SUBUNIT-SPECIFIC REGULATION OF PHOTORECEPTOR CNG CHANNELS BY PHOSPHOINOSITIDES

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ABSTRACT

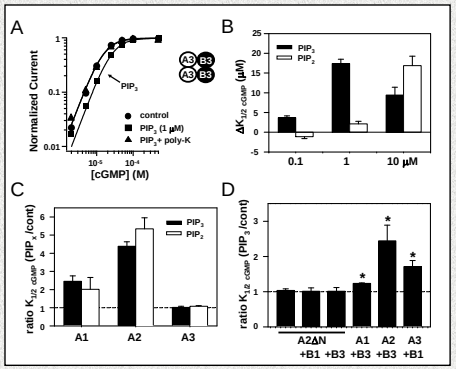
Cyclic nucleotide gated (CNG) channels in retinal photoreceptor cells play a key role in vertebrate phototransduction. The ligand sensitivity of photoreceptor CNG channels is adjusted during adaptation and in response to paracrine signals, but the mechanisms involved in channel regulation are only partly understood. Heteromeric A3+B3 (cone) and A1+B1 (rod) channels are sensitive to regulation by phosphoinositides (PIP_3 or PIP_2), demonstrating a decrease in apparent affinity for cGMP. To determine what subunit types are necessary for PIP_3 sensitivity, we generated heteromeric channels by co-expression of PIP_3-insensitive A2ΔN (Brady et al., 2006) with B3 or B1 subunits. Using patch-clamp recording in the inside-out configuration, we found that both channel types were insensitive to PIP_3 regulation, indicating that A3 or A1, but not B3 or B1 subunits, confer phosphoinositide sensitivity to heteromeric channels. Consistent with this idea, co-expression of A3 with B1, or A1 with B3, formed channels that were sensitive to PIP_3 regulation. Unlike homomeric A1 or A2 channels, A3-only channels paradoxically did not show a decrease in apparent affinity for cGMP after PIP_3. However, PIP_3 induced a nearly three-fold increase in cAMP efficacy for A3 channels, an effect that was reversed by poly-lysine application. The PIP_3-dependent change in cAMP efficacy was abolished by mutation of a critical ligand-discrimination residue (D609K) or by truncation of the channel distal to the cyclic nucleotide-binding domain (613stop). Furthermore, the apparent cGMP affinity of A3-613stop channels was reduced three-fold by PIP_3; this change in cGMP sensitivity also was reversed by poly-lysine. Together, these results suggest that regulation of A3 subunits by PIP_3 exhibits two components, one of which is unmasked either by assembly with B3 subunits or by deletion of the C-terminal region of A3.

METHODS

Molecular biology: Human CNGB3 was cloned as previously described (Peng et al., 2003). Human CNGA3 was a gift of K.-W. Yau. Subunits were subcloned in pGEMHE for heterologous expression in *Xenopus* oocytes. Point mutations were generated by overlapping PCR (cassette) mutagenesis. All mutations were confirmed by DNA sequencing. Recombinant GST-fusion protein expression in bacteria, and GST pull-down assays were carried out as previously described (Peng et al., 2003).
Heterologous expression: For expression in *Xenopus* oocytes, cRNA was synthesized *in vitro* from CNG channel cDNA using an upstream T-7 promoter. *Xenopus laevis* oocyes were isolated as previously described (Peng et al., 2003) and pressured injected with about 1 ng of cRNA.
Electrophysiology: Two to seven days after microinjection of *in vitro* transcribed mRNA (mMessage mMachine; Ambion) into *Xenopus* oocytes, patch-clamp experiments were performed with an Axopatch 200B amplifier (Axon Instruments) in the inside-out configuration. Recordings were made at 20 to 22°C. Data were acquired and analyzed using Pulse (Instrutech), Igor (Wavemetrics), and SigmaPlot/SigmaStat (SPSS). Initial pipette resistances were 0.40-0.80 Mohm. Intracellular and extracellular solutions contained 130 mM NaCl, 0.2 mM EDTA, and 3 mM HEPES (pH 7.2). Cyclic nucleotides were added to intracellular solution as needed. Intracellular solutions were changed using an RSC-160 rapid solution changer (Molecular Kinetics). Currents in the absence of cyclic nucleotide were subtracted. For the L-*cis*-diltiazem experiments, 1 mM cGMP was used at +80 mV with 25 μM L-*cis*-diltiazem in the intracellular solution. PIP_x solutions were prepared as previously described (Bright et al., 2007). PIP_3 and PIP_2 analogues were phosphatidylinositol tris-3,4,5 phosphate, dipalmitoyl, sodium salt and phosphatidylinositol -4,5 biphosphate, dipalmitoyl, sodium salt. Statistical significance was determined by using Student's *t* test or Mann–Whitney *U* test; a *p* value <0.05 was considered significant.

