

The delta subunit of type 6 phosphodiesterase reduces light-induced cGMP hydrolysis in rod outer segments.

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Abstract

The delta subunit of the rod photoreceptor PDE has previously been shown to co-purify with the soluble form of the enzyme and to solubilize the membrane-bound form(1). To determine the physiological effect of the delta subunit on the light response of bovine rod outer segments, we measured the real-time accumulation of the products of cGMP hydrolysis in a preparation of permeabilized rod outer segments. Addition of delta subunit GST fusion protein (δ -GST) to this preparation caused a reduction in the maximal rate of cGMP hydrolysis in response to light. The maximal reduction of the light response was about 80%, and the half-maximal effect occurred at 385 nM delta subunit. Several experiments suggest that this effect was not due to the effects of δ -GST on transducin or rhodopsin kinase. Immunoblots demonstrated that exogenous δ -GST solubilized the majority of the PDE in ROS, but did not affect the solubility of transducin. Therefore, changes in the solubility of transducin cannot account for the effects of δ -GST in the pH assay. The reduction in cGMP hydrolysis was independent of ATP, which indicates that it was not due to effects of δ -GST on rhodopsin kinase. In addition to the effect on cGMP hydrolysis, the δ -GST fusion protein slowed the turn-off of the system. This is likely due, at least in part, to an observed reduction in the GTPase rate of transducin in the presence of δ -GST. These results demonstrate that δ -GST can modify the activity of the phototransduction cascade in preparations of broken rod outer segments, probably due to a functional uncoupling of the transducin to PDE step of the signal transduction cascade, and suggest that the delta subunit may play a similar role in the intact outer segment.

Introduction

Retinal photoreceptor cells, rods and cones, can directly sense and respond to photons of light. In these cells, light induces a highly regulated and well-studied cascade of events that leads to changes in photoreceptor membrane potential and neurotransmitter release. PDE6¹ is an integral part of this cascade. When a photon of light hits the chromophore in the transmembrane protein rhodopsin, a conformational change occurs that allows the G protein, transducin, to be activated. Transducin then activates PDE6 by binding to its inhibitory gamma subunits. When active, PDE6 rapidly hydrolyzes cGMP, causing the closure of cGMP gated ion channels in the plasma membrane of the photoreceptor, reduced influx of sodium and calcium, and hyperpolarization of the photoreceptor cell. The light signal ends when transducin becomes inactive by hydrolyzing its GTP and rhodopsin kinase phosphorylates rhodopsin, blocking further interaction between transducin and rhodopsin. GDP bound transducin releases the gamma subunits of PDE6, allowing the catalytic subunits of PDE6 to become re-inhibited after transducin has hydrolyzed its GTP. Reductions in calcium caused by light stimulate guanylate cyclase to replace the hydrolyzed cGMP.

Rhodopsin is a transmembrane protein which is found in the internal membranous disks of photoreceptor cells. Therefore, it is logical that significant fractions of its effectors, transducin and PDE6, are also found on disk membranes. This colocalization presumably helps guarantee the most effective transfer of the light signal from one protein to the next. The localization of these effector molecules to the membrane is dependent on their post-translational modification with prenyl groups(2-4). In the case of PDE6, the modifications are found on the catalytic subunits: the alpha subunit is farnesylated, and the beta subunit is geranylgeranylated. In other proteins such as small G proteins, prenylation is sufficient to bind the protein firmly to the membrane. However, despite having two such

hydrophobic prenyl modifications, PDE6 catalytic subunits can easily be removed from the membrane by treatment with detergent-free hypotonic buffers.

In addition to the membrane bound PDE (mPDE), a soluble fraction of rod PDE6 (sPDE)(5) has been identified. Under isotonic conditions, approximately 30% of the PDE6 isolated from rod outer segments is found in this soluble fraction. sPDE appears identical to mPDE, except that sPDE is complexed with one or more 17 kDa delta subunits. Purified sPDE (which has bound delta subunit) is activated by transducin with the same dose/response as purified mPDE (without delta subunit) in solution(6), so the delta subunit does not appear to directly affect the enzyme's catalytic activity. At this point the functional roles of sPDE and the delta subunit in the photoreceptor are not clear; however, it is possible that regulation of the localization of PDE6's catalytic subunits could modify the response of the cell to light.

Several observations led us to hypothesize that the delta subunit might modulate the activity of the phototransduction cascade. The delta subunit is highly expressed in retina and is localized in the outer segments of the photoreceptors(1), so it is in the right location to have an effect on the cascade. Additionally, delta subunit-free PDE6 is activated to a higher level by transducin when the PDE is membrane bound than when it is in solution(7-10). Therefore, the delta subunit could alter PDE6's interaction with the membrane to cause a conformational change or a destabilization of the PDE6/transducin interaction. Alternatively, the delta subunit might simply move PDE6 away from the membrane where active transducin is bound, thus reducing the amount of PDE which is locally available to be activated by transducin. The delta subunit has also been shown to have an effect on the binding of cGMP to noncatalytic sites on PDE6(11). This could modify the PDE's interaction with its gamma subunit and thus its ability to be activated by transducin, or modify the gamma subunit's ability to act as a GAP (GTPase activating protein) co-factor for transducin. In addition, the delta subunit can interact with RPGR, a photoreceptor protein that has homology to the guanine nucleotide exchange factor RCC1(12), and can act

as a GDI (guanine nucleotide dissociation inhibitor) for Arl3, a small G protein(13).

Similar interactions could affect phototransduction.

Due to the effects of the delta subunit on the solubility of PDE6, we hypothesized that the delta subunit would reduce the PDE activity induced by a flash of light. In this paper, we test this hypothesis and demonstrate the effect of delta subunit-GST fusion protein on light-induced PDE activity in isolated rod outer segments. We use an assay that measures the real-time accumulation of the products of cyclic nucleotide hydrolysis, so the effect of δ -GST on both the rate of cGMP hydrolysis and the inactivation of the system is shown. Since this assay measures protons generated as a direct consequence of cGMP hydrolysis in homogenized rod outer segments, the effects we see cannot be affected by downstream changes in the phototransduction pathway, such as Ca^{++} influx and channel closure. We also perform experiments which suggest that the effects of δ -GST on cGMP hydrolysis are likely due to direct effects on the catalytic activity of the PDE, rather than effects on other parts of the phototransduction cascade.

Materials

Fresh bovine eyes were purchased from Schenk Packing (Stanwood, Washington).

[³²P]GTP was purchased from New England Nuclear (Boston, Massachusetts).

Ultrafree® filters were purchased from Millipore (Bedford, Massachusetts). Super Signal® West Pico chemiluminescent substrate was purchased from Pierce (Rockford, Illinois). All other chemicals were purchased from Sigma (St. Louis, MO).

Methods

Delta subunit expression and purification-- Recombinant delta subunit was expressed as a fusion protein with glutathione-S-transferase (-GST) and purified as described².

Concentrations of all proteins were determined using the Bradford assay(14) using BSA as a standard.

Peptide synthesis -- All peptides prepared in this study were prepared using the previously published general method for peptide synthesis with or without prenyl groups and C-terminal methyl esters(15). All peptides were purified to apparent homogeneity by HPLC on a C18 reverse phase column and their structures were confirmed by electrospray ionization mass spectrometry. Peptides used in this paper were previously described in detail² and include GKQPGGGPASKSC (-13-f-Me), PASKSC (-6-f-Me), and PRSSTC (-6-gg-Me), all with C-terminal methylation and prenylation. As negative controls, GKQPGGGPASKSC(S-farnesyl)-COOH without methylation (-13-f-OH) and PRSSTC-COOME without prenylation (-6-np-Me) were used.

pH assay – This assay is used to measure time-dependent cGMP hydrolysis in a mixture of ROS, nucleotides, and proteins. All procedures were performed in complete darkness using an infrared viewer unless otherwise indicated. ROS from fresh retinas(16) were

permeabilized by trituration 10 times through a 28 gauge needle attached to a 1 mL disposable insulin syringe (BD). ROS (final [Rh] = 7-10 μM in a total reaction size of 400 μL) were then added to a solution containing pH assay buffer (140 mM potassium aspartate, 7 mM KCl, 5 mM NaCl, 5 mM HEPES, 1 mM EGTA, 3.3 mM MgCl_2 , 0.986 mM CaCl_2 , pH = 8.0; this buffer is calculated to give 641 nM free Ca^{++}), 2 mM ATP, 0.5 mM GTP, and 2 μM delta-GST or control buffer (see purification procedure, above). This was incubated at room temperature for 5 minutes to allow full mixing of the components. Then cGMP was added to a final concentration of 5 mM and the pH electrode placed in the mixture. The mixture was stirred constantly. After a constant baseline (generally 0.1 to 1 mV/second) was achieved, light flashes were given as indicated in the figure legends. A 25 msec light flash with the 0.0 neutral density filter bleached ~0.1% of the rhodopsin. Data from the pH meter were collected and analyzed using the DataLogger program and the Serial Box Interface from Vernier Software, Beaverton, Oregon. Gain was adjusted so that an increase of 1 mV corresponded to about 3.4×10^{-10} moles of cGMP hydrolyzed. Two data points were collected per second. Derivatives shown are from data averaged over 7 points. The dark rate of cGMP hydrolysis is subtracted from all results shown in this paper. The pH of the reaction changed 0.3 pH units at most, which has little effect on the PDE's activity(17). A long light flash given at the end of every experiment to measure remaining cGMP showed that at least 2.5 mM cGMP remained after all data was collected--significantly above the K_m for the enzyme. In some experiments, neutral density filters were used to vary the light intensity of the flash. These filters are graded on a log scale so that a 1.0 filter lets in 10% as much light as a 0.0 filter. 0.3, 0.6, and 0.9 filters let in 50%, 25%, and 12.5% as much light, respectively, as the 0.0 filter.

