Calmodulin Binding to G Protein-coupling Domain of Opioid Receptors

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Calmodulin (CaM)1 is a ubiquitous Ca2+-sensitive regul-

ory protein found in virtually every class of living organism from plants to alga, from humans to protozoa (1). Originally identified as the Ca2+-dependent factor responsible for activating 3',5'-cyclic-nucleotide phosphodiesterase (2–5), the role of CaM as a regulator of cytoplasmic enzymes has broadened to include adenylyl cyclases, Ca2+/CaM-dependent kinases and phosphatases, ion channels, Ca2+-ATPases, and others (1, 6, 7). Mapping relationships between the multitude of enzymes regulated by CaM is essential to the understanding of biochemical cascades linked to activation of G protein-coupled receptors (GPCRs).

While previous studies have revealed broad involvement of CaM in downstream signaling pathways initiated by G protein-coupled receptors, progress is also being made toward uncovering interactions more proximal to the receptor. Recent examples include the discovery of a CaM binding site on a G protein b-subunit (8) and another report that describes a CaM binding site on the metabotropic glutamate subtype 5 receptor (9). The CaM binding domain found on the metabotropic glutamate subtype 5 receptor is located in the extended C-terminal region and appears unique to this receptor. Whereas core regions of the receptor involved in G protein coupling have not been previously implicated in CaM interactions, circumstantial evidence suggests such an involvement of CaM at the receptor level. The ability of certain CaM-binding peptides, like mastoparan and mellitin, to activate Go as well, in essence substituting for the receptor (10–12), leads to suspicion that GPCRs may contain core regions of structural similarities to CaM-binding domains.

In this report, we identify an i3 loop region within receptors of the opioid family, in particular OP1 and OP3, as a potential site for CaM interaction. We also demonstrate that CaM inversely affects G protein coupling by OP1 and OP3 and propose that CaM itself could function as an independent second messenger molecule.

EXPERIMENTAL PROCEDURES

Synthesis of OP3 Receptor i3 Loop Peptides—Peptides corresponding to sections of the i3 loop of the OP3 receptor (see Table I) were prepared on an Applied Biosystems 433A automated peptide synthesizer (Foster City, CA) using para-methylbenzhydramine resin and standard Fmoc (N-(9-fluorenylmethoxycarbonyl) solid phase methods. Peptides were purified on a Dynamax SD-200 high pressure liquid chromatograph (Varian Chromatography Systems, Walnut Creek, CA) using a reverse-phase C-18 column and eluting across a gradient of 10–50% acetonitrile in water containing 0.1% (v/v) trifluoroacetic acid.

Cell Culture and Transfections—HEK293 cells were maintained at mouse and human m-opioid receptor, respectively; HEK-rOP3 and hOP3, HEK cells stably transfected with rat or human OP3 receptors; PBR, polymerase chain reaction; PBS, phosphate-buffered saline; GTPγS, guanosine 5'-3-O-(thiotriphosphate; DPPE, [D-Pen2,5]-enkephalin; BAPTA, 1,2-bis(O-aminophenoxy)ethane-N,N',N'-tetraacetic acid, sodium.
37 °C and under 5% CO₂ in DME/H16/F12 medium supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, and 100 IU/ml penicillin. Stably transfected HEK293 cell lines were established as described by Arden et al. (13) containing the following opioid receptor subtypes: (i) the rat μ-opioid receptor (Oπ₁) (14); (ii) an N-terminally FLAG-tagged mouse μ-OP_3 (FLAG-mOP_3); (iii) a mutant rat κ-opioid receptor (mOP_3) (16, 17); (iv) an N-terminal FLAG-tagged human μ-opioid receptor (FLAG-hOP_3); and (v) a LyS<sup>145→A</sup> Ala FLAG-hOP_3 mutant receptor (K273A-OP_3) (described below). Cell lines established with cDNA encoding mouse, rat, and human OP_3 receptors (HEK-μOP_3, HEK-κOP_3, and HEK-δOP_3, respectively) were used interchangeably, since their i₃ loop sequences are identical. HEK-μOP_3, HEK-κOP_3, and HEK-δOP_3 cell lines were prepared using 1% to 2% total protein determined by [³H]diprenorphine (2 nM) binding in intact whole cell monolayers as described by Arden et al. (13).

