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Regulation of Human Cone Cyclic Nucleotide-Gated Channels by Endogenous Phospholipids and Exogenously Applied Phosphatidylinositol 3,4,5-trisphosphate

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ABSTRACT

Cyclic nucleotide-gated (CNG) channels are critical components of the vertebrate visual transduction cascade involved in converting light-induced changes in intracellular cGMP concentrations into electrical signals that can be interpreted by the brain as visual information. To characterize regulatory mechanisms capable of altering the apparent ligand affinity of cone channels, we have expressed heteromeric (CNGA3 + CNGB3) human cone CNG channels in *Xenopus laevis* oocytes and characterized the alterations in channel activity that occur after patch excision using patch-clamp recording in the inside-out configuration. We found that cone channels exhibit spontaneous changes in current at subsaturating cGMP concentrations; these changes are enhanced by application of ATP and seem to reflect alterations in channel gating. Similar to rod CNG channels, lavendustin A prevented this regulation, suggesting the involvement of a tyrosine phosphorylation event. However, the tyrosine residue in CNGB3 (Tyr545) that is equivalent to the critical tyrosine residues in rod and olfactory CNG channel subunits does not participate in cone channel regulation. Furthermore, the changes in ligand sensitivity of CNGA3 + CNGB3 channels were prevented by inhibition of phosphatidylinositol 3-kinase (PI3-kinase) using wortmannin or 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride (LY294002), which suggests that phospholipid metabolism can regulate the channels. Direct application of phosphatidylinositol 3,4,5-trisphosphate (PIP_3) to the intracellular face of excised patches also resulted in down-regulation of channel activity. Thus, phospholipid metabolism and exogenously applied PIP_3 can modulate heterologously expressed cone CNG channels.

In the vertebrate retina, absorption of light by opsin initiates a signal transduction cascade that produces a breakdown of cGMP by phosphodiesterase. The decrease in intracellular cGMP concentration results in the closure of CNG channels in the photoreceptor outer segment, leading to membrane hyperpolarization and decreased neurotransmitter release onto second-order neurons (Matulef and Zagotta, 2003). Native CNG channels from rods and cones are heterotetramers composed of CNGA1 + CNGB1 (Kaupp et al., 1989; Chen et al., 1993) or CNGA3 + CNGB3 (Bonigk et al., 1993; Gerstner et al., 2000) subunits, respectively. CNGA1 and CNGA3 can form functional homomeric channels; coassembly with CNGB1 or CNGB3 subunits, however, generates channels displaying properties that more closely resemble those of native channels, including sensitivity to block by L-*cis*-diltiazem, enhanced efficacy of the partial agonist cAMP, and sensitivity to regulation by Ca^{2+}-calmodulin (CaM) binding (Chen et al., 1994; Gerstner et al., 2000; Peng et al., 2003). Each channel subunit contains six transmembrane domains with a re-entrant P-loop that participates in pore formation and a cyclic nucleotide binding domain (CNBD) in the intracellular carboxyl-terminal region. Binding of cyclic nucleotide to this domain initiates an allosteric transition that results in channel opening (Matulef and Zagotta, 2003).

Considerable progress has been made in understanding physiological changes in the ligand sensitivity of native cone CNG channels (Ko et al., 2001; Kramer and Molokanova, 2001; Korenbrot and Rebrik, 2002), but the specific mecha-

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ABBREVIATIONS: CNG, cyclic nucleotide-gated; CNBD, cyclic nucleotide binding domain; IGF-1, insulin-like growth factor-1; CaM, calmodulin; PI3, phosphatidylinositol 3; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; ORN, olfactory receptor neuron; FVPP, sodium fluoride, sodium orthovanadate, and sodium pyrophosphate; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride.

nisms involved in channel regulation remain only partially understood. Treatment of patches excised from carp retinal cones with ATP, a presumed fuel for phosphorylation, results in changes in CNG channel ligand sensitivity (Watanabe and Shen, 1997). Although these results provide evidence that native cone channels can be regulated via phosphorylation-dependent pathways, they are difficult to interpret mechanistically in the absence of pharmacological or molecular manipulations.

In contrast to cone channels, much important progress has been made toward understanding the molecular mechanisms critical for rod channel regulation. The ligand sensitivity of native and heterologously expressed rod CNG channels has been shown to be modulated by the activity of kinases and phosphatases (Gordon et al., 1992; Molokanova et al., 1997, 2003; Savchenko et al., 2001) and by phospholipid signaling (Womack et al., 2000). Regulation of homomeric CNGA1 channels by tyrosine phosphorylation/dephosphorylation, for example, requires a specific tyrosine residue in the CNBD (Tyr498), whereas heteromeric channel regulation also involves an equivalent residue in CNGB1 (Tyr1097) (Molokanova et al., 1999, 2003). Insulin-like growth factor-1 (IGF-1), a molecule released by the retinal pigment epithelium (Waldbillig et al., 1991), altered the ligand sensitivity of native CNG channels via tyrosine dephosphorylation (Savchenko et al., 2001). Prior tyrosine phosphorylation of Tyr498 in CNGA1 prevented subsequent channel regulation by Ca^{2+}-CaM binding to CNGB1 (Krajewski et al., 2003). Furthermore, the tyrosine kinase inhibitor genistein seems to have an indirect negative allosteric effect on rod CNG channel gating that involves binding to a closely associated tyrosine kinase (Molokanova et al., 2000). Native CNG channels from rods, cones, and olfactory neurons are all sensitive to this allosteric effect of genistein, which suggests that all of these channel subtypes are associated with a tyrosine kinase (Molokanova et al., 2000). Together, these results illustrate how receptor signaling can alter the phosphorylation state and ligand sensitivity of CNG channels. The possible contribution of tyrosine phosphorylation or phospholipid metabolism to the regulation of cone CNG channels, however, has not been directly examined.