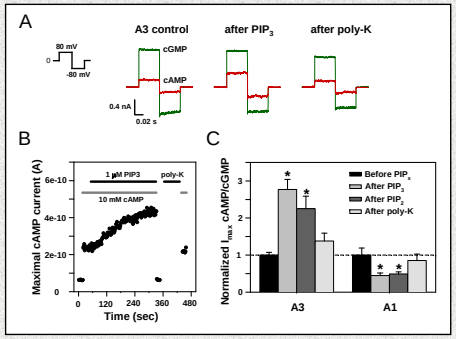
RESULTS

FIGURE 1: Structural elements necessary for phosphoinositide sensitivity of photoreceptor CNG channels appear to reside in CNGA3 and CNGA1 (not CNGB3 and CNGB1) subunits.



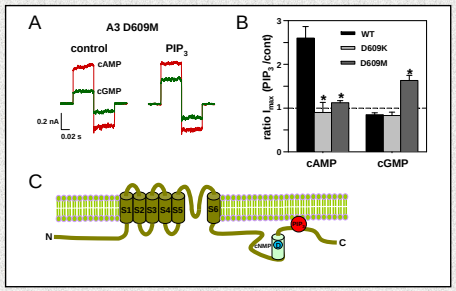
(A) Representative dose-response relationships for activation of heteromeric CNGA3 +CNGB3 channels before (circles), after PIP_3 (squares), and after subsequent poly-lysine (triangles). Currents were measured at +80 mV and were normalized to the maximum current elicited by a saturating concentration of cGMP. Continuous curves represent fits of the dose-response relationship to the Hill equation.
(B) Effects of different concentrations of PIP_2 or PIP_3 on $K_{1/2}$ cGMP for heteromeric CNGA3+CNGB3 channels.
(C) Effects of 10 μM PIP_2 or 1 μM PIP_3 on $K_{1/2}$ cGMP for CNGA1, CNGA2 and CNGA3 homomeric channels.
(D) Ratio $K_{1/2}$ cGMP (PIP_x/control) for various CNG channel subunit compositions. A2ΔN (see Brady et al., 2006), A2ΔN+B1, A2ΔN+B3 channels showed no sensitivity to PIP_3, while PIP_3 increased the $K_{1/2}$ cGMP of A1+B3, A2+B3 and A3+B1 channels. Data are means ± SEM of 3-4 independent experiments. *p < 0.05 for A1+B3, A2+B3, or A3+B1 compared to A2ΔN+B3 or A2ΔN+B1 channels, respectively.

FIGURE 2: PIP_3 application increased the relative cAMP efficacy for CNGA3 homomeric channels



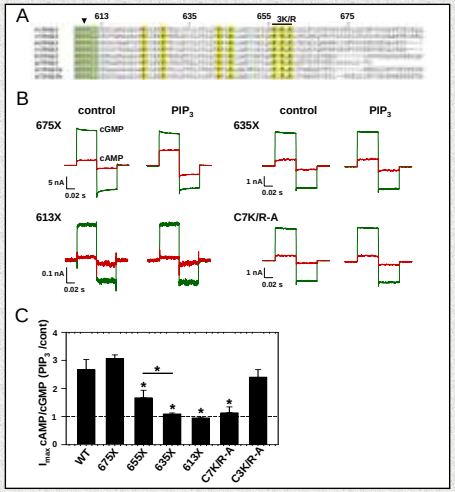
(A) Representative current traces for CNGA3 homomeric channels, elicited by saturating concentrations of cGMP (1 mM, green) or cAMP (10 mM, red), before and after PIP_3 application, and after subsequent poly-lysine (poly-K) application. Currents were elicited by voltage steps from a holding potential of 0 mV to +80 mV, then to -80 mV and 0 mV. Leak currents in the absence of cyclic nucleotide were subtracted.
(B) Time course for change in saturating cAMP current induced by 1 μM PIP_3; reversal by poly-lysine. Horizontal bars indicate times of application of respective agents.
(C) Summary illustrating both PIP_2- and PIP_3-dependent change in I_{max} cAMP/cGMP for A3 and A1 homomeric channels (ratio I_{max} cAMP/cGMP for wild-type A3 and A1 were normalized to 1). Data are means ± SEM of 4-9 independent experiments. *p < 0.05 compared to before PIP_x application.

