

Cyclic nucleotide Capture Compounds™ for the isolation of protein complexes and transmembrane proteins from complex samples

Capture Compound Mass Spectrometry (CCMS) is an innovative technology to reduce the complexity of biological samples through selective isolation of targeted protein or enzyme families. Small synthetic molecules (Capture Compounds™) interrogate and isolate native proteins, even lipophilic membrane proteins. The CCMS technology enables efficient complexity reduction of the proteome and allows discovering, isolating and profiling members of functional protein families within a variety of biological samples.

Introduction

Cyclic 3',5'- adenosine and guanosine monophosphate (cAMP and cGMP, respectively) are important second messenger molecules and involved in many cellular signal transduction cascades in a wide range of organisms (1). The identification and the profiling of cyclic nucleotide monophosphate (cNMP)-binding proteins is important in order to understand the molecular basis of these signaling events. Many cNMP-binding proteins are multispinning transmembrane proteins, such as the hyperpolarisation and cyclic nucleotide-gated (HCN) ion channels (2). This class of proteins is particularly difficult to access by conventional pull-down methods. Consequently, HCN channels have not yet been identified at the level of the endogenous proteins in mass spectrometry-based proteomics using small molecule affinity pull-down. Here, the application of CCMS for mass spectrometric identification of HCN channels and other cNMP-binding proteins from synaptosome samples is described. HCN channels have been reported to be located at synaptic structure (3).

Materials

The C8-cAMP caproKit™ contains the C8-cAMP Capture Compound™ (C8-cAMP-CC) with 8-aminohexylamino-(C8)-cAMP as the selectivity group, free cAMP as competitor solution, streptavidin magnetic beads, 5x concentrated capture buffer, and 5x concentrated wash buffer as well as purified regulatory subunit 1 of cAMP-dependent protein kinase (PRKAR1A_Human, [P10644](#)). The N²-cGMP caproKit™ contains the N²-cGMP Capture Compound™ with 2-aminohexyl-(N²)-cGMP as the selectivity group (Fig. 1) and cGMP as the competitor and otherwise the same components as described for the C8-cAMP caproKit™. Further PCR Tube strips for volumes up to 200 µl (Thermo Fisher, cat. No. AB-1114) as reaction vessels were used to prepare the samples and conduct the capturing experiments. Please note that at some stages of the experiment, centrifugation of the tube strips in a simple tabletop centrifuge with an appropriate butterfly rotor is desirable. Irradiation of the samples for photo cross-linking was performed with the caproBox™, and the beads were isolated using the caproMag™. As protein source, two rat brains were homogenized in a motor-driven glass-teflon

homogenizer using 12 strokes at 900 rpm in homogenization buffer containing 0.32 M sucrose, 5 mM HEPES/NaOH pH 7.4, supplemented with protease inhibitors. Ten volumes of homogenization buffer per gram of tissue wet weight were used. The homogenate was filtered through nylon gauze to remove debris, and the filtrate was centrifuged for 10 min at 1000 x g to separate the nuclei. The supernatant was centrifuged for 15 min at 4 °C at 12,000 x g to pellet organelles. The crude organelle pellet was resuspended in homogenization buffer, re-homogenized with 6 strokes at 900 rpm, and centrifuged for 20 min at 4 °C at 12,000 x g. Afterwards, the organelle pellet was resuspended in 1.5 ml/gram of tissue wet weight buffer B (0.32 M sucrose, 5 mM Tris/HCl pH 8.1) with a pastette and layered on top of a sucrose step gradient consisting of equal volumes of sucrose solution layers of 0.85 M sucrose, 1 M sucrose, and 1.2 M sucrose in an ultracentrifugation tube.

centrifugation at 16,000 x g at 4 °C and resuspended and solubilised in recommended mammalian cell lysate buffer (6.7 mM HEPES, 6.7 mM NaOAc, 6.7 mM MES, 200 mM NaCl, 10 mM β-mercaptoethanol, 1 mM EDTA, pH 7.5, supplemented to 0.1 % (w/v) n-dodecyl-β-maltoside). Solubilisation was carried out at room temperature for 30 min in a vortex. Insoluble material was pelleted at 16,000 x g in a tabletop centrifuge, and the supernatant used for the capture experiments. In a typical experiment, 80 μg of solubilised synaptosome proteins were used per assay.