We tested the hypothesis that phosphorylation-dependent regulatory pathways can modulate the ligand sensitivity of heterologously expressed cone CNG channels. To this end, we have characterized the changes in heteromeric channel ligand sensitivity that occur in excised patches from *Xenopus laevis* oocytes using pharmacological manipulations to identify some of the enzymes involved. Here we describe a putative pathway for down-regulation of cone CNG channels that is blocked by a tyrosine kinase inhibitor and inhibitors of PI3-kinase. Furthermore, direct application of PIP_3 to inside-out patches containing CNGA3 + CNGB3 channels similarly decreased channel ligand sensitivity. These studies may provide mechanistic insight into the physiological regulation of ligand affinity for native cone photoreceptor channels.

Materials and Methods

Molecular Biology. Human CNGA3 cDNA (AF065314) was a generous gift of Professor K.-W. Yau, and human CNGB3 cDNA (AF272900) was isolated as described previously (Peng et al., 2003). CNGA3 and CNGB3 were subcloned into pGEMHE for heterologous expression in *X. laevis* oocytes, and mRNA was synthesized in vitro using an upstream T-7 promoter and the mMessage mMachine kit (Ambion, Austin, TX). The $CNGB3_{Y545F}$ mutation was generated by overlapping polymerase chain reaction, and amplified cassettes were sequenced to confirm the fidelity of the polymerase chain reaction.

Electrophysiology. *X. laevis* oocytes were isolated as described previously, and RNA was injected at a ratio of CNGB3 to CNGA3 (2.5:1) that was shown previously to efficiently generate heteromeric channels (Peng et al., 2004). The animal-use protocols were consistent with the recommendations of the American Veterinary Medical Association and were approved by the Institutional Animal Care and Use Committee of Washington State University. Two to 7 days after injection, oocytes were subjected to patch-clamp recording in the inside-out configuration using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA). Initial pipette resistances were 0.40 to 0.75 MΩ. Currents were low-pass-filtered at 2 kHz and sampled at 25 kHz. Intracellular and extracellular solutions contained 130 mM NaCl, 0.2 mM EDTA, and 3 mM HEPES, pH 7.2. Cyclic nucleotides (Sigma-Aldrich, St. Louis, MO) were added to intracellular solutions as indicated, and currents in the absence of cyclic nucleotide were subtracted from all recordings. An RSC-160 rapid solution changer (Molecular Kinetics, Pullman, WA) was used for applying solutions to the intracellular face of the patch. Inhibition of current in 1 mM cGMP by 25 μM L-*cis*-diltiazem (BIOMOL, Plymouth Meeting, PA) was used to confirm the formation of heteromeric channels. Recordings were made at 20 to 22°C. Dose-response relationships were obtained by plotting the steady-state current at +80 mV as a function of cyclic-nucleotide concentration. Dose-response data were fitted with the Hill equation, $I/I_{max} = ([cNMP]^{nH}/(K_{1/2}^{nH} + [cNMP]^{nH}))$, where I is the current amplitude, I_{max} is the maximum current, [cNMP] is the ligand concentration, $K_{1/2}$ is the apparent affinity for ligand, and n_H is the Hill slope. Data were acquired using Pulse (HEKA Elektronik, Lambrecht, Germany) and analyzed using Igor Pro (Wavemetrics, Lake Oswego, OR) and SigmaPlot (Systat Software, Inc., Point Richmond, CA). For the phosphatase-inhibitor cocktail, 5 mM sodium fluoride, 0.1 mM sodium orthovanadate, and 10 mM sodium pyrophosphate (FVPP); sodium fluoride; and sodium pyrophosphate were obtained from Sigma-Aldrich, whereas sodium orthovanadate was obtained from LC Laboratories (Woburn, MA). Additional reagents were obtained as follows: Mg-ATP (Sigma-Aldrich), IGF-1 (Peprotech Inc., Rocky Hill, NJ), lavendustin A (LC Laboratories), wortmannin and LY294002 (EMD Biosciences Inc., Madison, WI), dipalmitoyl-PIP_3 (referred to as PIP_3 hereafter) (Matreya LLC, Pleasant Gap, PA), and poly(L-lysine) (Sigma-Aldrich). The data were expressed as mean ± S.E.M. unless otherwise indicated. Statistical significance was determined using a Student's t test or a Mann-Whitney rank sum test (SigmaStat; Systat Software, Inc.), and a p value of <0.05 was considered significant.

