Determining calmodulin binding to metabotropic glutamate receptors with distinct protein–interaction methods

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Abstract
mGluRs (metabotropic glutamate receptors) are G-protein-coupled receptors that modulate synaptic transmission. The eight mammalian mGluRs form three groups based on sequence and functional similarities: group I (1 and 5), group II (2 and 3) and group III (4, 6–8) mGluRs. In the present study, we used a Y2H (yeast two hybrid) screen to identify proteins that interact with the C-terminal intracellular tail of mGluR3. Prominent among the candidate receptor interacting proteins was calmodulin, a Ca2+ sensor known to bind identifiable sequences in group I and III mGluRs. The Y2H method was used to investigate calmodulin binding to mGluRs but failed to confirm the documented interaction with group III mGluRs. Furthermore, subsequent biochemical analysis showed that calmodulin does not interact with group II mGluRs. This illustrates that certain Ca2+-dependent interactions are not recapitulated in yeast. Moreover, it highlights the necessity for supporting biochemical data to substantiate interactions identified with Y2H methods.

Introduction
The metabotropic glutamate receptors belong to family C of the G-protein-coupled receptors. The signalling of group I (1 and 5), group II (2 and 3) and group III (4, 6–8) mGluRs [1] is regulated by protein interactions with intracellular domains of the receptor. Intracellular loop two is a major determinant of the interaction with the G-protein. The C-terminus binds a growing list of proteins that affect mGluR function. This region also shows the greatest sequence variation in mGluRs allowing interactions with subtype-specific regulators (see [2] for a review). In addition, there are proteins that are more generic regulators of functions, interacting across mGluR sub-groups. Particularly, group I and group III mGluRs bind the intracellular Ca2+ sensor CaM (calmodulin) [3–7]. Ca2+/CaM-binding domains contain two bulky hydrophobic amino acids with a 1–10, 1–14 or 1–16 spacing [8]. Group I mGluRs contain 1–14 motifs and group III mGluRs contain 1–10 motifs (Figure 1A). When screening for group II intracellular binding proteins, we identified CaM as a CRIP (candidate receptor interacting protein). This contrasted indications that Ca2+/CaM-binding determinants were not found in group II mGluRs [5]. Indeed, our studies show that group II receptors do not bind CaM, and highlight the unsuitability of Y2H (yeast two hybrid) methods for certain types of Ca2+/CaM-binding.

Materials and methods
Yeast two-hybrid screen
cDNA encoding the C-tail of mGluR3 (amino acids 829–879) was fused to LexA in pGilda (Clontech) and used as bait in a two-hybrid screen with a rat brain cDNA library (Origene). The screen was performed as detailed in [9], and prey plasmids were rescued from yeast colonies that satisfied three further rounds of selection on −Leu growth and β-galactosidase activity. Specificity of the bait and prey interaction included showing no transactivation of reporter genes by these plasmids alone. Semi-quantitative analysis of CaM-binding interaction to mGluR C-tails was performed using a liquid culture β-galactosidase assay [10].

CaM-binding experiments
Rat brain cerebellum membranes or membranes derived from HEK 293 cells transfected with pBkCMV-mGluR2 were solubilized (1 h at 4 °C) with PBS (pH 7.4) containing 1.5% (v/v) Triton X-100 and Complete protease inhibitor before centrifugation at 100 000 g for 45 min [9]. Solubilize (Load) was incubated with CaM–agarose in the presence of 1 mM CaCl2 or 5 mM EGTA for 2 h at 4°C. Unbound material was removed (Flow) and the beads washed three times with extraction buffer before bound protein was eluted with SDS sample buffer. The fractions were subjected to SDS/PAGE and Western blotting and probed for mGluR4a (Upstate Biotechnology, Lake Placid, NY, U.S.A.) or mGluR2/mGluR3 (Chemicon International, Temecula, CA, U.S.A.) immunoreactivity [9].

Key words: calmodulin, metabotropic glutamate receptor (mGluR), protein interaction, yeast two-hybrid (Y2H) screen.

Abbreviations used: CaM, calmodulin; CRIP, candidate receptor interacting protein; mGluR, metabotropic glutamate receptor; Y2H, yeast two hybrid.

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**Results and discussion**

A Y2H screen of a rat brain cDNA library, utilizing the C-tail of mGluR3 as bait, identified a number of CRIPs including a prey clone encoding full-length calmodulin. A semi-quantitative measurement of the interaction was made by assaying β-galactosidase activity using a liquid assay. The group III mGluRs, which have been previously shown to support a high-affinity interaction with Ca\(^{2+}\)/CaM, were tested in conjunction with mGluR2 and mGluR3 (Figure 1B). mGluR2 and mGluR3 produced significant β-galactosidase activity when compared with yeast transformed with empty bait vector alone. In contrast, the group III receptors, which harbour a defined high-affinity Ca\(^{2+}\)/CaM-binding site did not exhibit significant β-galactosidase activity. Indeed, the C-tail of mGluR6, the only group III receptor that does not bind CaM, gave a value similar to that for mGluR C-tails that harbour a CaM-binding site. The bait and prey interaction that drives the reporter expression occurs in the yeast nucleus, where Ca\(^{2+}\) levels may not be high enough to support Ca\(^{2+}\)-dependent binding. We attempted to increase nuclear calcium by adding 100 mM Ca\(^{2+}\) or the ionophore A21387 to the growth medium at the point of bait and prey induction [11]. These measures failed to change β-galactosidase activity (results not shown). This failure to report group III mGluR binding to CaM could be due to the bait proteins binding endogenous CaM precluding binding of the LexA-fused calmodulin. Indeed, in a Gal4-based yeast system (Clontech, Basingstoke, U.K.), where the bait protein is constitutively expressed, the C-tails of the group III mGluRs retard growth, but this toxicity is not seen with mutant C-tails that lack the 1–10 CaM-binding motif (results not shown). These difficulties seem to be specific to certain types of CaM-binding interactions, as CaM binding to sub-domains of other proteins has been faithfully produced using the Y2H method [12–14].

Accordingly, we used mGluR binding to immobilized-CaM beads [7] to investigate the CRIP status of CaM for group II mGluRs. CaM beads pulled down mGluR4a (Figure 2A) and mGluR7 (results not shown) immunoreactivity.
from solubilized rat brain membranes in a Ca\textsuperscript{2+}-dependent manner (Figure 2iiB). When the same fractions were probed with an antibody that recognizes a common epitope in mGluR2 and mGluR3, it appeared that mGluR2/mGluR3 bound to Ca\textsuperscript{2+}/CaM (Figures 2iiA and 2iiB). However, these results are confounded by documented cross-reactivity of these antibodies with mGluR5 [15], a known Ca\textsuperscript{2+}/CaM-binding receptor. We circumvented this by investigating if heterologously expressed mGluR2 bound to immobilized CaM and showed that mGluR2 did not interact with CaM in the presence or absence of Ca\textsuperscript{2+} (Figure 2iii). This was confirmed by binary assays in which immobilized CaM failed to pull down soluble glutathione-S-transferase mGluR2 or mGluR3 fusion proteins (results not shown; see [5]).

In conclusion, our results highlight a difficulty in defining certain kinds of CaM-binding determinants by the commonly used Y2H method. Furthermore, our results support the work of others [5], showing that the group II mGlRs do not undergo a CaM-binding interaction.

References
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