Capture experiment

The assays were performed according to the C8-cAMP and N²-cGMP caproKit™ Guideline OnBead and prepared as follows: for preparation of the caproBeads™, per assay 50 μl streptavidin magnetic beads were mixed with 25 μl of the C8-cAMP-CC solution and incubated for 2 min at room temperature under vigorous shaking. Afterwards, the tube strip was fitted in the caproMag™, the beads collected in the tube lids, and washed twice with wash buffer and ultimately collected in the tube lids according to the caproMag™ procedure. In the meantime, the assays were prepared in the following order: Water, 5x capture buffer, and lysate were mixed by vortexing to achieve a final reaction volume of 100 μl with a final protein concentration of 3.5 mg/ml. Note: for the competition sample add free competitor prior to the addition of the lysate. The caproBeads™ were thoroughly resuspended in the sample solutions and allowed to incubate for 3 hours at 4 °C under rotation. The samples were briefly centrifuged in a tabletop centrifuge with a butterfly rotor, to remove any liquid from the tube lids, but short enough to avoid pelleting of the caproBeads™. Afterwards, in 12 intervals of 2.5 min the samples were irradiated in the caproBox™ at 2 - 4 °C and washed with wash buffer. The beads were additionally washed twice with HPLC grade water and incubated with 10 μl of 50 mM NH₄HCO₃, 5 mM CaCl₂, and supplemented with 0.5 μg trypsin (promega sequencing grade). The beads were transferred into a new tube. Tryptic digest was allowed to proceed overnight at 37 °C under vigorous shaking. Beads were then fixed at the tube walls using caproMag™, and the supernatants with the peptides recovered, desalted via Stage Tips™ (Proxeon, Odense, Denmark), and subjected to LC-MS/MS analysis.

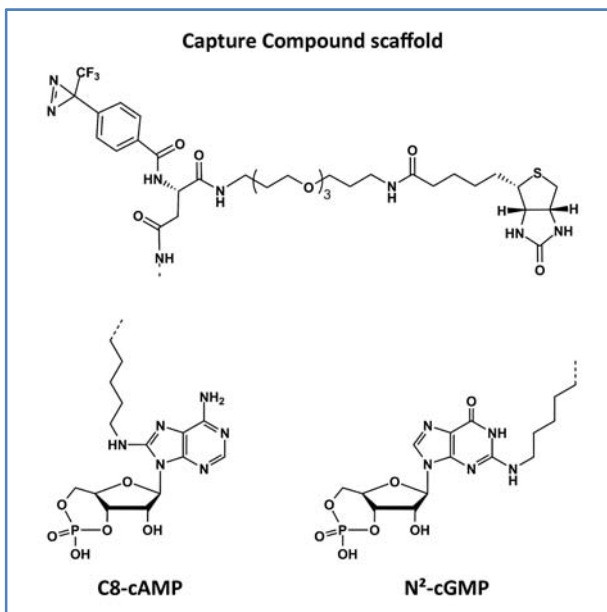


Figure 1 Structures of cAMP and cGMP Capture Compounds

Ultracentrifugation was carried out for 2 hours at 85,000 x g at 4 °C. The synaptosome fraction was recovered from the phase border between 1.0 M and 1.2 M sucrose, diluted with ≥4 volumes of PBS, and pelleted by centrifugation for 30 min at 40,000 x g. The synaptosome pellet was re-suspended in homogenization buffer, aliquoted, snap-frozen, and stored at -80 °C until further use. Immediately before setup of the capture experiments, the synaptosome suspension was thawed, the synaptosomes pelleted by

