [^{3}H]*N*-propylnorapomorphine. We found that this radioligand exhibited specific binding to membranes of cells expressing dopamine D1 receptors either with or without calcyon (Fig. 4). In both cases, the saturation data

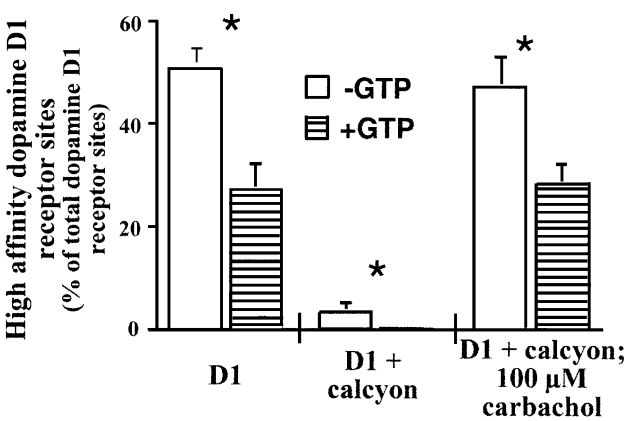

Fig. 3. Histogram representing the effect of the addition of 0.3 mM GTP on the percentage of high-affinity dopamine D1 receptor sites for agonists detectable in assays of [^{3}H]SCH23390 displacement by SKF38393 in membrane preparations containing either dopamine D1 receptor alone or dopamine D1 receptor together with calcyon in the presence or in the absence of 100 μM carbachol. Each bar represents a mean $\pm$S.E.M. of $n = 3$. Asterisks mark the statistically significant effects of GTP ($P < 0.05$). Note that the addition of GTP reduces the percentage of high-affinity sites for SKF38393 in all membrane preparations used in this study.

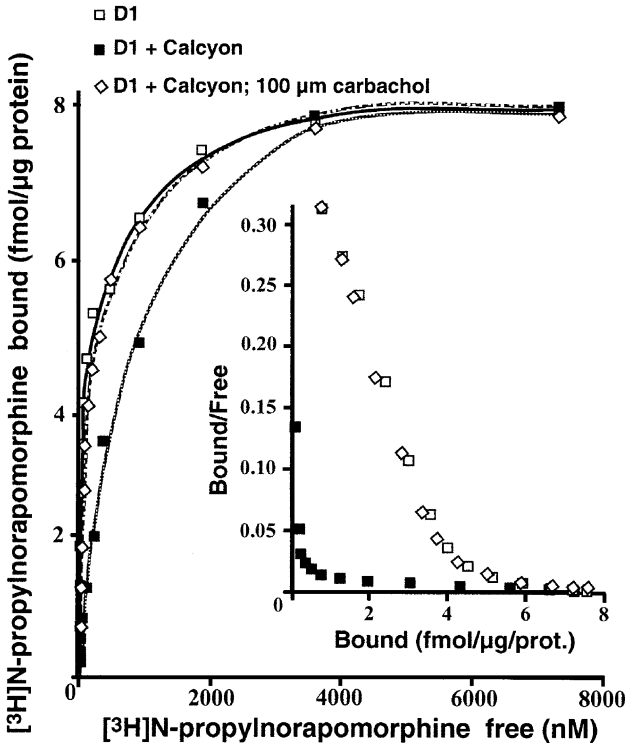

Fig. 4. Specific saturation binding of an agonist radioligand, [^{3}H]N-propylnorapomorphine (this dopamine D1/D2 radiologand selectively labels dopamine D1 receptors in our preparations lacking dopamine D2 receptors) to membranes from cells expressing D1 receptor alone or together with calcyon. In the latter membranes, the bindings were performed in the absence and in the presence of 100 μM of carbachol. The insert represents the corresponding Scatchard transformation of the data. Each point represents a mean value from three separate assays. Note saturability of the binding in all three assays' conditions. In all cases, the saturation data are best fitted by a model assuming specific binding to two affinity sites: $K_d = 9.1 \pm 0.6$ nM, $B_{max} = 3.8 \pm 0.3$ fmol/μg prot and $K_d = 853.3 \pm 17.5$ nM, $B_{max} = 4.3 \pm 1.7$ fmol/μg prot for calcyon-free membranes in the absence of carbachol; $K_d = 8.1 \pm 0.4$ nM, $B_{max} = 0.5 \pm 0.2$ fmol/μg/prot and $K_d = 827 \pm 31.0$ nM, $B_{max} = 7.7 \pm 1.2$ fmol/μg prot for calcyon-containing membranes in the absence of carbachol; $K_d = 11.0 \pm 0.7$ nM, $B_{max} = 3.4 \pm 0.5$ fmol/μg prot and $K_d = 810 \pm 27.9$ nM, $B_{max} = 4.4 \pm 0.7$ fmol/μg prot for calcyon-containing membranes in the presence of carbachol. The addition of carbachol to calcyon free membranes results in: $K_d = 7.9 \pm 0.6$ nM, $B_{max} = 3.6 \pm 0.4$ fmol/μg prot and $K_d = 833.7 \pm 19.8$ nM, $B_{max} = 4.2 \pm 1.0$ fmol/μg prot (not shown). Note that, while the total B_{max} values are similar between the calcyon-free and calcyon-containing membranes ($P > 0.05$), the latter membranes have significantly lower values for B_{max} of the high-affinity sites ($P < 0.05$). The addition of 100 μM carbachol to calcyon-containing membranes results in increased B_{max} of these sites to the levels seen in calcyon-free membranes, without appreciable changes in the total B_{max} value. The addition of carbachol to calcyon-free membranes does not produce significant changes either in total B_{max} or in B_{max} of the high-affinity sites ($P > 0.05$). We detected no significant calcyon or carbachol-induced alterations in K_d values for either high- or low-affinity sites ($P > 0.05$).