FIGURE 3: Mutation of critical ligand-selectivity residue (D609) in cyclic nucleotide-binding domain of CNGA3 alters ligand-specific response to PIP_3



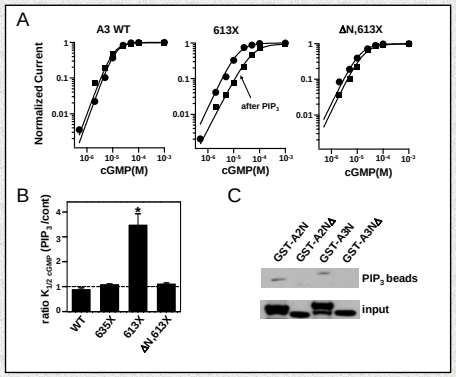
(A) Representative currents elicited by 1 mM cGMP (green) or 10 mM cAMP (red) before and after PIP_3, for CNGA3-D609M channels.
(B) Effects of PIP_3 application on saturating cAMP and cGMP current for CNGA3 channels with mutation of critical ligand discrimination residue (D609). Data are means ± SEM of 3-9 independent experiments. *p < 0.05 compared to wild-type channels.
(C) Cartoon showing possible mechanism for increase in cAMP efficacy for CNGA3: PIP_3 binds to the C-terminal region of CNGA3 (or a closely associated accessory protein) and alters interaction with ligand, making the ligand-binding domain better able to accommodate cAMP. One of the reasons cAMP is a partial agonist for A3 wild-type channels is the presence of a critical ligand-discrimination residue (D609) in the ligand-binding domain that interacts more favorably with cGMP, while presenting an unfavorable interaction with cAMP (Varnum et al., 1995; Peng et al., 2004). Mutation of this residue eliminates (D609K) or inverts (D609M) ligand-specific response to PIP_3.

FIGURE 4: C-terminal region of CNGA3 is necessary for PIP_3-dependent change in cAMP efficacy.



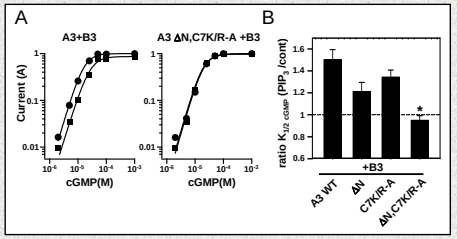
(A) Schematic diagram showing sites of truncations and charge neutralizations within the human CNGA3 C-terminal region, aligned with homologous proteins. Arrowhead points to critical ligand-discrimination site within the cyclic nucleotide-binding domain.
(B) Representative current traces, elicited by 1 mM cGMP (green) or 10 mM cAMP (red) before and after PIP_3, for CNGA3 675stop, 635stop, 613stop and C-terminal charge neutralization (C7K/R-A; seven residues highlighted above in yellow).
(C) Summary illustrating effect of CNGA3 C-terminal deletions and charge neutralizations on the PIP_3-induced increase in cAMP efficacy. Data are means ± SEM of 4-9 independent experiments. *p < 0.05 compared to wild-type A3.

FIGURE 5: The N-terminal region of CNGA3 mediates PIP_3–dependent change in apparent cGMP affinity, when unmasked by C-terminal truncation.