Results

CNGA3 + CNGB3 Channels Exhibit Current Run-Down. We excised patches from *X. laevis* oocytes expressing heteromeric (CNGA3 + CNGB3) cone CNG channels and characterized the subsequent changes in channel activity. Heteromeric channels exhibited an initial trend toward current run-down in subsaturating cGMP (10 μM) that frequently reversed with time (Fig. 1B, ○), but the observed change was somewhat variable. This regulation differs from that reported previously for rod CNG channels, which instead exhibit profound current run-up after patch excision (Molokanova et al., 1997). Because cone channel regulation might depend on protein phosphorylation or dephosphorylation, we examined the effect of Mg-ATP application (200 μM) to the intracellular face of excised patches on the observed

change in current. Because ATP acts as the source of phosphate groups for kinases, it is expected to maintain the activity of patch-associated kinases in the absence of endogenous ATP. We found that ATP application led to consistent current reductions after patch excision (Fig. 1, A–C).

Channel Regulation Involves Gating Alterations and Is State-Dependent. The changes in current described above could result either from a loss of active channels in the patch or a change in channel gating. To distinguish between these possibilities, we first compared the change in maximum current (I_{max}), the current elicited by a saturating concentration of cGMP (1 mM), for patches with or without ATP application. I_{max} was not altered appreciably for either group, and the ratio of final I_{max} to initial I_{max} was not significantly different between these groups (Fig. 2, A and B). Next, possible alterations in channel gating were examined. The current reduction in subsaturating cGMP was accompanied by reduced relative efficacy of the partial agonist cAMP (Fig. 2, A and C). ATP treatment also resulted in a rightward shift of the dose-response curve for cGMP, indicating a decrease in the apparent affinity for this ligand (Fig. 2, D and E). These changes were not altered significantly by concomitant application of FVPP, a phosphatase inhibitor cocktail ($p > 0.5$; data not shown). Together these results suggest that alterations in channel gating rather than a loss of active channels from the patch are responsible for the changes in current with time.

We next determined whether the state of the CNG channels (i.e., whether the channels were predominantly open or closed) influenced channel regulation. After obtaining the initial leak-subtracted current in 10 μM cGMP in the absence of ATP, ATP was applied to the intracellular face of the patch for 20 min in the presence or absence of a saturating concentration of cGMP (1 mM). ATP promoted greater current run-

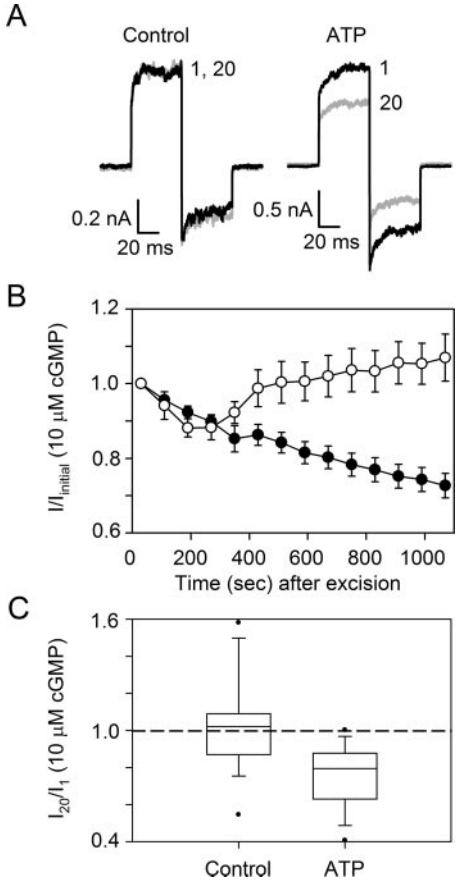

Fig. 1. ATP promotes current rundown of heteromeric cone CNG channels. A, representative current traces are shown for heteromeric (CNGA3 + CNGB3) cone CNG channels after activation by 10 μM cGMP either without (left) or with 200 μM Mg-ATP (right). Current traces were obtained both before (black) and after (gray) control or ATP treatment periods. The numbers 1 and 20 reflect time in minutes since patch excision. Current traces were elicited by voltage steps from a holding potential of 0 to +80 mV then to −80 and 0 mV. Leak currents in the absence of cyclic nucleotide were subtracted for all recordings. B, time courses for currents at +80 mV elicited by 10 μM cGMP, either in the absence (○) or continuous presence of ATP (●). All currents were leak-subtracted and normalized to initial current (n = 11–31). C, box plots are shown for the ratio of final current in 10 μM cGMP (current after 20-min control or ATP-treatment period) to the initial current for control (left, n = 23) and ATP-treated patches (right, n = 32). These groups are significantly different ($p < 0.001$). The line within the box represents the median; the box indicates the 25th and 75th percentiles, and the whiskers show the 5th and 95th percentiles.