were best fitted by a model assuming specific binding to two affinity sites (Fig. 4). Furthermore, similarly to what was observed in the displacement assays, calcyon-containing membranes have significantly lower percentage of high-affinity sites ($6 \pm 2\%$) as compared to calcyon-free membranes ($47 \pm 4\%$; Fig. 4). Also, similar to that seen in displacement assays, the addition of 100 μM carbachol to calcyon-containing membranes resulted in the increase of the percentage of high-affinity sites to $43 \pm 6\%$ (Fig. 4). In contrast, the addition carbachol to calcyon-free membranes failed to produce significant changes in the proportion of these sites ($45 \pm 5\%$). No alteration in the percentage of the high-affinity sites were accompanied by substantial changes either in the K_d values for high- and low-affinity sites or in the total number of the available specific binding sites (Fig. 4 legend).

4. Discussion

The present study demonstrates that, similar to G proteins (Hess et al., 1986), calcyon does not significantly affect either the affinity of dopamine D1 receptor for the antagonists or the affinity of agonist binding to the high and low-affinity states of the receptor. Calcyon, however, appears to regulate the proportion of dopamine D1 receptor high- and low-affinity states for agonists. In this respect, calcyon also resembles G proteins, which have been reported to affect the stability of agonist–receptor complex (Kent et al., 1980; Birnbaumer et al., 1990). In addition, the capacity to change the receptor affinity state for agonists has recently been demonstrated for another G protein-coupled receptor associated protein, arrestin (Gurevich et al., 1997). Arrestin is involved in switching these receptors from interaction with the G protein-associated pathways to a new set of signaling cascades (Hall et al., 1999). Therefore, the ability to affect the affinity state of receptors for agonists may be a common feature of receptor-associated proteins. However, not all proteins capable of binding to G protein-coupled receptors have been found to alter ligand binding affinity. For Example, no effect on the agonist binding to dopamine D4 receptor has been found for Grb2 and Nck adapter proteins, which have shown to be among the proteins coupled to this receptor (Oldenhof et al., 1998).

The data presented here also demonstrate that calcyon differentially affects the ability of D1 dopamine receptors to interact with agonist depending on whether the system has been primed. Specifically, we found that membranes of cells co-expressing dopamine D1 receptor and calcyon contained a significantly lower proportion of high-affinity sites for dopamine D1 receptor agonists as compared to membranes of cells expressing this receptors alone. However, this condition can be reversed in a dose-dependent manner by the crosstalk priming agent, carbachol. It is reasonable to assume that the effect of carbachol is related to its interaction with the $G_{q/11}$ protein-coupled muscarinic receptors endogenously expressed in HEK293 cells rather than to its interaction with dopamine D1 receptor binding sites. The latter scenario would likely to be accompanied by significant changes in observed K_i and K_d

values for dopamine D1 agonists, which were not observed in our study.

Our findings that in unprimed membrane preparations the co-expression of dopamine D1 receptor and calcyon leads to a substantial reduction in the proportion of the high-affinity binding sites for dopamine D1 receptor agonists suggest that priming is not a prerequisite for association of calcyon with dopamine D1 receptor. Previously, it was demonstrated that priming agents, together with dopamine D1 receptor agonists, could significantly increase the co-precipitation of calcyon and dopamine D1 receptor (Lezcano et al., 2000), thus presumably strengthening the interaction of this receptor with $G_{q/11}$ protein-coupled receptors. The results of the present study indicate that priming also boosts the high-affinity state of dopamine D1 receptor for agonists. This is consistent with the idea that priming promotes an increase in D1 dopamine receptor activation of Ca^{2+}-associated intracellular pathways by providing for more efficient linkage of this receptor with $G_{q/11}$-protein activated pathways and by shifting dopamine D1 receptor into high-affinity state for agonists. The latter can be accomplished either by an active stabilization of dopamine D1 receptor–agonist complex or by down-regulation of the 'negative influence' that calcyon exerts on dopamine D1 receptor binding sites. The fact that carbachol-induced increase in the proportion of the high-affinity sites asymptotes at the levels seen in the calcyon-free membranes points to a mechanism which overrides the influence of calcyon on dopamine D1 receptor agonist binding.