Single turnover GTPase assay-- GTPase activity was determined under the same conditions as those used for the pH assay, except that [^{32}P]GTP (~ 4×10^4 dpm/pmol, 50 nM) was added to the mixture, and ATP was omitted to remove rhodopsin phosphorylation

as a factor in the rate of GTP hydrolysis. Zero time values were determined by first adding 300 μM GTP S, then the [^{32}P]GTP. The assay was carried out as previously described(18). Briefly, ROS from fresh bovine eyes (7-10 μM rhodopsin) were mixed with delta subunit, pH assay buffer, and cGMP. This mixture was then incubated in the light or dark for 10 minutes to hydrolyze endogenous GTP. The reaction was then started by addition of [^{32}P]GTP, and 30 μL aliquots taken at the noted times. Reactions were stopped by adding the aliquots to 115 μL of 6% perchloric acid. After collection, aliquots were treated with 700 μL of 5% activated charcoal in 50 mM NaH_2PO_4 (pH=7.5). Free phosphate remaining in the supernatant after this treatment was counted in a Tri-Carb scintillation counter (Packard). At the zero time point, between 3-7% of the total radioactivity in these reactions remained in the supernatant.

Immunoblots-- Samples were prepared for immunoblot analysis by addition of 6x Laemmli sample buffer. Samples containing ROS were not boiled. After running these samples on 12% or 15% gels, the gels were blotted onto nitrocellulose. Nitrocellulose was blocked with 5% milk in TBST (20 mM Tris, pH 8, 140 mM NaCl, 0.05% (v/v) Tween 20). PDE catalytic subunit immunoreactivity was measured using the PDE6 cat pAb(1), a rabbit polyclonal antibody, at 1:3000 dilution. Transducin alpha subunit immunoreactivity was detected using antibody 5552 (kindly provided by Dr. Jim Hurley) at 1:3000 dilution. Transducin beta subunit was detected using the G (M-14) antibody from Santa Cruz Biotechnology at 1:1000 dilution. All primary antibodies were detected by incubation with a horseradish peroxidase linked goat anti-rabbit secondary antibody. The secondary antibody was visualized with Super Substrate West Pico chemiluminescent substrate.

Results

Based on the localization of the delta subunit and its ability to remove PDE6 from the membrane, we hypothesized that δ -GST would reduce the PDE activity seen in response to a flash of light. To test the effect of δ -GST on the light induced PDE activity, we used the pH assay to measure the real-time hydrolysis of cGMP in response to light in a rod outer segment preparation containing key components of the phototransduction cascade(19,20). In this assay, a pH meter was used to measure the generation of H^+ produced by the hydrolysis of cGMP into GMP by PDE. In the absence of δ -GST, ROS responded to light with a transient increase in cGMP hydrolysis due to activation of the phototransduction cascade (Figure 1, panel A, top trace). The rate of cGMP hydrolysis throughout the course of the reaction was measured by taking the derivative of the raw data shown in panel A of figure 1 (Figure 1, panel B). After the light flash, the rate of cGMP hydrolysis increased as the PDE was activated by transducin, and after about 50 seconds returned to the baseline dark rate of hydrolysis as the cascade was shut off by transducin's GTPase activity, the phosphorylation of rhodopsin by rhodopsin kinase, and reassociation of the inhibitory gamma subunit of PDE6.

Addition of δ -GST to this reaction resulted in a decrease in the total amount of cGMP hydrolyzed (Figure 1, panel A, bottom trace). This was associated with a reduction in the maximal rate of cGMP hydrolysis in the presence of δ -GST (Figure 1, panel B, bottom trace).

Peptides which can block δ -GST's ability to solublize PDE have been developed². These peptides block the solubilization of PDE6 by $2 \mu M$ δ -GST with IC_{50} s ranging from 1 to $10 \mu M$. To determine whether they would also block δ -GST's effect in the pH assay, these peptides ($10 \mu M$) were added to the pH assay reaction mixture (Figure 2). At this concentration, the peptides do block the effect of δ -GST (Figure 2, panels A and B). Peptides alone, without delta subunit, do not affect the response to light (Figure 2, panels

C and D). This is not surprising as immunoblots show that these ROS preparations contain little endogenous delta subunit (data not shown).

pH assay experiments were repeated to take into account variations between different preparations of ROS and δ -GST. Figures 1 and 2 show representative data from one experiment; Figure 3 shows the average maximum rate of cGMP hydrolysis from 3 separate experiments. Purified expressed GST alone had no effect in this assay (data not shown).

Immunoblots of ROS supernatants and pellets were performed to determine whether PDE6 catalytic subunits were solubilized under the same conditions used for the pH assay. These blots demonstrate that PDE6 catalytic subunits become soluble at the concentrations of δ -GST used, unless peptide is present to block the effect of δ -GST (Figure 3). Since the hypothesis being tested suggested that the reduction in cGMP hydrolysis would be caused by this solubilization of the PDE by δ -GST, we determined whether both of these effects occur in a similar concentration range of δ -GST (Figure 4). Dose/response curves of the solubilization of PDE catalytic subunits by δ -GST (Figure 4A) and the δ -GST-associated reduction in cGMP hydrolysis (Figure 4B) were performed. Both effects occur in a similar concentration range. This strongly suggests that the two effects are causally linked.

The dose/response curve using various amounts of δ -GST (Figure 4B) showed that the IC_{50} of δ -GST's effect in the pH assay was 385 nM. Based on calculations of the rhodopsin:PDE ratio in bovine rod outer segments(21), there should be about 140 nM holo-PDE in this preparation—280 nM catalytic subunits. Therefore, the IC_{50} of δ -GST was in the range of the amount of PDE catalytic subunits in this experiment. Since other work has suggested that the delta subunit interacts with the catalytic subunits with high affinity (less than or equal to 14 nM)(1), it is likely that this IC_{50} represents titration of the catalytic subunits by the delta subunit and underestimates the true affinity of the interaction.

The magnitude of the δ -GST effect on the maximum rate of cGMP hydrolysis was dependent on light intensity (Figure 5). Using a 1.0 neutral density filter, which blocks 90% of the light that will come through a 0.0 filter ($\sim 0.01\%$ rhodopsin bleached), the maximal rate of cGMP hydrolysis in the presence of δ -GST was 80% of the maximal rate without δ -GST (panels A and C). Using a 0.0 neutral density filter ($\sim 0.1\%$ rhodopsin bleached) the maximal rate of cGMP hydrolysis in the presence of δ -GST was 18% of the maximal rate without delta subunit (panels A and B). Immunoblots showed that δ -GST brought approximately the same amount of PDE into the supernatant with and without light exposure (data not shown), so the variable effect of δ -GST was not due to different amounts of PDE being solubilized under different light conditions. One interpretation of these results is that in the presence of delta subunit, the response of the ROS is basically independent of light-- a similar, low level response is always seen. Another possibility is that the dose/response curve for light has been shifted well to the right. Due to high noise at low light intensities and the lack of a brighter light source, we could not distinguish between these two possibilities by using higher or lower light levels.

In addition to its effect on the maximal rate of cGMP hydrolysis, δ -GST prolonged the time to turn-off of the system (Figure 6). The amount of time that it took for the rate of cGMP hydrolysis to be reduced from its maximal value to half of the maximal value (over baseline) was calculated. In the presence of $2\ \mu\text{M}$ δ -GST, it took approximately 2.5 times as long for the signal from a 0.0 flash to be reduced by half. Due to the larger amount of noise in derivatives of lower light flashes, it was not possible to determine whether this effect of δ -GST was also reduced at lower levels of light.

It was important to determine whether the effects on cGMP hydrolysis and inactivation were due to action on the PDE itself, to the effects of δ -GST on some other part of the signal transduction cascade, or a combination of these two possibilities. Previous studies have shown that δ -GST can solubilize mPDE and that the delta subunit appears to have no direct effect on the catalytic activity of the PDE. Figure 4 shows that

solubilization of PDE is associated with the reduction of cGMP hydrolysis by γ -GST, and both effects of γ -GST were negated by pre-incubation with peptides that block the solubilization of PDE activity² by γ -GST (Figure 2 and Figure 3). These observations suggest that the effects of γ -GST in the pH assay may be due to PDE solubilization, but they do not rule out that γ -GST's effects could be due to actions on other proteins in the signal transduction cascade. To investigate these possibilities further, we performed experiments designed to determine whether reduction of cGMP hydrolysis by γ -GST could be due to effects on transducin or rhodopsin kinase.