Site-directed Mutagenesis of the hOP_3 Receptor—The FLAG epitope was attached to N terminus of the hOP_3 receptor (18) using Pfu DNA polymerase (Stratagene, La Jolla, CA) and confirmed by DNA sequencing (Biomo-
**μ-Opioid Receptor-Calmodulin Interactions**

**RESULTS**

**CaM-binding Motif Search**—Binding of CaM to CaM-binding proteins does not involve a universal, conserved sequence motif (26), therefore it remains difficult to define CaM binding domains in distinct protein families using sequence alignment data. To identify possible CaM binding domains in GPCRs, we used a series of sequence motifs derived from distinct protein families with known CaM binding domains for a PatScan (27) pattern search of the SwissProt protein data base. Scanning for possible binding motifs produced matches within a number of known CaM binding domains and a limited number of additional proteins, including GPCR sequences located in the i3 loop, among the opioid receptors emerged with several motifs searching similarities to myosin light chain kinases, Ca2+/CaM kinases, and phosphodiesterases. Shown in Fig. 1 are representative alignments, with key motif residues underlined, suggesting the possibility that the i3 loop might interact not only with G proteins (10, 19, 28, 29) but also with CaM.

**Binding of OP3 Receptor-derived Peptides to CaM**—The proposed site for interaction with CaM in the i3 loop of opioid receptor was first examined by observing mobility shifts of CaM using nondenaturing gel electrophoresis (8). CaM was incubated in varying molar ratios with selected peptides derived from the sequence of the OP3 receptor i3 loop (Table I; Fig. 2A). Three peptides containing the C-terminal portion of the i3 loop predicted to interact with CaM, namely i3-1, i3-2, and i3-3, caused a notable shift in the electrophoretic mobility of CaM, whereas the N-terminal peptide i3-4 did not (Fig. 2B). The small gel shift caused by the low mass i3-1 peptide is better visualized in Fig. 2B. These results indicate that the C-terminal portion of the OP3 receptor i3 loop is a suitable candidate for interaction with CaM. Mobility shifts were not observed in the absence of Ca2+ (not shown), suggesting that peptide-CaM interactions are strengthened in the presence of Ca2+.

In contrast to mobility shifts observed with Ca2+/CaM kinase II-(290–309) or -(281–309), which were complete at molar ratios of 1:1 (Table I), shifts in CaM mobility caused by i3 loop peptides were incomplete even at substantially higher ratios. The i3-2 peptide, which lacks the N-terminal portion of the i3 loop, for example, produced a visible gel shift only at a 100-fold excess over CaM (Fig. 2A). The inability of i3 loop peptides to cause a complete shift in CaM under these conditions is similar to results reported for peptides derived from the CaM binding region of the metabotropic glutamate subtype 5 receptor (9).

Since motif analysis indicated that Lys277 of the OP3 receptor may be important for binding interaction with CaM, two modified i3-1 peptides (one in which the N-terminal Lys is removed (i3-5) and another in which Lys is replaced with Ala (i3-6)) were also synthesized and tested (Fig. 2B, Table I). Both of these modified peptides failed to induce mobility shifts of CaM, demonstrating that modifications to this critical residue can affect the ability of i3 loop peptides to interact with CaM.

Shifts in the mobility of Ca2+/CaM kinase II peptides reflect the strong interaction that occurs with CaM, consistent with previous reports (30). In contrast, several peptides representing mostly the autophosphorylation substrate domain of Ca2+/CaM kinases failed to shift CaM. Moreover, autacamide-2 and mastoparan, two peptides with sequence motifs and predicted secondary structures resembling those of CaM binding domains also failed to shift the CaM band (Table I), although mastoparan is known to bind to CaM with high affinity when determined by fluorescence shifts of dansylated CaM (11). This suggests that unlike the i3 loop peptides, binding stability of mastoparan to CaM appears insufficient to induce a detectable shift in this gel assay.

