

G-protein-mediated inhibition of the Trp channel TRPM1 requires the G $\beta\gamma$ dimer

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ON bipolar cells are critical for the function of the ON pathway in the visual system. They express a metabotropic glutamate receptor (mGluR6) that, when activated, couples to the G_o class of G protein. The channel that is primarily responsible for the synaptic response has been recently identified as the transient receptor potential cation channel subfamily M member 1 (TRPM1); TRPM1 is negatively coupled to the mGluR6/Go cascade such that activation of the cascade results in closure of the channel. Light indirectly opens TRPM1 by reducing transmitter release from presynaptic photoreceptors, resulting in a decrease in mGluR6 activation. Conversely, in the dark, binding of synaptic glutamate to mGluR6 inhibits TRPM1 current. Closure of TRPM1 by G-protein activation in the dark is a critical step in the process of ON bipolar cell signal transduction, but the precise pathway linking these two events is not understood. To address this question, we measured TRPM1 activity in retinal bipolar cells, in human ependymal melanocytes (HEMs) that endogenously express TRPM1, and in HEK293 cells transfected with TRPM1. Dialysis of the G $\beta\gamma$ subunit dimer, but not G α_o , closed TRPM1 channels in every cell type that we tested. In addition, activation of an endogenous G-protein-coupled receptor pathway in HEK293 cells that releases G $\beta\gamma$ without activating Go protein also closed TRPM1 channels. These results suggest a model in which the G $\beta\gamma$ dimer that is released as a result of the dissociation from G α_o upon activation of mGluR6 closes the TRPM1 channel, perhaps via a direct interaction.

patch clamp | calcium imaging

Retinal ON bipolar cells are connected to photoreceptors through a sign-inverting synapse. At this synapse, glutamate binds to the metabotropic glutamate receptor mGluR6, which couples to the closure of a cation-selective transduction channel. Insight into the molecular identity of the transduction channel was gained from the observation that low levels of mRNA encoding the transient receptor potential ion channel TRPM1 was associated with a form of congenital stationary night blindness (CSNB) in a population of Appaloosa horses (1). Soon after, it was shown that ON bipolar cell function was absent or severely impaired in a mouse model that lacked TRPM1 expression (2–4), although there is evidence that one or more classes of cone-driven ON bipolar cells may use an additional channel (3). Mutations in TRPM1 have now been positively linked to CSNB in humans as well (5–7). Taken together, studies of mouse, horse, and human provide convincing evidence that the ON bipolar cell transduction channel is composed of TRPM1, either alone or in conjunction with other channels. However, all these studies do not provide insight into the mechanism by which activation of mGluR6 leads to closure of TRPM1.

The metabotropic receptor mGluR6 preferentially couples to the G_o class of G protein, both in bipolar cells (8, 9) and expression systems (10, 11). Activation of mGluR6 and, correspondingly, Go, results in closure of TRPM1. However, it is unclear whether the G α_o subunit itself is necessary for closing TRPM1. In other examples of channel modulation by the Go class of G protein, the principle effector is the G $\beta\gamma$ dimer, rather than the α subunit (12). These channels include the G-protein-

gated inwardly rectifying K⁺ channel (GIRK), which is activated by G $\beta\gamma$ (13), and the high-voltage-activated Ca²⁺ channels, which are inhibited by G $\beta\gamma$ subunits (14, 15). This cascade is widely used by transmitter receptors, such as the GABA_B receptor, that serve to regulate the excitability of presynaptic terminals (16). Interestingly, Koike et al. (4) recently expressed the TRPM1 channel in CHO cells and reported that the channel is closed by application of exogenous G α_o , but not by the G $\beta\gamma$ dimer (17).

Because of its importance, we have revisited this question by comparing the effect of exogenous G $\beta\gamma$ and G α_o on native TRPM1 channels expressed in ON bipolar cells. Slices of mouse retina were bathed in the mGluR6 agonist L-AP4, and TRPM1 channels were transiently opened either by brief puffs of an mGluR6 antagonist or with light stimulus. We also performed experiments on TRPM1-expressing cell lines. We find that G $\beta\gamma$ subunits decreased channel opening in all cell types, whereas activated G α_o had little effect. Our findings, although different from recent reports by Koike et al. (4, 17), support a mechanism whereby activation of mGluR6 closes TRPM1 directly through the effector G $\beta\gamma$, consistent with previously established mechanisms of the activity of this dimer on other ion channels.