If γ -GST moved transducin away from the membrane, it might be expected to reduce the gain of the signal transduction pathway and result in reduced cGMP hydrolysis. This was a distinct possibility, as part of the γ -GST binding site on the PDE catalytic subunits are the prenylated C-termini², and the gamma subunit of transducin is prenylated (farnesylated) as well. We performed immunoblots on aliquots of ROS incubated with various concentrations of γ -GST (Figure 7). At concentrations where PDE is moved almost completely into the supernatant by γ -GST, transducin's alpha and beta subunits are unaffected. Therefore, solubilization of transducin is unlikely to be the mechanism for the reduction in cGMP hydrolysis by γ -GST. This result is also interesting as it demonstrates the specificity of γ -GST action. γ -GST selectively solubilizes some prenylated proteins and not others.

To determine whether changes in the ability of transducin to hydrolyze GTP were involved in the effects on the light response, the effect of γ -GST on the GTPase rate of transducin was examined directly. Single turnover GTPase activity was measured under conditions of limiting GTP, which was added to start the reaction. In the light under these conditions, only a small fraction of the activated transducin should have been able to bind and hydrolyze GTP, so there was only one round of GTP hydrolysis by transducin(18). GTP was hydrolyzed more slowly in the presence of γ -GST (Figure 8). The rate constants calculated from multiple experiments show that γ -GST slowed the rate of GTP hydrolysis

to 43% of its original rate, from an average half-time of 5.25 seconds to an average half-time of 11.9 seconds (Figure 8, panel B). This effect of γ -GST was also blocked by preincubation with peptides that block the ability of γ -GST to make the catalytic subunits soluble. The dark rate of GTP hydrolysis, which is probably due to other GTPases such as small G proteins as well as transducin, was not significantly altered by γ -GST (Figure 8, panel A). These results show that γ -GST does not cause its reduction in cGMP hydrolysis by accelerating transducin's rate of GTP hydrolysis; however, this result could explain the increased amount of time it takes the system to turn off in the presence of γ -GST (Figure 6).

To determine whether γ -GST might reduce the maximum rate of cGMP hydrolysis by increasing the activity of rhodopsin kinase, the pH assay was performed without ATP (Figure 9). After a light flash under these conditions, the phototransduction cascade should activate as usual. However in the absence of ATP, rhodopsin is not phosphorylated, so the cascade will not turn off until the limiting substrate, in this case cGMP, is depleted. Incubation with γ -GST decreased the maximum rate of cGMP hydrolysis to a similar extent as was seen in the presence of ATP. Therefore, the effect of γ -GST on cGMP hydrolysis in response to light was not ATP dependent, and was unlikely to be mediated by a kinase.

Discussion

It has been established that the recombinant purified delta subunit solubilizes membrane-bound type 6 PDE(1), but the physiological implications of this solubilization were unknown. Much recent research has pointed out the importance of compartmentalization of signal transduction components for proper function of regulatory cascades. For these reasons it was interesting to determine the physiological effects of the delta subunit in the photoreceptor. It is likely that the delta subunit, which is expressed in a number of other tissues outside of the retina and is highly conserved, may play similar roles in tissues outside of the photoreceptor.

Localization studies(1) demonstrate that the delta subunit in the retina is primarily localized in the outer segments of the rod cells, where other components of the phototransduction cascade are also present at high concentrations. A significant fraction (~30%) of the PDE purified from rod outer segments is soluble. These results suggest that one of the delta subunit's primary actions in the retina may be to modify the phototransduction cascade by affecting PDE activity. One obvious possibility was that by moving PDE away from the membrane, the delta subunit would reduce the ability of transducin to activate the PDE, thus reducing the gain of the system in response to a flash of light. One must keep in mind, however, that in the intact rod, the concentration of membranes is much higher than it is in the experiments shown in this paper. Therefore, it is possible that in the intact photoreceptor, the delta subunit does not actually make the PDE catalytic subunits soluble. Nonetheless, it is well documented that the ability of transducin to activate the PDE is sensitively dependent on the presence and/or composition of membranes in the assay(8,10,22). Several studies have also shown that PDE is more tightly membrane bound when activated by transducin(22,23). Therefore it is very likely that the delta subunit could affect the transducin/PDE interaction by altering the PDE's interaction with the membrane even without making the PDE soluble. We decided to

examine the effects of γ -GST on the photoreceptor's light response, and to confirm that peptides that block the solubilization of the PDE² could block these physiological effects. Data from the pH assay shows that γ -GST had several effects on the rod's response to light. As we expected, it reduced the total amount of cGMP that was hydrolyzed in response to a flash of light, and decreased the maximum rate of cGMP hydrolysis. The delta subunit had a larger-fold effect at high (although not saturating) levels of light than at lower levels of light. This may represent a shift in the sensitivity of the light response. Unexpectedly, γ -GST also increased the amount of time it took the system to turn off.

Based on both previously published data and data presented in this paper, we feel it is likely that the reduction in cGMP hydrolysis in the presence of γ -GST was due to the interaction of γ -GST with the PDE and not some other component of the signal transduction pathway. The delta subunit has not been reported to be co-purified with any of the other proteins of the signal transduction pathway, suggesting that the PDE may be the delta subunit's most common partner in this pathway, or at least its most tightly interacting partner. Peptides that block the ability of γ -GST to make the PDE soluble² also blocked the reduction in the rate of cGMP hydrolysis. However, the delta subunit may also interact with other prenylated proteins in the photoreceptor, and if γ -GST's effects were due to actions on these proteins, the peptides might be expected to block these effects as well. Therefore, we made more direct measurements of γ -GST's effects on transducin and rhodopsin kinase, the two other components of the signal transduction pathway that could reduce cGMP hydrolysis in this assay. The effects of γ -GST on these other elements of the phototransduction cascade cannot explain the reduction in cGMP hydrolysis presented here. Namely, reduction of the GTPase activity of transducin by γ -GST would be expected to increase, not decrease, the amount of cGMP hydrolyzed by increasing the lifetime of active transducin. The reduction in cGMP hydrolysis also occurred in the absence of ATP, which shows that an effect on rhodopsin kinase was not responsible for this result. We therefore feel that the reduction in the rate of cGMP hydrolysis was most

likely due to a functional uncoupling of the rhodopsin-transducin-PDE pathway and a resultant reduction in the gain of the transducin to PDE step of the phototransduction cascade.

One circumstance in which the gain of the phototransduction cascade is reduced is during light adaptation. Two types of light adaptation have been identified: background adaptation and bleaching adaptation. Background light adaptation in the rod outer segment occurs when a rod is exposed to a very low level (<0.01% rhodopsin bleached) of background light. Bleaching adaptation occurs after a bright flash of light which bleaches significantly more of the rhodopsin. In both cases, adaptation results in a response with a smaller amplitude (reduced gain) than the same flash given to a non-adapted rod. Background adaptation has a large calcium dependent component(24). One model to explain background adaptation proposes that it occurs as a result of guanylyl cyclase activation at low levels of calcium(25-27). This calcium-dependent change in the recovery phase of the cascade resets the background level of cGMP to a higher concentration, and probably accounts for much of the change seen with adaptation. However, there are data to suggest that a slowing of the excitation stages of the flash response may also be involved in light adaptation. For example, a reduction in the gain of rhodopsin to PDE activation(28-30) has been noted during light adaptation. It has also been shown that during the rising phase of the light adapted response, which occurs before significant changes in intracellular calcium have occurred, there is a reduced rate of activation of the PDE as compared to the dark-adapted response(31). A molecular mechanism for this slowed activation has not yet been determined, but the reduction in the hydrolysis of cGMP in response to a flash of light that we demonstrate in the presence of β -GST could be responsible.

Bleaching adaptation appears to occur due to an activation of the phototransduction cascade by bleached photopigment(32,33) or arrestin-bound meta-II rhodopsin (34). The “inactivated” rhodopsin activates the visual signal transduction cascade, but to a lesser extent than unphosphorylated meta II rhodopsin does. The low level activation of the

cascade mimics the changes seen in background light adaptation. However, not all of the characteristics of bleaching adaptation are the same as those seen as background adaptation. For instance, bleached rhodopsin does not turn on the signal transduction cascade as well as unbleached rhodopsin in equivalent background light(31,35). It is not clear whether this reduction in gain is calcium dependent. This could potentially be caused by a reduction in the pool of activatable PDE by the delta subunit. Further experiments to determine whether the effects of the delta subunit are calcium dependent may help to discern whether the delta subunit may be involved in background adaptation, bleaching adaptation, or some other process in the retina.

Although the reduced hydrolysis of cGMP that is seen in the presence of δ -GST is suggestive of a role for the subunit in light adaptation, there are some aspects of δ -GST's effects that do not incorporate well into this model. Prolongation of the turn-off of the system is not one of the electrophysiological features of light adaptation. Several factors could contribute to this prolongation. The reduction in GTP hydrolysis in the presence of δ -GST could contribute to prolongation of the light signal. This reduced rate of GTPase may be a secondary effect that results from reduced interaction between transducin's alpha subunit and the PDE gamma subunit, which can promote acceleration of the GTPase activity of transducin(36,37). Other work has shown that δ -GST may reduce the PDE catalytic subunit's affinity for the gamma subunit(11). This could potentially prolong the amount of time that it takes for the PDE to be re-inhibited. The prolongation of turn-off could also be due to the interaction of δ -GST with other proteins in the retina, such as RPGR.