**Interaction of i3 Loop Peptides with Dansyl-CaM**—CaM binding of i3 loop peptides was also examined by measuring changes in the dansyl-CaM fluorescence spectrum (20). The i3-3 peptide, which represents the amino acid sequence of the entire OP3 receptor i3 loop, caused a notable increase in dansyl-CaM fluorescence among the i3 loop peptides tested, and changes in fluorescence intensity occurred both in the presence and absence of Ca2+ (Fig. 3A). This suggests that i3-3 is capable of inducing a substantial conformational change in CaM (12) and that its interaction is at least partially Ca2+-independent. Each of the other i3 loop peptides was also able to induce detectable, although smaller, changes in dansyl-CaM fluorescence at higher concentrations, although some of these peptides failed to induce a CaM gel shift, and no response was observed with the control FLAG peptide.

To estimate the relative potency of the i3 loop peptides, changes in fluorescence intensity were measured when Ca2+/CaM kinase II-(290–309) is complexed with dansyl-CaM, an interaction that has an approximate binding K_d of 50 nM (30), in the presence of test peptides. In the presence of Ca2+, i3 loop...
The numbering of the OP3-derived peptides is according to the human OP3 sequence (single letter code). The shown sequences are identical between human and rodent OP3. Substitutions of the wild-type sequence are indicated by single letter amino acid code with a right superscript number indicating the substituted position.

| Peptide name | Sequence | CaM binding* |
|--------------|----------|--------------|
| OP3-i3-1 (273–286) | KDRNLRRTRRMLVL | ++ |
| OP3-i3-2 (267–286) | LGSKERKLRRTRRMLVL | + |
| OP3-i3-3 (258–286) | ILRLKVRMLGSKERKLRRTRRMLVL | ++ |
| OP3-i3-4 (258–286) | ILRLKVRMLGSKERKLRRTRRMLVL | ++ |
| OP3-i3-5 (274–286) | ADRNLRRTRRMLVL | - |
| OP3-i3-6 (A273–273–286) | ADRNLRRTRRMLVL | - |
| Ca2+/CaM kinase II (281–291) substrate | MHQRETVDCLK-NH2 | - |
| Ca2+/CaM kinase II (A286–281–301) inhibitor | MKKNARML-GAILTTMLA | - |
| Ca2+/CaM kinase II (290–309) inhibitor | MKKNARML-GAILTTMLA | - |
| Autocamtide-2 | KKLRLQRTETVNL | - |
| CaM-dependent protein kinase substrate | PLSRTLSSV-NH2 | - |
| CaM-dependent protein kinase substrate analogue | PLRRTLSVAA-NH2 | - |
| Mastoparan | INLAKAAAKL-NH2 | - |
| FLAG peptide | DYYDNDQDK | - |

*++, partial gel shift at >1:10 CaM:peptide ratio; ++, partial gel shift at 1:1 CaM:peptide ratio; +++, complete gel shift at a 1:1 ratio.

Fig. 2. Binding of OP3 receptor i3 loop-derived peptides to CaM using a nondenaturing gel shift assay. Calmodulin was incubated with peptides derived from the OP3 receptor i3 loop in molar ratios of 1:1, 1:10, and 1:100 (CaM:peptide as indicated) in the presence of Ca2+ and separated by nondenaturing polyacrylamide gel electrophoresis including Ca2+. The addition of EGTA to chelate Ca2+ suppressed the gel shift (not shown). For peptide designations see Table I. A, lane 1, CaM alone; lanes 2–13, CaM plus the indicated peptides, i3-1, -2, -3, and -4. B, lane 1, CaM alone; lanes 2–4, CaM plus peptides i3-1, -3, -5, and -6.