Results

Exogenous G $\beta\gamma$ Inhibits TRPM1 Current in RBCs. There is general agreement that activation of the mGluR6 receptor in ON bipolar cells results in closure of the downstream TRPM1 channel and that this effect is mediated by a G protein. TRPM1 is a cation-selective channel, and will therefore depolarize ON bipolar cells when it opens. One way to open TRPM1 is to use a pharmacological approach, as we have done in most of the experiments presented here. To close the TRPM1 channel, an mGluR6 agonist L-AP4, (4 μ M), was included in the bath solution. This concentration is sufficient to fully activate mGluR6 and close TRPM1. A high concentration of the mGluR6 antagonist LY34149 (100 μ M) was then “puffed” onto the dendrites of an ON bipolar cell, resulting in a transient displacement of L-AP4 from mGluR6, and the opening of TRPM1 channels. At physiological membrane potentials, the opening of TRPM1 produces an inward current, but we typically voltage clamp cells at positive voltages, as this tends to reduce run down of the response (18, 19), and so the current generated by the opening of TRPM1 is outward. An example of the TRPM1 current generated using this approach is shown in Fig. 1A. In this study, we targeted a specific class of ON bipolar cells that receive synaptic input from rods (RBCs). Morphologically, they can be distinguished by the length of their axons, which terminate in the deepest layer of the inner plexiform layer (20). An example of this type of cell, filled with Alexa 488, is also shown in Fig. 1A.

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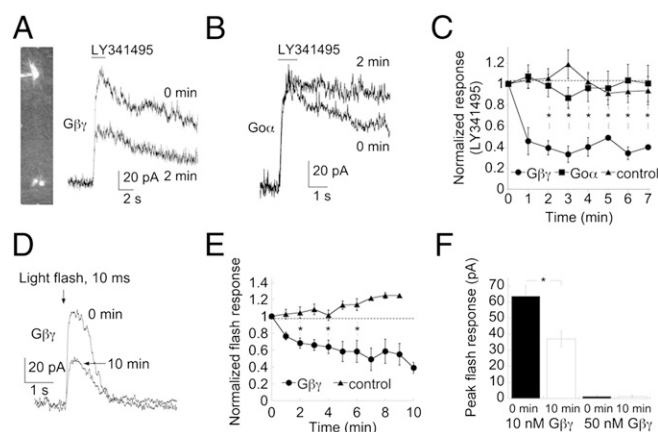


Fig. 1. TRPM1 channels in mouse RBCs are negatively controlled by G $\beta\gamma$ subunits. (A) Representative RBC response to a puff of the mGluR6 receptor antagonist, 100 μ M LY341495, when dialyzed with 50 nM G $\beta\gamma$ protein. (Upper) Break-in response. (Lower) Response at 2 min later after break-in at holding potential of +40 mV. (B) LY341495 induced response from a representative RBC that was dialyzed with 100 nM G α_o protein. Responses are shown at break-in and after 2 min of dialysis, as indicated. Note the different kinetics of the decay responses. (C) Summary of the time course of the normalized LY341495-evoked response in RBCs that were dialyzed with G $\beta\gamma$ (filled circles), G α_o (filled squares), and in control cells (filled triangles). Asterisks indicate statistical significance ($P < 0.01$) between the normalized current amplitude of cells dialyzed with G $\beta\gamma$ compared with control cells at 2–7 min. (D) Representative light-evoked response of an RBC at break-in and after dialysis with 10 nM G $\beta\gamma$; V_{hold} = +50 mV. (E) Time course of the light response (normalized to the response amplitude at break-in) in control conditions (filled triangles) and after dialysis of 10 nM G $\beta\gamma$ (filled circles). Asterisks indicate a significant difference ($P < 0.01$) between the normalized response of dialyzed cells versus the response of control cells at 2, 4, and 6 min of recording. (F) Summary of peak light-response amplitudes at break-in and after 10 min of dialysis of 10 and 50 nM G $\beta\gamma$. Although the response in the presence of 10 nM G $\beta\gamma$ showed a clear decline, no response was detected at any time point in the presence of 50 nM G $\beta\gamma$.

An important and unresolved issue in mGluR6 transduction is the mechanism by which activation of the G protein, thought to be a member of the Go family (8, 9), results in closure of TRPM1. One possibility is that the channel is closed by one or more of the G-protein subunits that couple to mGluR6. To test this we first dialyzed G $\beta\gamma$ dimers (50 nM) into RBCs through the recording pipette. After 2 min of recording, the TRPM1 current decreased by $\sim 60\%$ (Fig. 1A and C). Overall, dialysis with G $\beta\gamma$ protein reduced TRPM1 currents with an exponential time course, reaching steady state roughly 2 min after break-in. The average response decreased to $39.3 \pm 7.0\%$ of the response at break-in (Fig. 1C, $*P < 0.001$ at 2 min for G $\beta\gamma$ -dialyzed vs. control cells). The decrease of the TRPM1 response is not due to recording rundown, because we saw no such reduction in control cells that were not dialyzed with the G $\beta\gamma$ protein.