If the delta subunit is involved in light adaptation or some other dynamic process in the outer segment, it would be expected that its interaction with the catalytic subunits of PDE6 would be regulated. Recent work by our group² has suggested that the delta subunit interacts more strongly with methylated PDE than with demethylated PDE. As methylation is a reversible process, it could regulate the PDE/delta subunit interaction. However, at this

point this model is purely hypothetical, as light-induced regulation of either methylation or PDE solubility have not been reported.

In conclusion, data presented in this paper demonstrates that δ -GST can have significant effects on the phototransduction cascade, and that it may be involved in the light adaptation process. The action of the delta subunit is likely not limited to its effects on the visual signal transduction cascade. The delta subunit is present in a number of tissues outside of the retina ((1,38) and T.A.C., unpublished observations), where none of the other components of the visual signal transduction pathway are known to localize. It has been cloned from organisms as varied as mouse, human, and *C. elegans* (39,40), and is very highly conserved, which suggests that its functional role may be quite important. It is possible that the delta subunit may regulate the gain of other signal transduction pathways, such as those involving small G-proteins(12,13,38) by changing the localization of their necessary components; alternately, the delta subunit could have a different role in these other tissues. We hope that future studies can more definitively determine the physiological purpose of this subunit in the retina as well as in other tissues.

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Figure Legends

Figure 1. The effect of recombinant delta subunit (2 μM) on the light response of broken rod outer segments. ROS membranes ($[\text{rhodopsin}] = 7 \mu\text{M}$ in final reaction) were incubated in the dark in pH assay buffer with 0.5 mM GTP, 2 mM ATP, and 2 μM delta subunit-GST fusion protein or control buffer for 5 min at room temperature. 5 mM cGMP was added, a pH electrode was put into the reaction mixture, and stirring begun. After changes in pH had stabilized, a 25 millisecond flash of light was given (flash given at 10 seconds on these graphs). Panel A shows data from these experiments. An increase of one millivolt corresponds to 3.42×10^{-10} moles of cGMP hydrolyzed. Data shown has the low dark rate of cGMP hydrolysis (generally 0.1 to 1 mV/second) subtracted out. Panel B shows the derivative over time of the data shown in A. Panels A and B: the response of the preparation to light in the absence (top trace, solid) and presence (bottom trace, dashed) of δ -GST. Data shown in all panels is representative of at least 3 experiments performed with different batches of ROS and delta subunit.

Figure 2: Block of δ -GST effect by prenylated peptides. Experiment performed as in Figure 1, except that some samples were also incubated with 10 μM prenylated, methylated peptide corresponding to the C-terminus of the alpha or beta catalytic subunit. A and B. Peptides block δ -GST effect. ROS alone, thick dashed line. With delta subunit, lower solid line. With 10 μM δ -6-gg-Me, upper dashed line. With 10 μM δ -13-f-Me, dotted line. C and D. Peptides alone (10 μM of each) have no effect in the absence of delta subunit. ROS alone, lower dashed line. ROS + 10 μM δ -13-f-Me + 10 μM δ -6-gg-Me, upper solid line. Unprenylated or unmethylated peptides cannot block δ -GST's effects in this assay (data not shown).

Figure 3. δ -GST's effect on the maximum rate of cGMP hydrolysis corresponds with the movement of PDE from the pellet fraction into the supernatant. pH assays were performed as described in Figure 1. Graph shows the average maximal rate of cGMP hydrolysis from three separate experiments. Error bars represent the standard deviation. Aliquots (25 μ L) of each reaction were spun down at 14,000 x g for 10 minutes to separate membrane-bound from soluble fractions. These fractions were separated using SDS-PAGE, blotted onto nitrocellulose, and the localization of the PDE catalytic subunits was determined by doing a Western blot with the PDE6 cat pAb(1) at 1:1000 dilution (insets-- S is soluble fraction; P is pellet fraction). The peptide used in this western blot experiment was -6-f-Me. -13-f-OH had no effect on the delta subunit activity (data not shown).

Figure 4. Dose-response of δ -GST actions. A. Solubilization of PDE6 by - GST. ROS aliquots (7 μ M) were incubated with the indicated concentrations of -GST for 5 minutes. Supernatants were separated from pellets by centrifugation at 100,000 x g and immunoblots to show PDE6 catalytic subunit immunoreactivity performed as described in Methods. S, supernatant fraction; P, pellet fraction. B. Assay was performed as in Figure 1 with varying amounts of delta subunit. The maximum rate of cGMP hydrolysis as a percent of the maximum rate seen without delta subunit is shown. Data shown is the average of 3 experiments done with 2 separate delta subunit preparations. Data was fitted with an exponential equation to determine IC₅₀. Error bars represent standard deviation.

Figure 5. δ -GST's effect on maximal rate of cGMP hydrolysis is more pronounced at higher light levels. pH assays were performed as described in Figure 2. 25 msec flashes were given using neutral density filters which block up to 90% of the light. Panel A: Comparison of - GST's effects on the maximal rate of cGMP hydrolysis over a range of flash intensities (white bars: no delta subunit; gray bars: with delta subunit).

Panels B and C: traces shown are the derivative of raw data. Panel B: δ -GST's effect at higher light levels. Flashes without a neutral density filter (0.0; ~0.1% rhodopsin bleached), and with a 0.3 neutral density filter (~0.05% rhodopsin bleached) were given with (lower two lines) and without (upper two lines) δ -GST. Panel C: δ -GST's effect at lower light levels. Flashes were given with 0.6 (~0.025% rhodopsin bleached) and 0.9 (~0.0125% rhodopsin bleached) filters with (lower two lines) and without (upper two lines) δ -GST. These results are representative of experiments performed three times.

Figure 6. δ -GST increases the amount of time it takes to reduce the maximum rate of cGMP hydrolysis by half. pH assays were performed as described in figure 2 using 0.0 neutral density filter. Maximum rates of cGMP hydrolysis were calculated, and the time it took for responses to return halfway to baseline was measured. Data shown is average of 3 experiments; error bars represent the standard deviation.

Figure 7. δ -GST does not make transducin soluble at the concentrations where it solubilizes PDE6 catalytic subunits. Experiment was performed as in Figure 4A. Samples were probed for both PDE6 and transducin subunit localization. S, supernatant fraction; P, pellet fraction.

Figure 8. δ -GST reduces light activated GTPase activity. Assays were performed as described in Methods. A. Hydrolysis of GTP over time. Open symbols: hydrolysis of GTP in light. Filled symbols: hydrolysis of GTP in dark. Data shown are combined results of multiple experiments performed with different batches of ROS and delta subunit; error bars represent standard deviations. B. Rate constants calculated from exponential fits of data from multiple experiments (numbers in parenthesis = n; error bars represent standard deviations). Exponential fit was used to calculate the half-time of the

reaction. Half lives for GTP were 5.2 sec, 11.7 sec., 6.9 sec., and 5.9 sec for ROS alone, ROD + δ -GST, ROS + GST, and ROS + δ -GST + peptide, respectively. k was calculated using the equation $k = 0.693/(t_{1/2})(41)$. Peptide used in these experiments was 10 μ M δ -GG-Me.

Figure 9. δ -GST's effect on maximal rate of cGMP hydrolysis is independent of ATP. Experiments were performed as in figure 1 only without the addition of ATP. Panel A: cGMP hydrolysis for one representative experiment. Solid trace—without delta subunit; dashed trace, with delta subunit. Panel B: maximal rate of cGMP hydrolysis as a percent of the maximal rate without delta subunit from 3 experiments done with 2 separate delta subunit preparations. Error bars represent standard deviation.

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Footnotes

1. Abbreviations used: ATP, adenosine 5'-triphosphate; BSA, bovine serum albumin; cGMP, guanosine 3':5'-cyclic monophosphate; GAP, GTPase accelerating protein; GDI, GDP dissociation inhibitor; GDP, guanosine diphosphate; GST, glutathione-S-transferase; GTP, guanosine triphosphate; GTP S, guanosine 5'-O-(3-thiotriphosphate); G-protein, GTP binding protein; HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); HPLC, high performance liquid chromatography; mPDE, Type 6 3'-5' cyclic nucleotide phosphodiesterase found bound to the membrane; PDE6, Type 6 3'-5' cyclic nucleotide phosphodiesterase; ROS, rod outer segment(s); sPDE, Type 6 3'-5' cyclic nucleotide phosphodiesterase found in the soluble fraction; Tris, tris(hydroxymethyl)aminomethane.