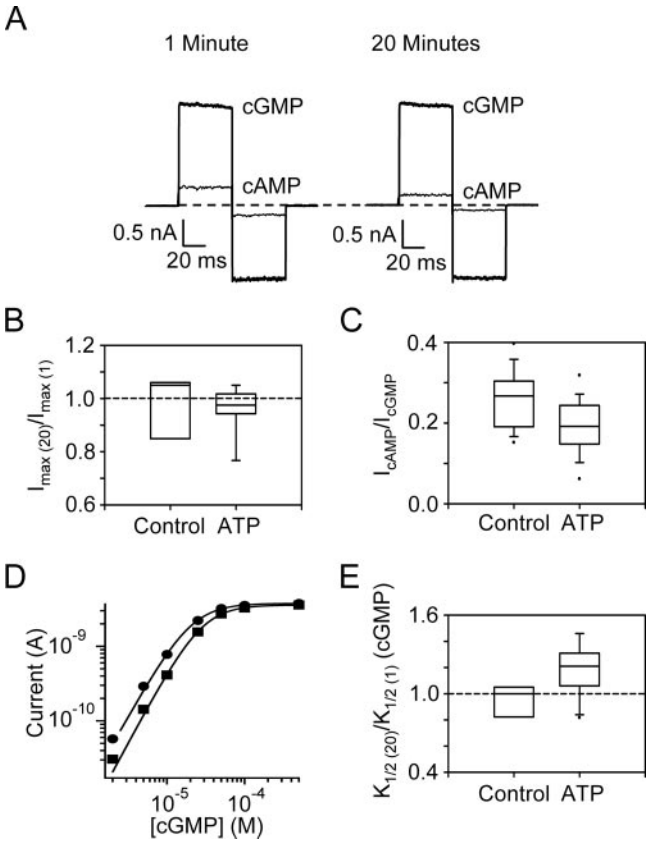

Fig. 2. ATP-induced current rundown is associated with alterations in channel gating. A, representative current traces are shown for heteromeric channels activated by 10 mM cAMP (thin trace) or 1 mM cGMP (thick trace). Traces are shown before (left) and after ATP treatment (right) for a single patch. B, box plots for the I_{max} ratio (final/initial) for control (left, n = 7) and ATP-treated patches (right, n = 16). These groups were not significantly different (p = 0.49). C, box plots are shown for the final ratio of current in saturating cAMP to current in saturating cGMP for both control (left, n = 23) and ATP-treated patches (right, n = 31). These groups were significantly different ($p < 0.001$). D, representative dose-response relationships are shown for the activation of heteromeric channels by cGMP at +80 mV, both before (●) and after ATP treatment (■). Continuous curves represent fits of the dose-response relation to the Hill equation, $I/I_{max} = ([cNMP]^{nH}/(K_{1/2}^{nH} + [cNMP]^{n}))$. Initial Hill fit (●): I_{max} = 3.76 nA, $K_{1/2}$ = 20.3 μM, and n = 1.9. Final Hill fit (■): I_{max} = 3.58 nA, $K_{1/2}$ = 28.3 μM, and n = 1.9. E, box plots are shown for the $K_{1/2}$ ratio for cGMP (final/initial) for control (left, n = 7) and ATP-treated patches (right, n = 19). These groups were significantly different ($p < 0.01$).

down when applied in the absence of cyclic nucleotide than in the presence of saturating cGMP (Fig. 3, A and B). Therefore, the channels were regulated more efficiently when they were in the closed state compared with the open state. Consistent with this observation, average current rundown in 10 μM cGMP (where approximately 20% of the channels are open) was intermediate between these two groups (Fig. 1C).

Regulation Depends on the Activity of an Unknown Tyrosine Kinase. We tested for the involvement of tyrosine kinases in cone channel regulation because of the strong evidence for rod channel regulation by these enzymes (Molokanova et al., 1997, 1999, 2000, 2003). We found that lavendustin A (10 μM), a general tyrosine kinase inhibitor, prevented both the current reduction in subsaturating cGMP (Fig. 4, A and B) and the increase in the $K_{1/2}$ for cGMP (Fig. 4C). IGF-1 pretreatment, which promotes tyrosine dephosphorylation of rod CNG channels in *X. laevis* oocytes and native rod photoreceptors (Savchenko et al., 2001), did not significantly alter the effect of subsequent ATP application to expressed cone CNG channels (Fig. 4C). Similar to rod CNG channels, the activity of an unknown tyrosine kinase seems to be involved in the changes in cone channel gating that occur after patch excision.

We next sought to determine whether cone CNG channel regulation involves phosphorylation at a site equivalent to the residues previously shown to be necessary for rod and olfactory channel regulation. Molokanova and coworkers (1999, 2003) have demonstrated that rod CNG channel regulation depends on tyrosine residues in CNGA1 (Tyr498) and CNGB1 (Tyr1097) at equivalent positions within the CNBD. Furthermore, this site is also critical to olfactory channel regulation, because mutation of the phenylalanine at this position in CNGA2 to tyrosine (F477Y) confers regulation to the otherwise insensitive homomeric CNGA2 channels (Molokanova et al., 1999). Whereas CNGB3 presents a tyrosine at the corresponding position (Tyr545), CNGA3 instead exhibits a phenylalanine (Fig. 5A). We mutated this residue in CNGB3 to phenylalanine, expressed the $CNGB3_{Y545F}$ subunits in combination with wild-type CNGA3 subunits, and treated the resulting patches with ATP. Current run-down in 10 μM cGMP was not significantly less than that of wild-type heteromeric channels (Fig. 5B), and the change in the $K_{1/2}$ for cGMP was not significantly different (Fig. 5C). Furthermore, we did not detect a phosphotyrosine immunoreactive band in Western blots of immunoprecipitated CNGA3 and CNGB3 subunits (data not shown). These results do not exclude the possibility of direct tyrosine