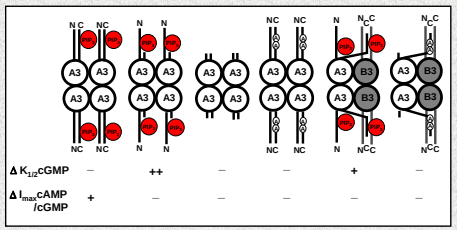
(A) Representative dose-response relationships for activation of homomeric CNGA3 wild-type, C-terminal deletion (613X), or combined N- and C-terminal deletion (ΔN,613X) channels by cGMP.
(B) Summary showing PIP_3-induced increase in $K_{1/2}$ cGMP for CNGA3-613X channels; this effect was abolished by further N-terminal deletion. Data are means ± SEM of 3-9 independent experiments. *p < 0.05 compared to wild-type A3.
(C) N-terminal regions of CNGA2 and CNGA3 were expressed as GST-fusion proteins and tested for PIP_3 binding in vitro by using PIP_3-agarose beads. Input proteins (lower) and bound proteins (upper) were identified by immunoblotting with anti-GST antibodies. GST-A2N, N-terminal region (amino acids 1–138) of rat CNGA2; GST-A2NΔ, amino acids 61–90 deleted; GST-A3N, N-terminal region (amino acids 1–164) of human CNGA3; GST-A3NΔ, amino acids 51–108 deleted. Data are representative of four independent experiments (see Brady et al., 2006).

FIGURE 6: Both N- and C-terminal regions of CNGA3 contribute to PIP_3 regulation of heteromeric CNGA3+CNGB3 channels.



(A) Representative dose-response relationships before and after PIP_3, for activation of heteromeric wild type A3+B3 channels and heteromeric channels with the combination of A3 N-terminal deletion and A3 C-terminal charge neutralizations (C7K/R-A).
(B) Effects of PIP_3 on heteromeric A3+B3 channels having deletions and/or mutation in A3 subunits. All subunit combinations shown exhibited sensitivity to block by L-cis-diltiazem that is characteristic of heteromeric A3+B3 channels (data not shown). Data are means ± SEM of 3-5 independent experiments. *p < 0.05 compared to wild-type channels.

FIGURE 7: Simple model showing two components for PIP_3 regulation of cone CNG channel gating.



SUMMARY

(1) The structural elements necessary for phosphoinositide sensitivity of photoreceptor CNG channels appear to reside in CNGA3 and CNGA1 (not CNGB3 and CNGB1) subunits.

(2) The carboxyl-terminal region of CNGA3 is necessary for a PIP_3- or PIP_2-dependent increase in cAMP efficacy.

(3) Regulation of CNGA3 channels by PIP_3 exhibits two components, one of which is unmasked either by assembly with CNGB3 subunits or by deletion of the C-terminal region of CNGA3. Presumably, an interaction between N- and C-terminal regions (intra- or inter-subunit) controls the nature of PIP_3 sensitivity.

(4) The N-terminal region of CNGA3 can bind PIP_3 *in vitro*.

(5) Regulation of heteromeric CNGA3+CNGB3 channels by PIP_3 depends on N- and C-terminal regions of CNGA3.

REFERENCES

Brady JD, Rich ED, Martens JR, Karpen JW, Varnum MD and Brown RL. (2006) Proc. Natl. Acad. Sci. 103:15635-40.

Bright SR, Rich ED, and Varnum MD. (2007) Mol Pharmacol. 71, 176-183.

He F, Mao M, Wensel TG J Biol Chem. (2004) 279(10):8986-90.

Li G, Rajala A, Wiechmann AF, Anderson RE, Rajala RV. (2008) J Neurochem. 107(5):1382-97.

Peng C, Rich ED, Thor CA and Varnum MD. (2003) J Biol Chem.278:24617–24623.

Peng C, Rich ED, and Varnum MD. (2004). Neuron 42, 401-410.

Rajala RV, McClellan ME, Ash JD, Anderson RE. (2002) J Biol Chem. 277(45):43319-26.

Varnum MD, Black KD, and Zagotta WN. (1995). Neuron 15, 619-625.

Womack KB, Gordon SE, He F, Wensel TG, Lu CC, Hilgemann DW. (2000) J Neurosci. 20:2792–2799.

Supported by NIH R01-EY012836