2. Terry A. Cook, Farideh Ghomashchi, Michael H. Gelb, Stephanie K. Florio, and Joseph A. Beavo, accepted, *Biochemistry*

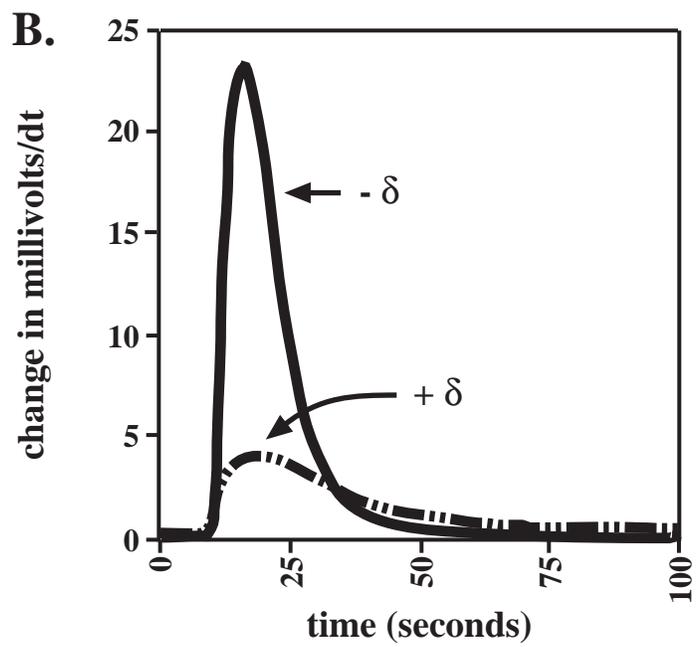
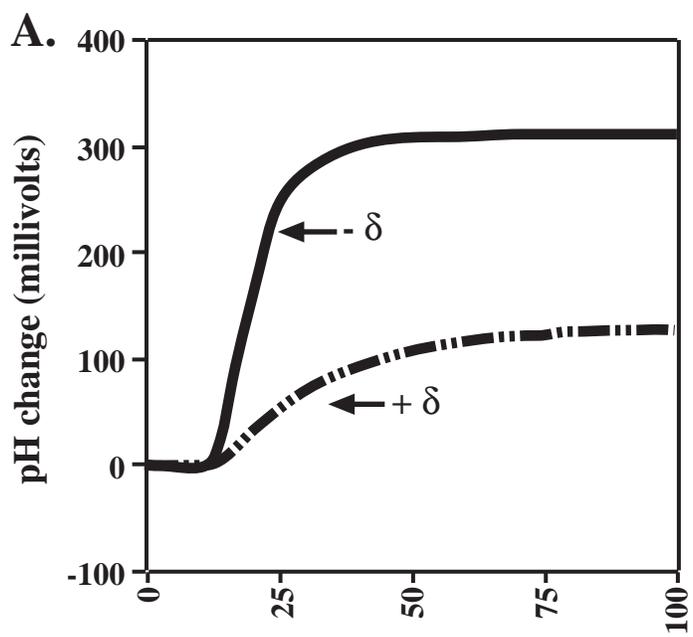


Figure 1

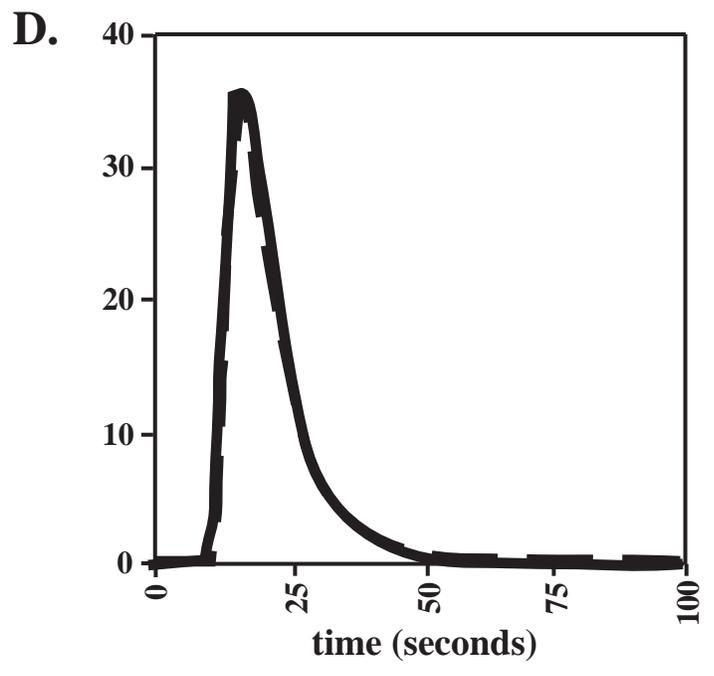
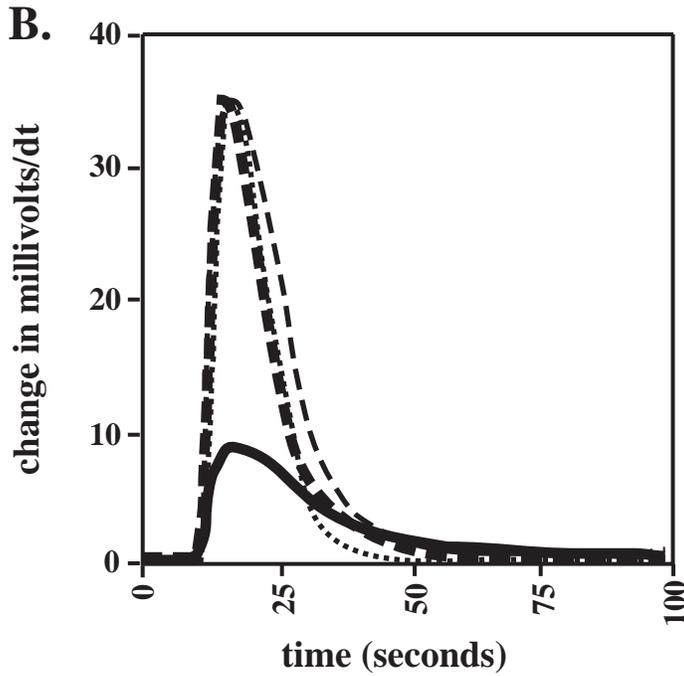
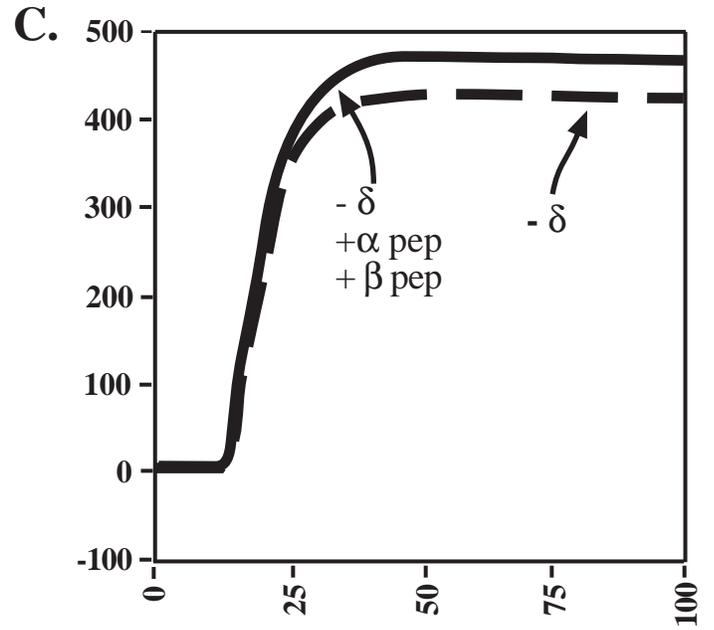
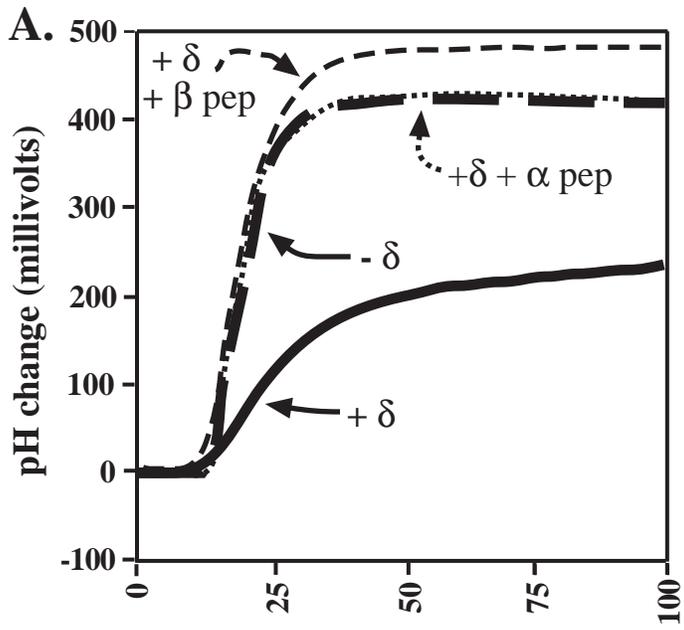