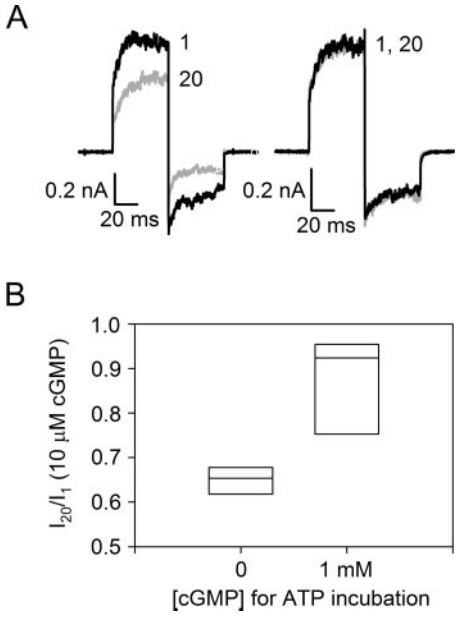

Fig. 3. Regulation of cone CNG channels is state-dependent. A, representative traces are shown for current elicited by 10 μM cGMP both before (black) and after ATP treatment (gray). Initial and final 10 μM currents were elicited in the absence of ATP. ATP was applied either in the absence of cyclic nucleotide (left) or in the presence of a saturating concentration of cGMP (1 mM, right) for 20 min. B, box plots are shown for the ratio of final to initial current in 10 μM cGMP for patches treated with ATP, either in the absence of cyclic nucleotide ($n = 5$) or in the presence of a saturating concentration of cGMP ($n = 4$). These groups were significantly different ($p < 0.01$).

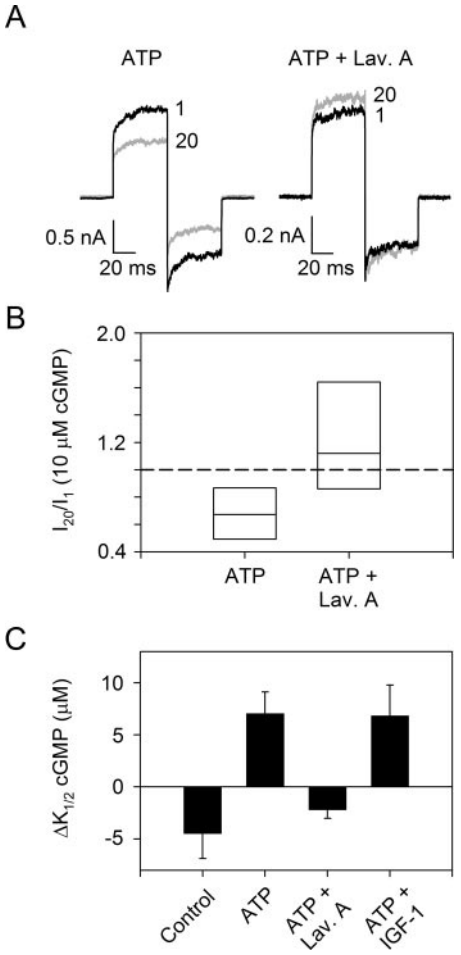

Fig. 4. Cone CNG channel regulation is hindered by a tyrosine kinase inhibitor. A, representative initial (black) and final (gray) current traces elicited by 10 μM cGMP are shown for heteromeric (CNGA3 + CNGB3) cone CNG channels. Patches were treated for 20 min with either ATP alone (left) or ATP and 10 μM lavendustin A (right). The latter group also received a 20-min pretreatment of the intact oocytes with lavendustin A (10 μM). B, box plots are shown for the ratio of final current to initial current in 10 μM cGMP for heteromeric channels. Both ATP-treated (left, $n = 5$) and patches cotreated with ATP and lavendustin A (right, $n = 5$) are shown. These groups were significantly different ($p < 0.05$). C, bar graph of $\Delta K_{1/2,\ cGMP}$ for the following groups: control (left, $n = 4$), ATP-treated (middle left, $n = 6$), pretreated for 30 min with lavendustin A and continuously treated with ATP + lavendustin A (middle right, $n = 4$), and pretreated with IGF-1 for 30 min and continuously treated with ATP (right, $n = 5$). The ATP and ATP + lavendustin A groups were significantly different ($p < 0.01$). The whiskers represent the standard error for the individual groups. Lav. A, lavendustin A.

phosphorylation of CNGB3 or CNGA3 subunits, but the robust regulation of $CNGB3_{Y545F}$-containing channels provides evidence that unlike rod and olfactory channels, phosphorylation of this site is not the cause of the changes in cone channel ligand affinity observed in the presence of ATP.

Channel Regulation Depends on the Activity of PI3-Kinase. The results described above led us to consider indirect mechanisms for the involvement of tyrosine phosphorylation in cone channel regulation. In particular, we considered the potential role of PI3-kinase, because this enzyme has been shown to be activated in photoreceptor outer segments after tyrosine phosphorylation of the insulin receptor β-subunit (Rajala et al., 2002). Furthermore, phospholipid signaling, particularly the production of PIP_2, has been shown to play a role in the regulation of many classes of ion channels (Suh and Hille, 2005). For heteromeric cone CNG channels, prior and concomitant application of 100 nM wortmannin prevented both the reduction in current in subsaturating cGMP (Fig. 6, A and B) and the increase in the $K_{1/2}$ for cGMP (Fig. 6C) that were associated with ATP application. This low concentration of wortmannin is expected to target PI3-kinase rather than other lipid kinases. Another PI3-kinase inhibitor, LY294002, prevented the change in current in subsaturating cGMP (Fig. 6B) without significantly altering the change in apparent ligand affinity (Fig. 6C). Taken together, these results support the involvement of phospholipid metabolism in cone CNG channel regulation.