Fig. 3. Emission fluorescence spectra of dansyl-CaM. A, emission fluorescence of dansyl-CaM (0.6 μM) in 10 mM HEPES (pH 7.0) buffer, in the absence and presence of 200 μM Ca2+, respectively, was determined with and without the i3 loop peptide i3-3 added (2 μM). Excitation was at 340 nm. The i3-3 peptide alone did not afford measurable fluorescence at 400–600 nm. B, C, and D, competition between Ca2+/CaM Kinase II-(290–309) peptide (100 nM) and various concentrations of i3 loop-derived peptides for binding to dansyl-CaM (150 nM), measured by fluorescence excitation/emission at 340/490 nm in a fluorescence plate reader. B, in the presence of 10 μM free Ca2+, C and D, in the absence of Ca2+.

Peptide-calmodulin interactions measured with a gel shift assay (see Fig. 2).

The numbering of the OP3-derived peptides is according to the human OP3 sequence (single letter code). The shown sequences are identical between human and rodent OP3. Substitutions of the wild-type sequence are indicated by single letter amino acid code with a right superscript number indicating the substituted position.
detectable by Western blot analysis when extracts of HEK-FLAG-mOP₃ cells were applied (data not shown). This suggests that if these proteins were associated with OP₃ receptor in the intact cells, they had dissociated during the solubilization process. On the other hand, externally added biotinylated CaM did not bind to immobilized FLAG-mOP₃ receptor in the presence of Ca²⁺ (Fig. 4D). In contrast, CaM was not recovered when using extracts from vector-transfected cells treated in the same fashion. Moreover, in the absence of Ca²⁺, CaM was not retained by FLAG-rOP₃ receptor, indicating that the binding is Ca²⁺-sensitive (Fig. 4D).

CaM Blocks G Protein Activation by i3 Loop Peptide i3-3—Peptides derived from both the N and C terminus of the i3 loop of various GPCRs have been shown to activate G proteins directly (29). Therefore, the ability of peptides derived from the i3 loop of OP₃ receptor to stimulate [³⁵S]GTPyS binding to membranes of HEK-rOP₃ cells was examined. Each of the peptides tested, i3-1, -2, -3, and -4 (30 µM each), caused a significant 10–20% increase in [³⁵S]GTPyS binding to membranes. The full-length i3 loop peptide, i3-3, produced the largest effect of 22% (SD = 2%, n = 6, p < 0.001, Student’s t test, unpaired). The addition of an equimolar CaM concentration completely blocked G protein activation by i3-3, reducing [³⁵S]GTPyS incorporation to the same level observed with CaM alone (n = 9, p < 0.001, Student’s t test, unpaired). This result indicates that CaM binding to i3-3 prevents G protein activation.

Effects of CaM on Opioid Receptor-G Protein Coupling—The effect of CaM on G protein activation was examined in membranes prepared from HEK-mOP₁ (δ-opioid) and HEK-rOP₃ (μ-opioid) cells. Washing in Ca²⁺-free pH 8/EGTA buffer reduced CaM levels in plasma membranes (expressed as CaM activity/mg of protein) to 58% (S.D. = 14%, n = 9, p < 0.001, t test, unpaired) of its original level. [³⁵S]GTPyS binding activity of washed membranes, on the other hand, was significantly elevated over unwashed controls (Fig. 5). The increase in [³⁵S]GTPyS binding activity (of washed versus unwashed membranes) was observed in both absence (basal activity) and presence of agonists (OP₁ and OP₃ membranes stimulated with 1 µM DPDPE and morphine, respectively) (Fig. 5). Washing
membranes containing OP3 receptors, therefore, caused an increase in both basal and stimulated levels of [35S]GTPγS incorporation that is reflected in an upward shift of concentration-response curves illustrated in Fig. 6. Both basal and stimulated increases in [35S]GTPγS binding activity were abolished by pertussis toxin pretreatment in opioid receptor-containing membranes, and no change in activity was observed in mock-transfected (pRC/CMV) membranes under any of the conditions described above (Fig. 5). This suggests that CaM may interfere with or reduce G protein coupling in membranes containing OP1 and OP3 receptors. Furthermore, elevated [35S]GTPγS binding activity is observed in membranes containing either opioid receptor when compared with mock-transfected control under agonist-free conditions (Fig. 5). This enhanced activity was abolished by pertussis toxin, indicating that it reflects an increase in basal activity caused by the presence of opioid receptors.