Figure 2

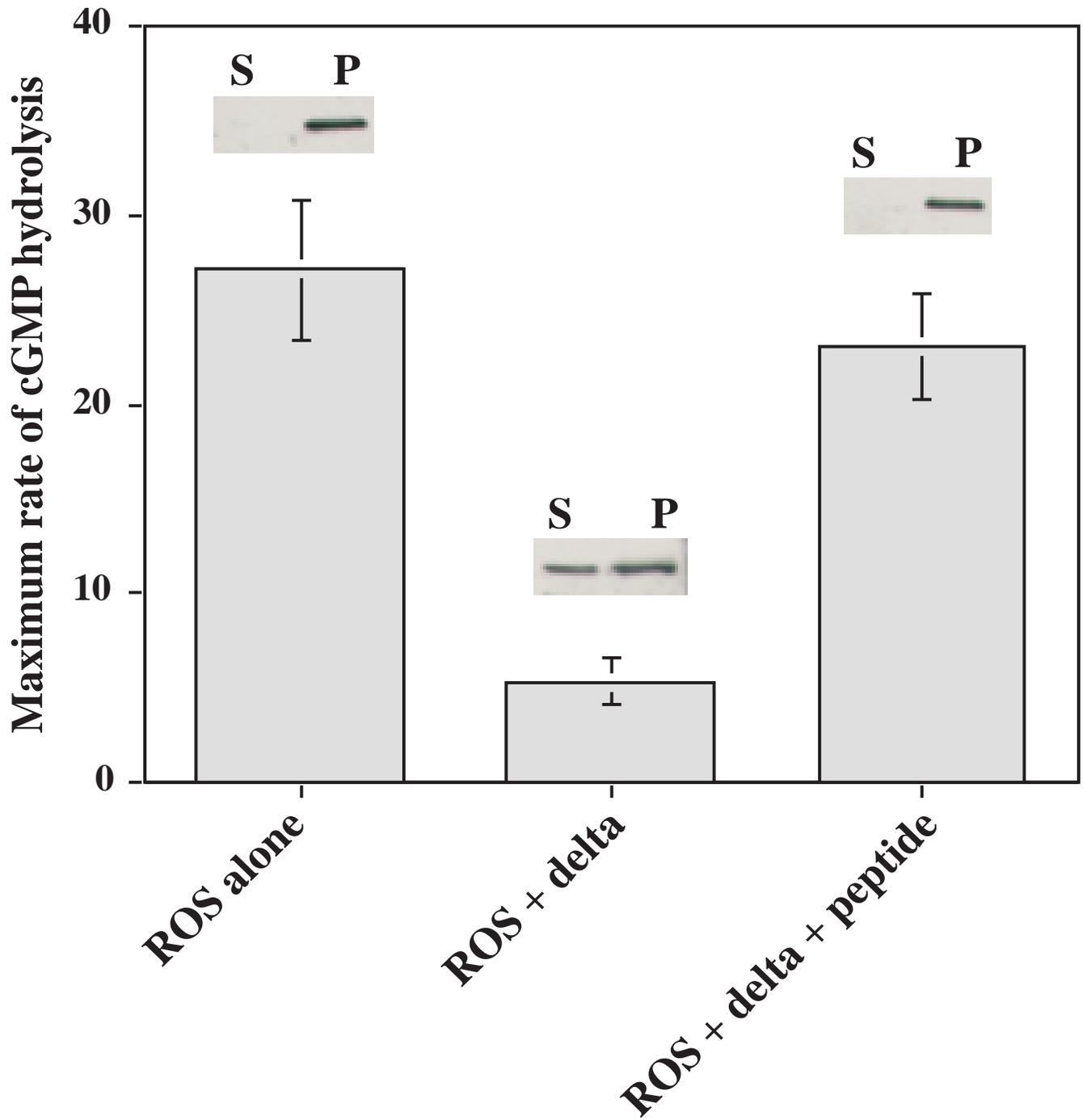
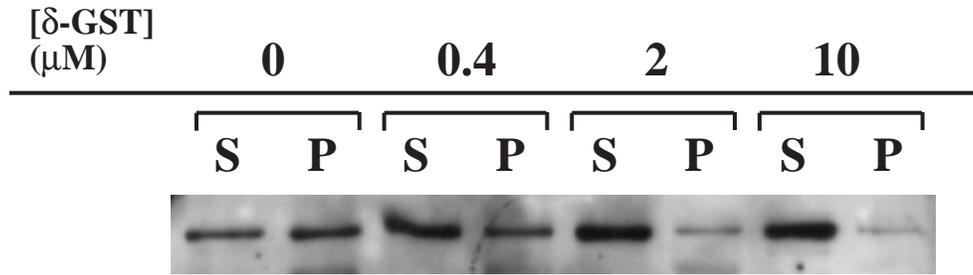


Figure 3

A.



B.