Cone CNG Channels are Modulated by Direct Application of PIP_3. To directly test the impact of phospholipid signaling on cone CNG channels, we applied PIP_3 (1 μM) to the intracellular face of excised patches and monitored changes in channel activity. This treatment did not significantly alter I_{max} in 1 mM cGMP (Fig. 7A) (mean $I_{max,final}/I_{max,initial}$ = 107% ± 2.6, n = 13), but the current elicited by 10 μM cGMP was reduced (Fig. 7B) (mean $I_{final}/I_{initial}$ = 79.8% ± 3.8, n = 13). The PIP_3-mediated reduction in current was rapid, typically observed in less than 1 min. PIP_3 treatment also led to a prominent rightward shift in the dose-

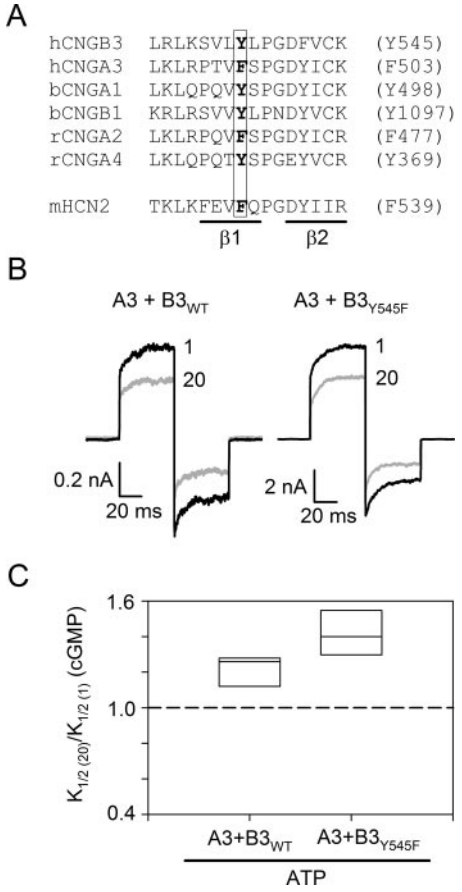

Fig. 5. Cone CNG channel regulation does not depend on Tyr545. A, sequence alignment is shown for each of the CNG channel subunits and the related hyperpolarization-gated, cyclic nucleotide-modulated channel HCN2. Listed below this alignment are known structural elements from the crystal structure of mHCN2 (Zagotta et al., 2003). The residues equivalent to the critical tyrosine residues in CNGA1 (Tyr498) and CNGB1 (Tyr1097) are boxed. B, representative initial (black) and final (gray) current traces elicited by 10 μM cGMP with ATP treatment for heteromeric channels containing wild-type CNGB3 (left) or $CNGB3_{Y545F}$ subunits (right). C, box plots depicting the ratio of final over initial $K_{1/2,cGMP}$ for wild-type (left, n = 4) or mutant channels (right, n = 4) treated with ATP. These groups were not significantly different ($p > 0.05$).

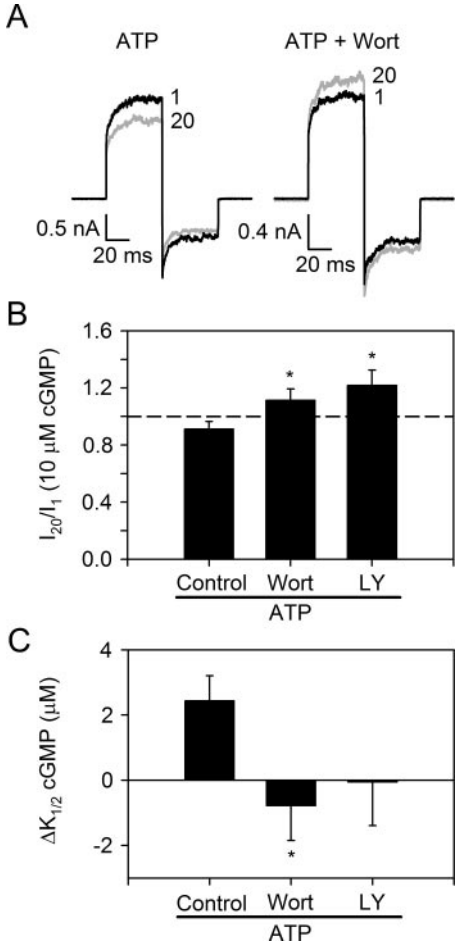

Fig. 6. Cone CNG channel regulation depends on the activity of PI3-kinase. A, representative initial (black) and final (gray) current traces elicited by 10 μM cGMP for patches treated with either ATP (left) or ATP and 100 nM wortmannin (right). The latter group also received a 10-min pretreatment with wortmannin (100 nM). Both groups also received a 10-min pretreatment with IGF-1, and ATP was applied in the absence of cyclic nucleotide. B, bar graph is shown for the ratio of final-to-initial current in 10 μM cGMP for patches treated with ATP either without (left, n = 14) or with wortmannin (middle, n = 9) or 20 μM LY294002 (right, n = 6). Both the wortmannin- and LY294002-treated groups were significantly different from the control-ATP patches ($p < 0.05$). C, bar graph is shown for the change in apparent affinity for cGMP for control-ATP (left, n = 14), wortmannin- (middle, n = 9), and LY294002-treated patches (right, n = 7). Only the wortmannin group was significantly different from the control ($p < 0.05$). Wort, wortmannin.