Mass spectrometry

LC-MS/MS experiments were performed using a nanoflow HPLC system (Proxeon) coupled on-line to an ESI-LTQ-Orbitrap system. From the LC column, peptides were eluted during an 80-min linear gradient from 5 % ACN/0.1 % FA to 40 % ACN/0.1 % FA followed by additional 2 min to 100 % ACN/0.1 % FA and remaining at 100 % for another 8 min with a controlled flow rate of 300 nl/min. MS/MS fragmentation was performed in a data-dependent mode using one survey MS scan followed by four MS/MS scans per second. MS/MS spectra were matched to the protein database using SEQUEST allowing a mass tolerance of 5 ppm. The SEQUEST peptide identifications were required to satisfy minimum XCorr values of 2, 2.5, and 3 for singly, doubly, and triply charged peptides, a minimum ΔC_n of 0.1, and a P (pep) of ≤ 0.001 . Peptides with a better score than this were accepted for analysis without further validation. The estimated false discovery rate of peptide identifications was determined using the reversed protein database approach and was $< 0.5\%$.

Results

Comparison of the capture profile of cNMP-binding protein kinase subunits (KAPs, PKG) and interaction partners (AKAP) obtained with the C8-cAMP (red) or N²-cGMP (blue) caproKit™, reveals that the C8-cAMP caproKit™ robustly isolates the KAPs, the regulatory subunits of the cAMP-dependent protein kinase and some associated AKAP proteins (Fig. 2). The N²-cGMP caproKit™ also isolates KAPs to a certain extent due to some promiscuity between cAMP- and cGMP binding, but less than the C8-cAMP caproKit™.

A comparison of the capture profiles for cNMP-binding hyperpolarisation and cyclic nucleotide-gated ion channels (HCNs) with the C8-cAMP caproKit™ (red) and the N²-cGMP caproKit™ (blue) is shown in Figure 3. Some voltage-gated calcium channels (CACs) were also captured, potentially due to their presence in cNMP-binding protein signaling complexes. The HCN ion channel proteins are particularly robustly captured using the C8-cAMP caproKit™.

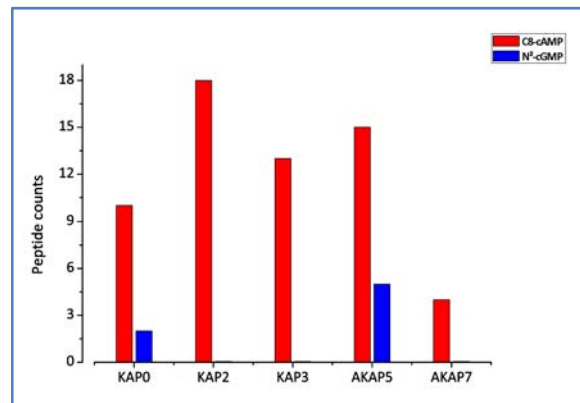


Figure 2 Peptide counts of KAP and AKAP peptides identified from rat brain preparations using C8-cAMP (red) and N²-cGMP (blue) caproKits, respectively.

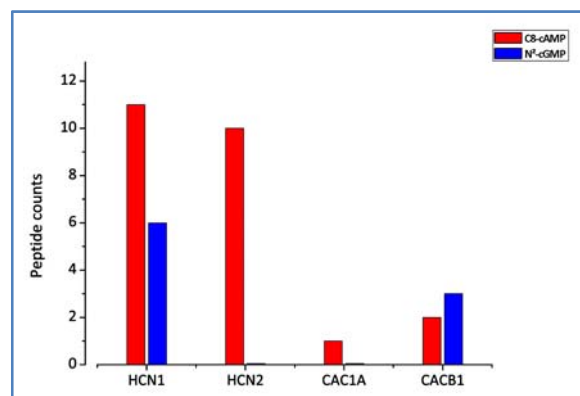


Figure 3 Peptide counts of transmembrane and membrane associated proteins identified from rat brain preparations using C8-cAMP (red) and N²-cGMP (blue) caproKits, respectively.