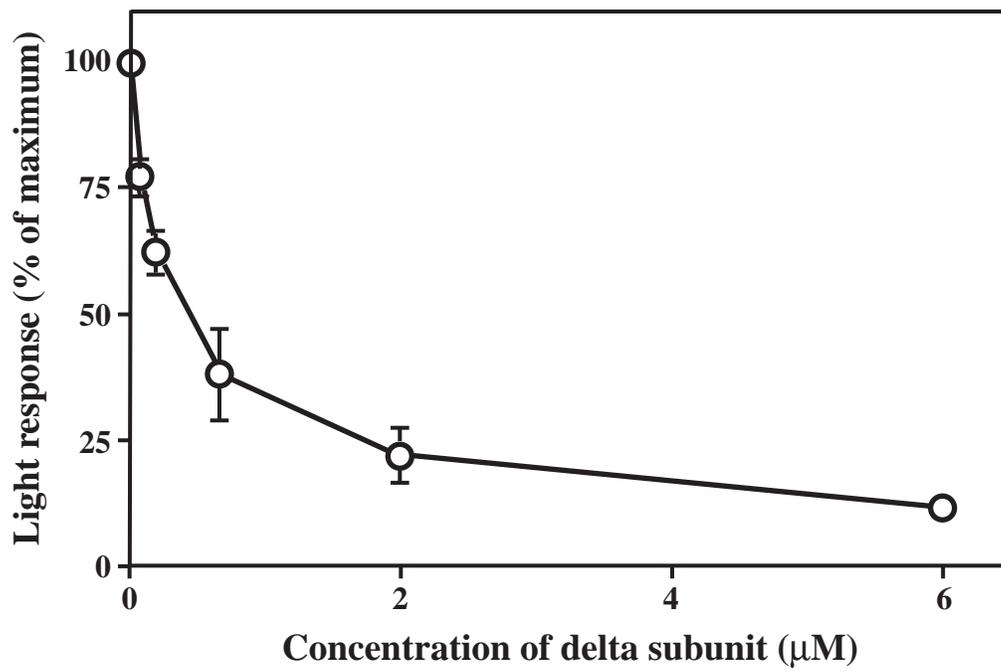


Figure 4

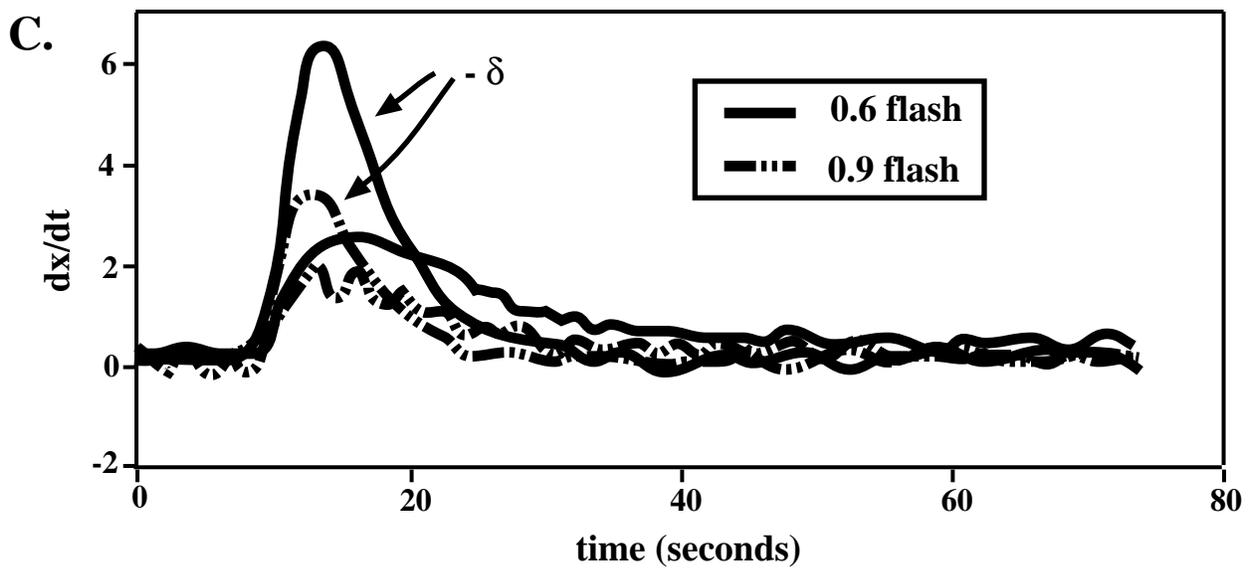
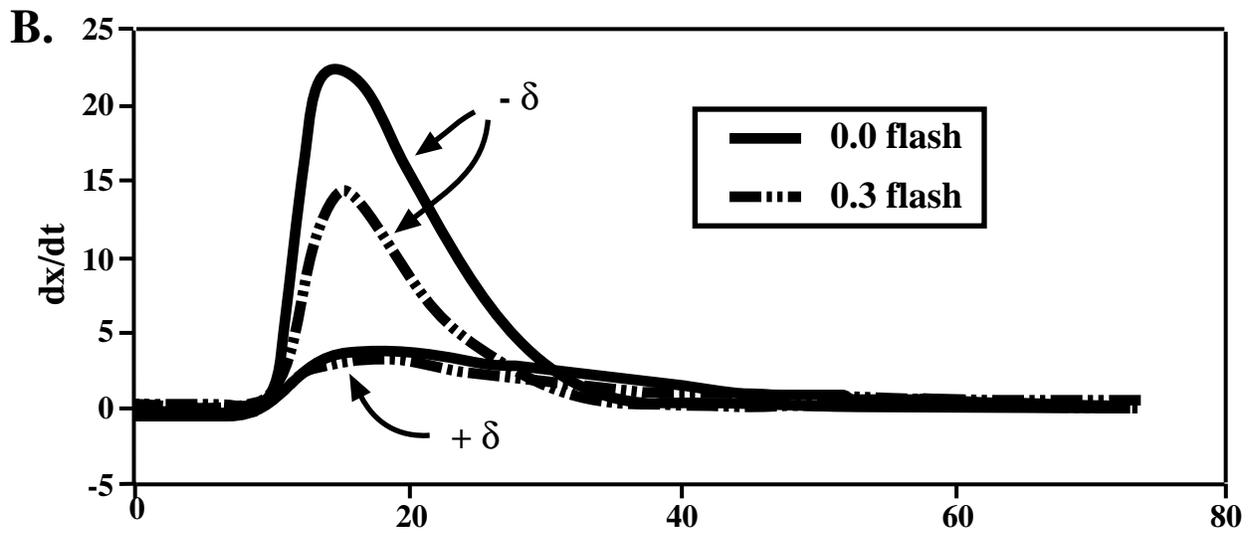
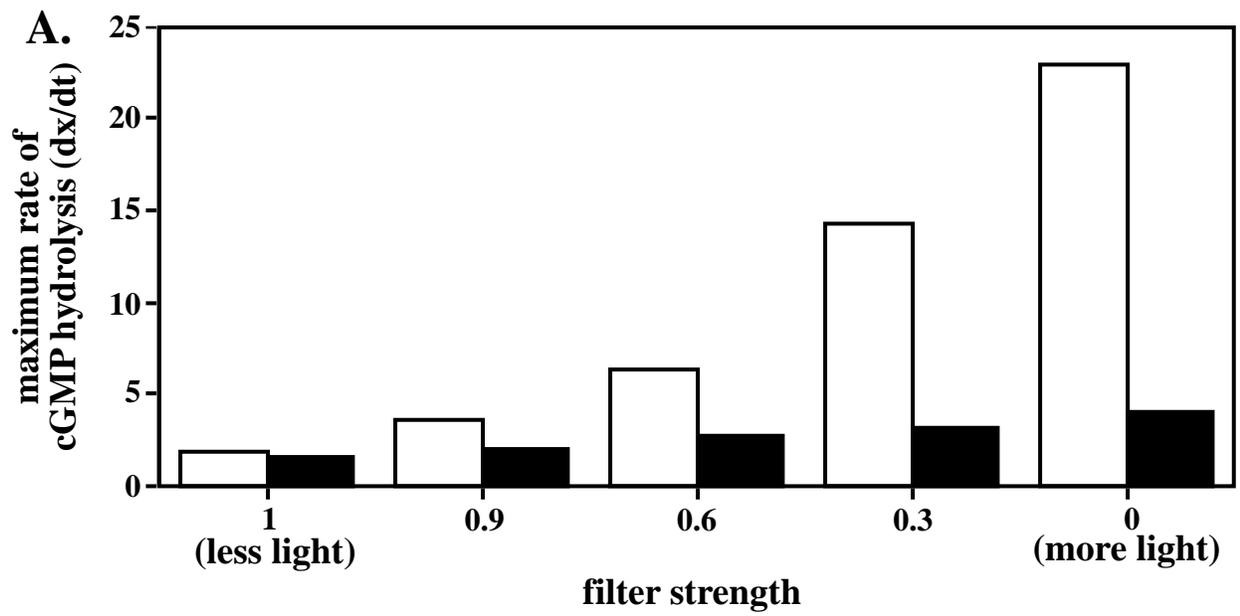


Figure 5

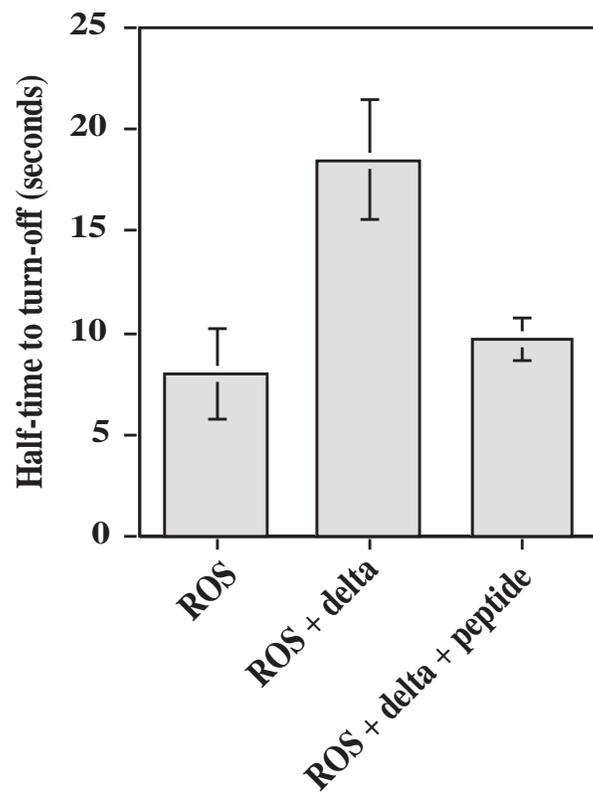


Figure 6

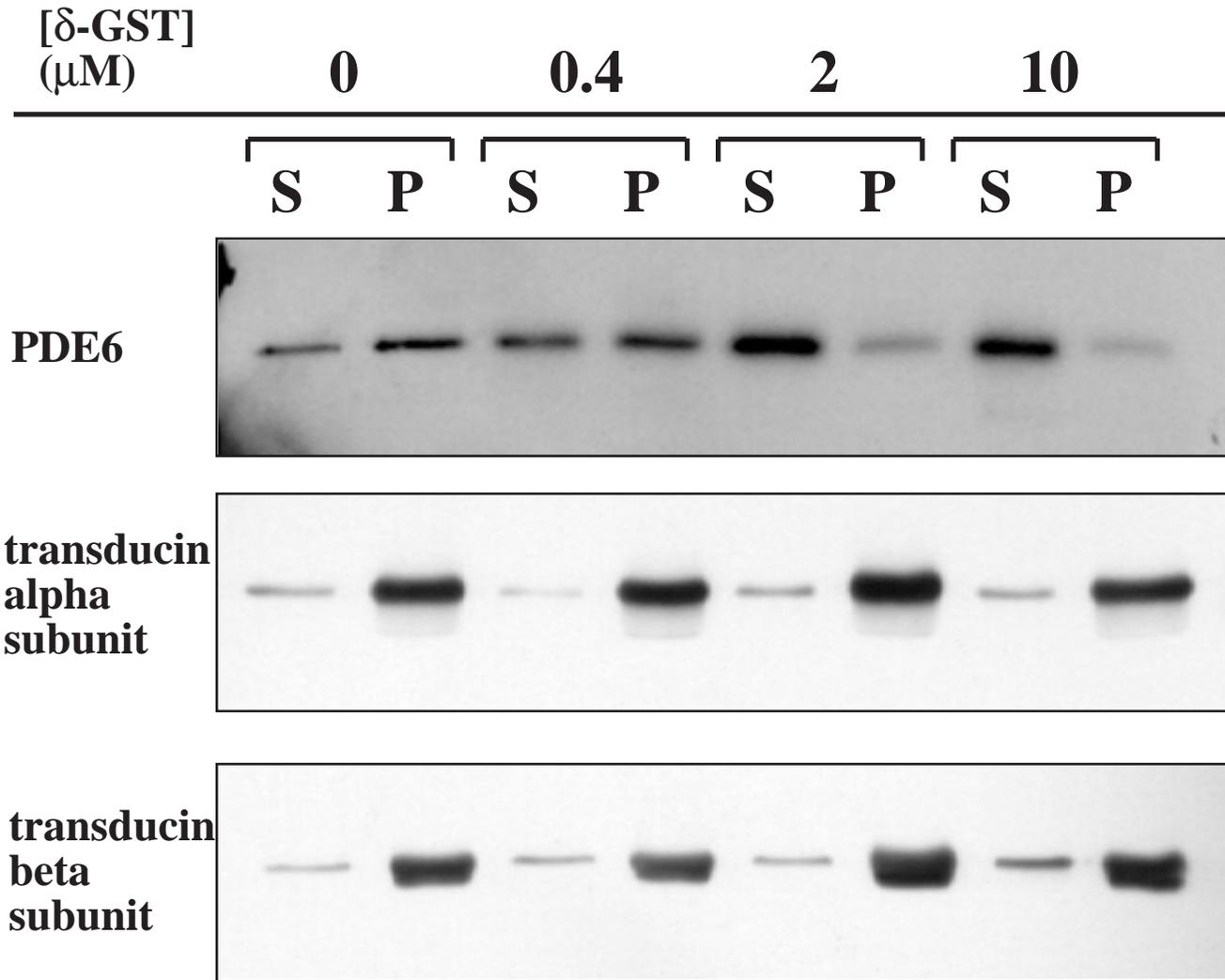
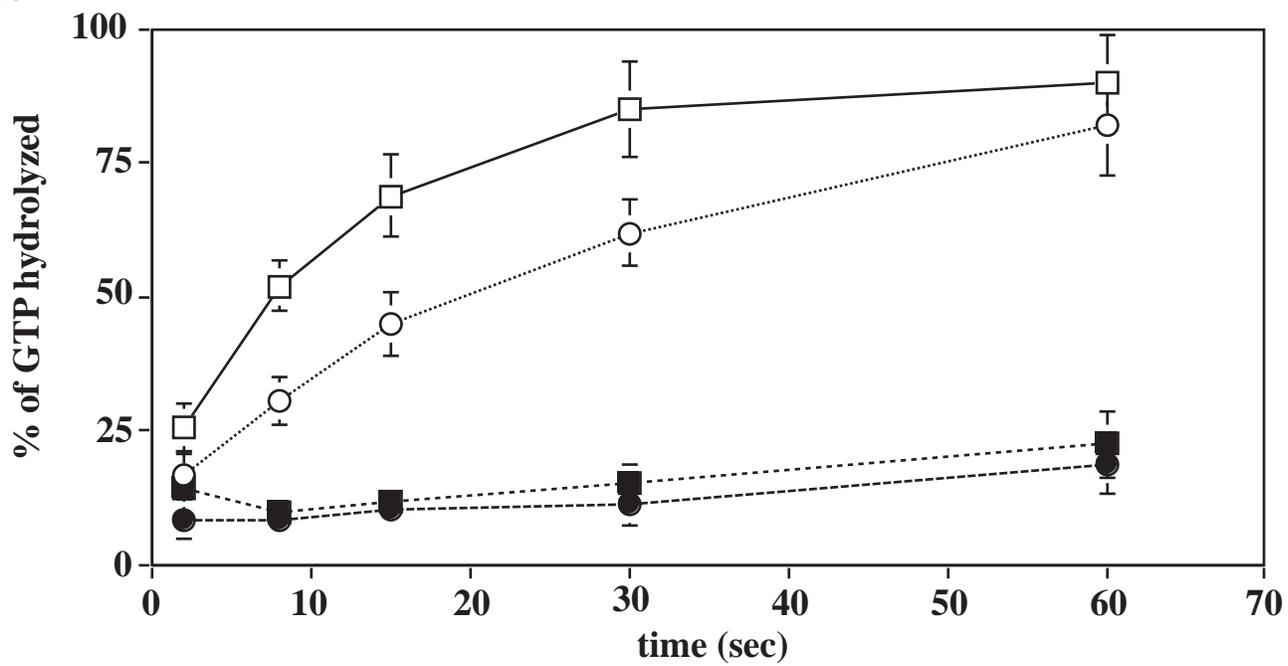