response curve for channel activation by cGMP (Fig. 7, C, ▲, and D). To test whether the putative interaction between the channels and phospholipids could be disrupted, poly(L-lysine) was applied at a concentration of 25 μg/ml for 2 min. This manipulation resulted in apparent block of CNG channels, but the block largely reversed (~75%) after a 2-min wash in control solution. Dose-response data obtained after this wash revealed that the apparent affinity of the channels for cGMP returned to the initial levels observed before PIP_3 treatment (Fig. 7, C and D). PIP_3 was also applied in the presence of FVPP to examine the impact of phosphatases on sensitivity to lipid regulation. FVPP decreased variability and enhanced channel regulation by PIP_3 (Fig. 7D), but the latter effect was not statistically significant ($P > 0.1$). Together, these results provide evidence for direct regulation of cone CNG channels by PIP_3.

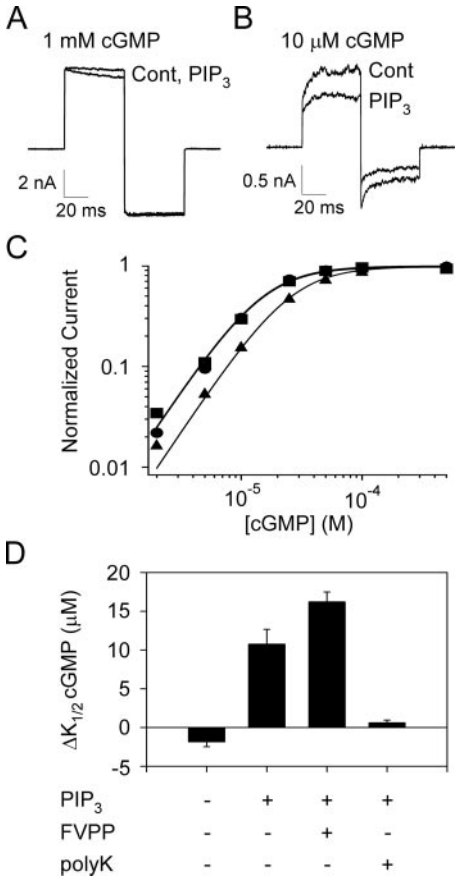

Fig. 7. Direct application of PIP_3 reduces apparent ligand affinity of channels. A, representative maximum current traces elicited by 1 mM cGMP are shown both before (black) and after (gray) PIP_3 treatment (1 μM). B, representative current traces elicited by 10 μM cGMP are shown both before (black) and after (gray) PIP_3 treatment. C, representative dose-response relationships for the activation of heteromeric channels by cGMP at +80 mV are shown before PIP_3 treatment (●), after PIP_3 treatment (▲), and after both PIP_3 and subsequent poly(L-lysine) (25 μg/ml) application (■). Current values are normalized to the corresponding maximum current. For control Hill fit (●): $K_{1/2} = 15.2$ μM and $n = 1.8$; for PIP_3 Hill fit (▲): $K_{1/2} = 25.7$ μM and $n = 1.8$; for PIP_3 + poly(L-lysine) Hill fit (■): $K_{1/2} = 15.1$ μM and $n = 1.8$. D, bar graph for the change in the $K_{1/2}$ for cGMP relative to the initial value is shown for control ($n = 13$), PIP_3-treated ($n = 13$), PIP_3 and FVPP (see *Results*)-treated ($n = 5$), and PIP_3- and poly(L-lysine)-treated ($n = 7$) dose-response curves.

Discussion

We have demonstrated here that heterologously expressed cone CNG channels exhibit spontaneous changes in channel activity after patch excision that probably reflect changes in channel gating and that such regulation occurs more consistently in the presence of ATP. Similar to rod CNG channels, this regulation could be prevented by coapplication of the tyrosine kinase inhibitor lavendustin A, but the target residues are not the same. For cone CNG channels, regulation also seems to depend on the activity of PI3-kinase. Furthermore, channel modulation is recapitulated by direct application of PIP_3, which provides additional evidence that lipid phosphorylation and the production of PIP_3 may represent the important underlying event driven by application of ATP. Whereas the exact physiological significance of phospholipid-dependent regulation of cone CNG channels remains to be determined, these results suggest that alterations in lipid metabolism in photoreceptors could potentially alter the function of native cone CNG channels.

The observed rightward shift in the dose-response relationship for channel activation by cGMP after ATP treatment or direct PIP_3 application was relatively small, but such regulation could still be relevant to the physiology of phototransduction. The low physiological concentration of cGMP in photoreceptors (approximately 2–4 μM in the dark) (Pugh and Lamb, 1990) in conjunction with the steep dependence of channel opening on cyclic nucleotide concentration make the activity of native channels highly sensitive to changes in gating. Thus, even small changes in the apparent affinity of the channels for ligand could result in dramatic alterations of the cyclic nucleotide-dependent current in photoreceptors.