The effect of adding CaM to washed membranes was also considered and is illustrated in Fig. 6A. The addition of CaM to washed HEK-rOP3 membranes caused a lowering of [35S]GTPγS binding activity to levels observed before washing, across the entire concentration range of morphine, without significant changes in EC50 values (0.84–1.94 nM). These results reinforce the contention that CaM is responsible for the changes observed and inversely affects G protein coupling. Guanine nucleotides (GDP) were omitted in the experiment illustrated in Fig. 6A to facilitate [35S]GTPγS binding and possible competition between CaM and G proteins. In the presence of 1 μM GDP, pH 8/EGTA washing similarly enhanced [35S]GTPγS binding activity over the entire concentration range of morphine used, but the addition of CaM was unable to fully reverse this enhanced activity (data not shown). This suggests that a rather stable OP3 receptor-G protein interaction may have formed in the membranes (31).

Effect of Alteration of CaM Levels in Intact Cells on Opioid Receptor G protein Coupling—CaM levels were also altered in intact cells by expressing plasmids encoding sense and antisense CaM cDNA. Membranes prepared from HEK-rOP3 cells transiently transfected with sense CaM cDNA, expressing CaM at levels of 186% (CaM activity/mg protein; S.D. = 24%; n = 9, p < 0.001, t test, unpaired) over mock-transfected cells, had a significantly reduced level of basal and agonist-stimulated [35S]GTPγS binding activity (illustrated in Fig. 6B). This was reversed upon washing cell membrane with pH 8/EGTA buffer (Fig. 6B), which lowered CaM levels by 46% (S.D. = 8%, n = 9, p < 0.001, t test, unpaired). EC50 values for morphine did not change significantly under any condition (3.5–4.3 nM). (Higher EC50 values observed in Fig. 6B compared with Fig. 6A are due to the presence of GDP in the assay incubations.) CaM transfection not only lowered the entire dose-response curve but also blunted the maximal morphine effect on [35S]GTPγS binding activity (Emax from 120 ± 2 to 94 ± 9% over basal, means ± S.D., p < 0.05, t test, unpaired); this effect was also reversed upon washing (Emax 126 ± 7%).

In contrast to cell lines with sense CaM vectors, stable expression of antisense CaM reduced CaM levels to 73% (S.D. = 13%, n = 9, p < 0.001, t test, unpaired) of the control, and these cells were found to have significantly elevated basal and morphine-activated [35S]GTPγS binding activity (Fig. 6C). Importantly, washing of the antisense CaM cell membranes failed to affect the morphine concentration-response curve of [35S]GTPγS binding activity (Fig. 6C). Moreover, the maximal effect of morphine was enhanced by antisense CaM expression from 110 ± 11 to 147 ± 21% over basal (means ± S.D., n = 9, p < 0.05, t test, unpaired), and the EC50 value was significantly reduced from 5.5 ± 1.4 to 1.7 ± 0.2 nM (means ± S.D., n = 9, p < 0.05, t test, unpaired). The activity of mock-transfected cell membranes was equivalent to untransfected controls (data not shown), ruling out a nonspecific effect of plasmid transfection.