Figure 7

A.



B.

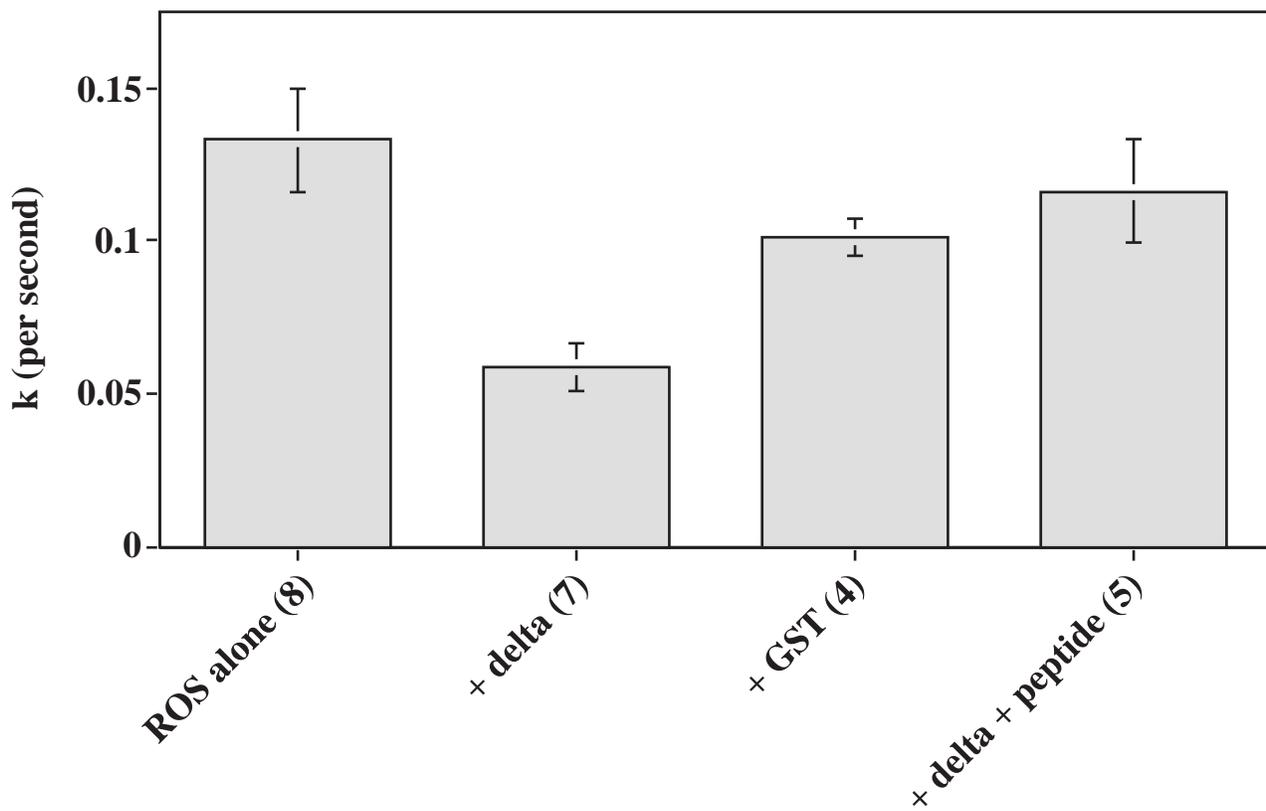


Figure 8

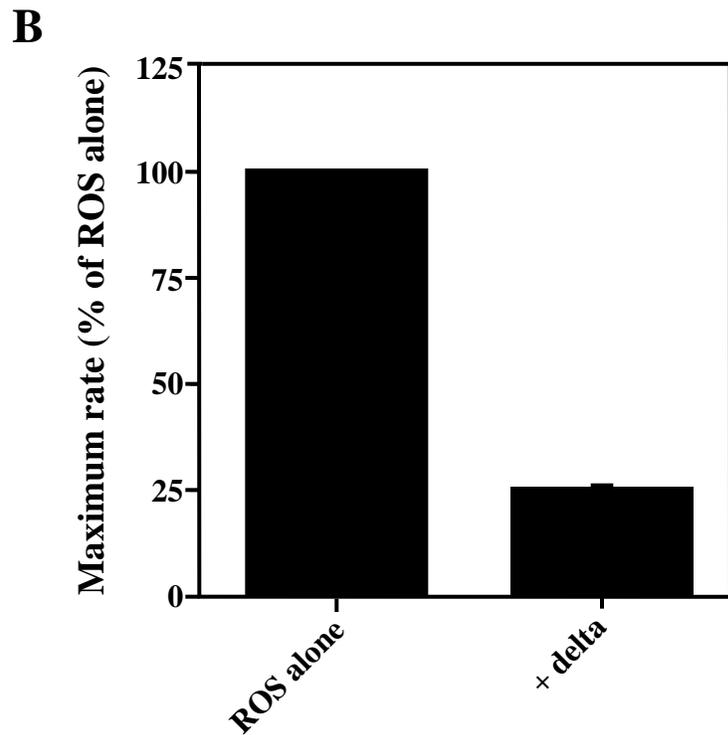
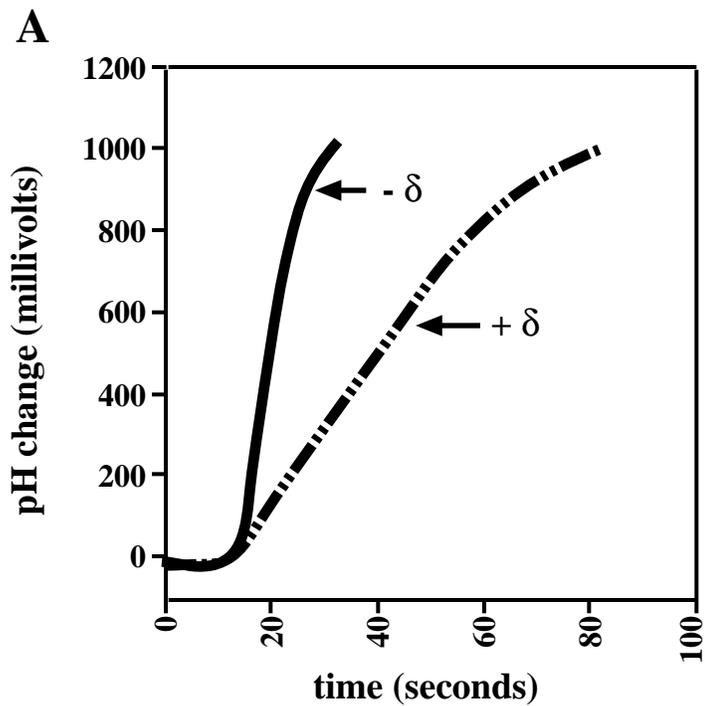


Figure 9