These ATP-driven alterations in channel gating could be prevented by wortmannin and LY294002, the inhibitors of PI3-kinase. Furthermore, direct application of PIP_3 resulted in a similar reduction in ligand sensitivity. Thus, we have provided evidence that an increase in the production of PIP_3 could result in the channel down-regulation that we have observed in the presence of ATP. In addition, the ATP-driven change in ligand sensitivity was prevented by lavendustin A, a tyrosine kinase inhibitor. Although this result suggests that tyrosine phosphorylation is involved in the regulation of channel activity, this conclusion is based on a single pharmacological reagent that could have nonspecific effects on lipid metabolism. It is also possible that pretreatment of intact oocytes with lavendustin A affected tyrosine phosphorylation of membrane proteins and thereby influenced the activation and/or recruitment of PI3-kinase via SH2 domains (Martin, 1998; Guo et al., 2000). We have not ruled out the possibility of direct tyrosine phosphorylation of channel subunits at residues other than Tyr545 in CNGB3, but the simplest explanation for our results is that the tyrosine phosphorylation-dependent event occurs upstream of direct regulation of the channels by phospholipid binding.

Heterologously expressed, homomeric CNGA3 channels have been shown to be regulated by direct serine phosphorylation after activation of protein kinase C by the phorbol ester phorbol 12-myristate 13-acetate (Muller et al., 2001). It remains to be determined whether heteromeric CNGA3 plus CNGB3 channels can be regulated by protein kinase C or by the activity of some other serine/threonine kinase or phosphatase. For native rod channels, Gordon and coworkers

(1992) have described an increase in apparent affinity for cGMP after patch excision that was dependent on the activity of an unknown serine/threonine phosphatase. However, it is not clear whether this regulation involves direct phosphorylation of the channel or of some closely associated protein.

The production of phospholipids influences the activity of both rod and olfactory CNG channels (Womack et al., 2000; Spehr et al., 2002; Zhainazarov et al., 2004; Brady et al., 2006). For example, inhibition of PI3-kinase has been shown to enhance odorant transduction in olfactory receptor neurons (ORNs) (Spehr et al., 2002). PIP_3 application to inside-out patches excised from ORNs or cells expressing olfactory CNG channel subunits inhibits channel activation (Zhainazarov et al., 2004; Brady et al., 2006) and interferes with channel modulation by Ca^{2+}-CaM (Brady et al., 2006). Furthermore, olfactory CNG channel regulation seems to depend on direct binding of PIP_3 to CNGA2 subunits (Brady et al., 2006). Thus, phospholipid production in ORNs tunes olfactory signaling, and direct modulation of olfactory CNG channels seems to be at least partially responsible for this effect.

ATP application to patches excised from *X. laevis* oocytes expressing heteromeric rod CNG channels results in significant inhibition of channel activity, and this effect was rapidly reversed upon application of an anti-PIP_2 antibody (Womack et al., 2000). Furthermore, direct application of PIP_2 to patches resulted in a dramatic reduction in channel activity (Womack et al., 2000). Thus, it seems that ATP can affect rod CNG channel activity by enhancing the production of PIP_2. Likewise, we have found that ATP-driven cone channel regulation depends on phospholipid metabolism and can be mimicked by direct application of PIP_3 to patches. The results indicate that phospholipid regulation of heteromeric cone CNG channels is qualitatively similar to phospholipid regulation of olfactory and rod CNG channels.

Although the physiological significance of this regulatory pathway has not yet been determined, tyrosine phosphorylation has been linked to the recruitment of PI3-kinase to cell membranes (Martin, 1998) and specifically to the activation of PI3-kinase in rod outer segments (Guo et al., 2000). Furthermore, such a pathway may be important for the response of photoreceptors to light, because light exposure stimulates tyrosine phosphorylation of the insulin receptor β subunit and thereby increases the activity of PI3-kinase in rod photoreceptors (Rajala et al., 2002). In addition, phosphodiesterase activity is stimulated by exposure to vesicles containing phospholipids (He et al., 2004). Thus, phospholipid signaling could potentially amplify the photoresponse, resulting in a greater reduction in channel activity than would occur via activation of the phototransduction cascade alone.

It is also possible that phospholipid signaling participates in calcium-feedback regulation in photoreceptors. CaM can bind the regulatory subunit (p85) of PI3-kinase in a calcium-dependent fashion and thereby enhance the activity of PI3 kinase (Joyal et al., 1997). Hence, calcium entry through open CNG channels could result in activation of PI3-kinase and subsequent phospholipid-dependent down-regulation of channel activity. Such a mechanism would help explain the discrepancy between the profound degree of calcium sensitivity observed for native cone CNG channels and the relatively small impact of direct Ca^{2+}-CaM binding on channel activity (Hackos and Korenbrot, 1997; Fain et al., 2001; Korenbrot and Rebrik, 2002; Peng et al., 2003; Rebrik and Korenbrot, 2004). In addition, PI3-kinase signaling can enhance cell survival via the Akt pathway (Brunet et al., 2001). PI3-kinase activity can be neuroprotective in the retina; for example, the inhibition of retinal cell death by ciliary neurotrophic factor treatment is prevented by LY294002 (Ikeda et al., 2004). The significance of phospholipid-dependent regulation of CNG channels remains to be determined, but phospholipid signaling is clearly important to the function and survival of photoreceptors (Yu et al., 2004).

To our knowledge, this is the first study to examine phosphorylation- and lipid-dependent regulation of heteromeric cone CNG channels using pharmacological manipulations. We have shown that the spontaneous regulation of cone CNG channels involves phospholipid metabolism and that direct application of PIP_3 can initiate down-regulation of cone channel activity. Future studies will be directed toward elucidating the structural features and molecular mechanisms involved in phospholipid modulation of cone CNG channels.

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