Figure 4 shows the comparison of the capture profiles of the cyclic nucleotide caproKits on other cNMP binding proteins: cGMP-dependent protein kinase PKG, the phosphodiesterase PDE2A, and Epac 4, all directly captured from the mixture of solubilised synaptosome proteins. PKG can be captured by both the C8-cAMP and the N²-cGMP caproKit™, due to some promiscuity in nucleotide binding at the Capture Compound concentration chosen. However, the preference of the N²-cGMP Capture Compound™ for PKG over the cAMP-binding KAPs is evident (compare to Fig. 2). The N²-cGMP caproKit™ is well suited to isolate phosphodiesterases (e.g. PDE2A) from the synaptosome preparation. Interestingly, PDE2A is known to be a phosphodiesterase that is stimulated selectively by cGMP through binding to a cyclic nucleotide binding domain (4). The cAMP-binding protein Epac4 is in turn captured exclusively by the C8-cAMP caproKit™.

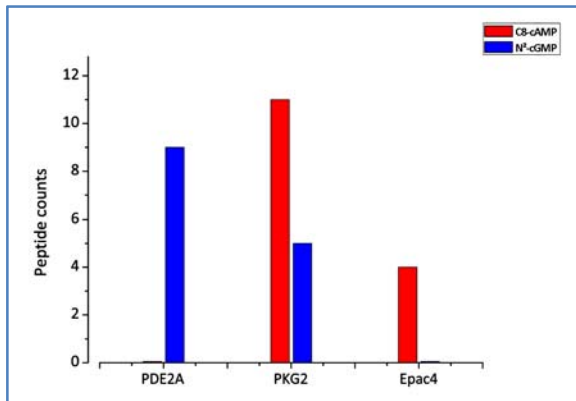


Figure 4 Peptide counts of PDE, PKG and Epac obtained with C8-cAMP (red) and N²-cGMP (blue) caproKits, respectively.

Conclusion

We compared the capture profiles of the C8-cAMP caproKit™ and the N²-cGMP caproKit™ with respect to cyclic nucleotide monophosphate (cNMP) binding proteins. In order to cover a wide range of different cNMP-binding proteins and, in particular, to assess the utility of the caproKits™ to isolate also low-abundant transmembrane proteins, we chose rat synaptosome proteins as a protein source. All reported identifications were from capture experiments with very small amounts of complex protein mixture of solubilised synaptosome proteins to start with. The C8-cAMP caproKit™ and the N²-cGMP caproKit™ can be effectively used for the isolation of cNMP binding proteins from crude protein mixtures, with significantly distinct selectivity profiles for the different Capture Compounds™. Remarkably, the C8-cAMP caproKit™ permits the isolation of HCN ion channels at the endogenous protein level from very small amounts of synaptosome protein mixture. This has not been achieved in cAMP- or cGMP affinity pull-down approaches reported in the literature.

References:

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- 2) Kaupp, U. B., and Seifert, R. (2002) *Physiol Rev* **82**, 769- 824
- 3) Bender, R. A., Kirschstein, T., Kretz, O., Brewster, A. L., Richichi, C., Ruschenschmidt, C., Shigemoto, R., Beck, H., Frotscher, M., and Baram, T. Z. (2007) *J Neurosci* **27**, 4697-4706.
- 4) Martins, T.J., Mumby, M.C., and Beavo, J.A. (1982) *J. Biol. Chem.* **257**, 1973-1979

Contact and order information:

Headquarters

caprotec bioanalytics GmbH

Volmerstrasse 5
D-12489 Berlin

Phone: +49 30 63 92 39 90

Fax: +49 30 63 92 39 89

Web: www.caprotec.com

Email: sales@caprotec.com

Ordering information:

Part Nr.	Description
1-1030-050	C8-cAMP caproKit™ 50 reactions
1-1030-010	C8-cAMP caproKit™ 10 reactions
1-1040-050	N ² -cGMP caproKit™ 50 reactions
1-1040-010	N ² -cGMP caproKit™ 10 reactions

The caproKit includes the respective specific Capture Compound™, all buffers, protein controls, competitor, and Streptavidin magnetic beads.

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Or contact us. Email: info@caprotec.com

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