G Protein Coupling of the Mutant K273A-FLAG-hOP3 Receptor—Since the motif search and experimental results with i3 loop-derived peptides indicated that Lys273 of OP3 receptor could play a role in the proposed interaction with CaM, a K273A-OP3 receptor mutant was constructed, and its ability to stimulate [35S]GTPγS binding activity was compared with wild-type FLAG-hOP3 receptor. The FLAG-K273A-hOP3 receptor was still retained on a CaM affinity gel, as determined by immunoblotting, but retention was relatively small and not easily quantifiable (data not shown). Thus, CaM interaction with this mutated receptor does not appear to be completely abrogated, in agreement with the i3-6 peptide’s partial ability to block FLAG-mOP3 binding to CaM (Fig. 4C).

Compared with the wild-type FLAG-hOP3 receptor, basal and morphine-activated [35S]GTPγS binding activity for the FLAG-K273A-hOP3 receptor were elevated regardless of washing with pH 8/EGTA buffer (see Fig. 6D). These results indicate that substitution of Lys273 with Ala induces elevated levels of basal G protein stimulation and that the receptor is no longer responsive to changes in CaM content.

CaM Affects High Affinity Binding of Morphine to the OP3 Receptor—The addition of CaM to HEK-rOP3 membranes had no significant effect on binding of the antagonist tracer, [3H]naloxone, which is thought to bind to the G protein-coupled and -uncoupled states of the receptor equally well. In contrast, morphine-[3H]naloxone competition curves in HEK-rOP3 membranes revealed the presence of low and high affinity sites (Fig.
7A). The displacement curve is fitted better by a two-site, rather than a one-site, receptor binding model, indicating a distribution of 34 ± 2% high affinity and 64 ± 8% (means ± S.D.) low affinity sites.

If the high affinity site were to represent the G protein-coupled state, one would expect displacement of G proteins by CaM to abolish the high affinity site. Indeed, incubation of the membranes with CaM (final concentration, 15 μM) in the presence of 50 μM CaCl2 largely eliminated the high affinity site (Fig. 7A), and the resultant data fit a one-site model (Fig. 7A). The binding curve in the presence of CaM is nearly identical to that obtained in the presence of GTPγS, which uncouples the OP3 receptor from its G proteins (Fig. 7A). Chelation of Ca2+ by EGTA prevented the effect of CaM on the high affinity binding site, another indication that CaM-OP3 interactions are Ca2+-sensitive.

We also measured [3H]naloxone-morphine displacement curves in membrane obtained from HEK-rOP3 cell that over-expressed CaM (HEK-rOP3/CaM, stably transfected, in which membrane CaM activity was 134 ± 16% of that of mock-transfected cells). Overexpressing CaM eliminated the high affinity binding site (Fig. 7B). Moreover, the addition of GTPγS had no further effect on the morphine displacement curve (Fig. 7B). Thus, elevated CaM levels abolished the high affinity agonist binding site.

To address the question of whether CaM effects on OP3 receptor arise from direct binding to the receptor or indirectly to another protein attached to the receptor such as Gα, we determined ligand binding of the mutant receptor K273A-FLAG-hOP3, which effectively couples to G proteins but is functionally insensitive to CaM. Shown in Fig. 7C, the high affinity site of the K273A-FLAG-hOP3 receptor remained unaffected by CaM addition, although the addition of GTPγS eliminated this high affinity site. This supports the view that the changes in high affinity morphine binding to OP3 receptors were mediated by direct CaM-OP3 receptor interactions.

Morphine-induced CaM Release from Plasma Membranes—To determine whether OP3 receptor activation can cause dissociation of CaM from the plasma membrane, we measured changes of CaM content in plasma membranes prepared from HEK-rOP3 that had been exposed to 1 μM morphine (Fig. 8A). There was a significant reduction of membrane CaM content within 1 min, leveling off at 15 min. A similar effect was observed with HEK-FLAG-hOP3 and with HEK-mOP3 cells stimulated with 1 μM DPDPF (Fig. 8B). Moreover, treatment with pertussis toxin (100 ng/ml for 18 h) failed to affect the morphine-induced loss of CaM from plasma membranes in HEK-rOP3 cells, whereas morphine had no effect on CaM levels in K273A-FLAG-hOP3 cells (Fig. 8B). These results suggest that G proteins do not play a role in CaM translocation and, further, that CaM depletion from the membrane might be a direct result of CaM dissociation from the receptor.