An important observation is that addition of GTP to calcyon-expressing membranes further reduces the proportion of high-affinity dopamine D1 receptor binding sites for agonists. In addition, GTP counteracts the increase in the proportion of these sites produced by carbachol in the calcyon-containing preparation. These data are consistent with several models of the receptor—(G-protein)—calcyon interaction. For example, as mentioned above, it is possible that calcyon in unprimed membrane preparations prevents G protein from affecting dopamine D1 receptor affinity state. In this model, the observed effect of GTP would be due largely to its interference with the G protein-induced high-affinity state in the receptors, which either escaped the interaction with calcyon and/or were released from calcyon's regulation by priming membrane preparations with carbachol. Alternatively, it is conceivable that both calcyon and G protein jointly regulate the affinity state of the entire dopamine D1 receptor population. Such a model suggest that GTP might produce its effects by altering the contribution of G protein to this combined regulatory activity. Finally, at least some of the GTP regulatory effects in carbachol-treated preparations could be due to its actions on $G_{q/11}$ proteins crosslinked to dopamine D1 receptors, which might interfere with the effect of carbachol. In any case, our data point to the fact that the ability of dopamine D1 receptors to form the high-affinity state for agonists in samples are likely to be a reflection of combined regulatory influences of G protein, calcyon, and possibly other receptor-associated proteins present in these samples. Furthermore, based on the observations of these and other studies (Schulz et al., 1985; Hess et al., 1986; Urwyler, 1987; Ferre et al., 1993), it is reasonable to expect that the contribution of individual proteins to regulation of the receptor affinity state would depend on the presence of specific chemicals, such as nucleotides, ligands (priming agents), and ions, in the milieu.

In this respect, it is interesting that there are indications that calcyon may indeed participate in the regulation of the proportion of the high and low-affinity dopamine D1 receptor sites for agonists in samples from calcyon-rich areas of the brain. For example, it has been demonstrated that calcyon is much more abundant in the prefrontal cortex than in the basal ganglia (Lezcano et al., 2000). This observation predicts a lower proportion of the high-affinity dopamine D1 receptor sites in the former structure, which is exactly what has been reported by De Keyser et al. (1988).

Finally, while the present paper reports that expression of calcyon in D1-HEK293 cells results in a significant reduction in the proportion of the high-affinity binding sites for dopamine D1 receptor agonists, our previous studies showed that calcyon expression does not interfere with the levels of adenosine 3′,5′-cyclic monophosphate (cAMP) production induced by such agonists, either in the presence or in the absence of carbachol (Lezcano et al., 2000; Lezcano and Bergson, unpublished observations). This contradicts a popular view that the ability of agonists to actuate their G proteins should correlate closely with the levels of receptor high-affinity state formed in the presence of these agonists (Kent et al., 1980; Assie et al., 1999; Waelbroeck, 1999). There is, however, a growing list of instances demonstrating a lack of relationship between the detectable high-affinity state of a G protein-coupled receptor and the efficacy of second messenger activation by the agonists acting on this receptor (Childers et al., 1993; Johnson et al., 1994; Van Rampelbergh et al., 1996; Roth et al., 1997; Seifert et al., 1998; Di Salvo et al., 2000). Therefore, it is tempting to speculate that the ability to exist in a high-affinity state may not be essential for the capacity of dopamine D1 receptors to activate G_s proteins and increase production of cAMP. However, it is possible that for a dopamine D1 receptor to initiate a robust intracellular Ca^{2+} release through the calcyon-mediated receptor crosstalk may actually require this receptor to achieve a high-affinity state. In this case, in the systems containing calcyon and, therefore, capable of receptor crosstalk, the downregulation of the proportion of high-affinity dopamine D1 receptor sites by this protein, in the absence of priming, may serve as a safety mechanism that prevents accidental activation of receptor crosstalk. Priming makes an effective calcyon-mediated crosstalk possible in part by allowing dopamine D1 receptors to achieve a high-affinity

state. Furthermore, the dopamine D1 receptor-associated G_s proteins may participate in regulation of the efficacy of the calcyon-mediated receptor crosstalk by limiting the proportion of high-affinity dopamine D1 receptor sites achievable in a given system. We hope that future studies will be able to directly address this hypothesis.

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