Effect of CaM Levels on OP3 Receptor Regulation of cAMP in Intact Cells—The ability of morphine to lower forskolin-stimulated cAMP levels was measured in HEK-rOP3 and in sense and antisense CaM-expressing HEK-rOP3 cells. Changing the CaM content failed to affect the EC50 value of morphine significantly (range between 4 and 8 nM). However, in HEK-rOP3 cells, as-CaM cells with reduced CaM content, maximum inhibition of cAMP accumulation by morphine increased from 73 ± 3% (normal CaM level) to 97 ± 4% (means ± S.D., n = 6, p < 0.05, t test, unpaired). Similarly, morphine was slightly but significantly more efficacious in HEK-K273A-FLAG-hOP3 than in HEK-FLAG-hOP3 cells (maximum inhibition of cAMP accumulation was 82 ± 3% versus 68 ± 2%; means ± S.D., n = 6, p < 0.01, t test, unpaired), again without a significant change in the EC50 (2 nM in each case). These results parallel those observed with [35S]GTPγS binding to cell membranes.

**DISCUSSION**

This study provides evidence in support of the hypothesis that CaM interacts with the i3 loop of OP3. We have demonstrated that solubilized OP3 interacts with CaM in a Ca2+-sensitive manner. Moreover, peptides derived from the i3 loop of OP3 bound to CaM and suppressed interactions between CaM and solubilized OP3. As expected from these results, CaM content of plasma membranes inversely affected G protein coupling by OP3 and suppressed high affinity binding sites of morphine to OP3, thought to represent the G protein-coupled state of the receptor. However, these results taken alone did not rule out the possibility that CaM could modulate receptor-G protein coupling indirectly, for example, by binding to the β subunit of G proteins (8). However, the solubilized and immunopurified OP3 appeared devoid of associated G protein, since we were unable to detect any Gαi3 (a major Gα-coupling protein of OP3) in the immunopurified rOP3 preparation; therefore, G proteins are unlikely to mediate the observed CaM binding to purified OP3. To resolve the question whether a direct interaction occurs, we screened a number of OP3 mutants with single residue substitutions in the i3 loop for loss of CaM interaction, but with preserved G protein coupling. Importantly, the OP3 mutant K273A (selected on the basis of the role of this Lys residue in motif structure and i3-loop peptide binding to CaM) displayed enhanced G protein coupling, but it was insensitive to CaM. This result strongly supports a direct interaction between OP3 and CaM.

Taken together, our results suggest that CaM and G proteins compete for a shared binding domain in the i3 loop of OP3. As proposed for G protein binding domains, both the N terminus and the C terminus of i3 may play a role in CaM binding,
although the C terminus is the dominant factor. While the binding mechanism between CaM and the i3 loop remains to be resolved, it is possible that a distortion of the i3 loop upon receptor activation could lower the affinity to CaM and cause CaM dissociation. CaM binding to OP3 was enhanced by Ca2+, but studies with i3 loop peptides indicate that Ca2+ is not essential. At resting conditions with low intracellular Ca2+ levels, OP3 may thus be occupied either by CaM or by G proteins. OP3 activation could cause dissociation of CaM so that the unoccupied receptor can subsequently rebind G proteins. Therefore, elevated CaM levels failed to abolish agonist-induced G protein coupling.

This study further establishes a significant level of basal G protein coupling in cell membranes for both OP3 and OP1, as suggested previously (31–33). Basal G protein signaling was highly sensitive to CaM content; thus, overexpression of CaM suggested previously (31–33). Basal G protein signaling was CaM-insensitive mutant receptor K273A-hOP3 displayed increased basal activity over that of wild-type hOP3.

In conclusion, we present evidence in support of the hypothesis that CaM binds to the i3 loop of opioid receptors and that CaM may serve as a second messenger molecule upon receptor activation.

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