In seven other cells, all dialyzed with 100 nM G $\beta\gamma$, we did not observe a discernible response to LY341495, even at break-in, suggesting the occurrence of rapid dialysis of the subunits. Such a lack of response to LY341495 was never observed in cells that were not dialyzed with G $\beta\gamma$.

Our data are consistent with the idea that G $\beta\gamma$ can close TRPM1. However, an alternative hypothesis is that the other G-protein subunit, G α_o is responsible for closing TRPM1. TRPM1 channels expressed in CHO cells have been reported to be closed by the G α_o subunit (4, 17). In this scenario, dialysis of exogenous G $\beta\gamma$ binds to endogenous G α_o , preventing the α subunit from closing the channel, during activation of mGluR6. To test this possibility, we examined the baseline holding current in the presence of L-AP4 (i.e., when mGluR6 is activated). If

G $\beta\gamma$ dialysis prevents closure of TRPM1 during mGluR6 activation, this should be observed as a progressive increase in holding current as the G-protein subunit is dialyzed into the cell. However, the average change in holding current was in the opposite direction than the predicted change (-6.5 ± 3.8 pA) and was not significantly different from no change at all ($P = 0.11$ compared with 0 pA). These data are consistent with the idea that G $\beta\gamma$ prevents the opening of the TRPM1 channel in the presence of mGluR6 antagonists rather than interfering with its closure.

As a more direct test of the idea that the G α_o subunit closes TRPM1, we dialyzed purified G α_o protein into RBCs. G α_o protein was first activated by incubation in 100 nM 5'-guanylyl imidodiphosphate (GMP-PNP) for 30 min at 4 $^{\circ}$ C. Dialysis of activated G α_o subunit had no effect on the amplitude of the TRPM1 current (Fig. 1B and C, $P = 0.78$ for control vs. G α_o after 2 min of recording). Interestingly, G α_o had a significant effect on the rate of decay of the response to LY341495 (Fig. 1B, $P = 0.003$ compared with control cells) without any effect on rise time. The reason for this effect is unclear, but could result from the buffering of endogenous G $\beta\gamma$ by exogenous G α_o that was not bound to GMP-PNP.

As an independent approach, we tested the effects of G $\beta\gamma$ on the light-evoked RBC response. Fig. 1D illustrates the response of an RBC to a 10-ms full-field flash of 425 nm light delivering 3.6 photons/ μm^2 , a flash strength that will activate only rods (21–23). At a concentration of 10 nM, dialysis of G $\beta\gamma$ resulted in a slow depression of the light response, reaching a steady-state value of $39.0 \pm 6.5\%$ of the response at break-in after 10 min of dialysis, (Fig. 1D and E; $P = 0.003$). In contrast, the response of nondialyzed control cells showed no significant decline (Fig. 1E; response after 6 min of recording = 113.9% of the response at break-in). Dialysis of RBCs with a higher concentration of G $\beta\gamma$ (50 nM, $n = 3$, Fig. 1F) yielded no detectable light response, even at break-in, suggesting rapid dialysis of the dimer and complete inability of the channel to open.

TRPM1 Channels in HEK293 and HEM Cells Are Sensitive to Inhibition by Capsazepine. It has been suggested that capsaicin can open the TRPM1 channel in RBCs (2). As a further test of this idea, we measured responses to capsaicin as well as LY341495 in mice lacking functional TRPM1 channels (*trpm1*^{tm1Lex}). As expected, application of LY341495 did not produce a detectable response in RBCs (Fig. 2A, Left), as mGluR6 transduction is absent in this mouse model (2–4). Moreover, we were unable to detect responses to capsaicin in RBCs from *trpm1*^{tm1Lex} mice (Fig. 2A, Right), suggesting that the action of capsaicin is specific to TRPM1 in this cell type. Similarly, capsaicin fails to evoke a response in mice lacking nyctalopin, a membrane-bound protein that is required for proper expression of TRPM1 (24). The observation that TRPM1 can be activated by capsaicin is critical as it paves the way for the pharmacological isolation of TRPM1 presented below.

TRPM1 is expressed in peripheral tissue as well as in ON bipolar cells, specifically in melanocytes (25, 26). We studied the properties of TRPM1 in neonatal human ependymal melanocytes (HEMs). Using whole cell recording, we found that HEMs were unresponsive to application of capsaicin (Fig. 2C). We have shown previously that the transient receptor potential cation channel subfamily V member 1 (TRPV1) antagonist capsazepine blocks capsaicin-evoked currents in RBCs (2), providing for the possibility that capsazepine may also be an antagonist of the TRPM1 receptor. TRPM1 channels expressed in HEMs have been reported to open constitutively (25). If so, we reasoned that capsazepine might be able to elicit a current in HEMs by antagonizing TRPM1 and blocking the constitutive flow of current through the channel. In support of this idea, we observed that capsazepine reduced a standing current in the cells that were unresponsive to capsaicin. At a holding potential of +40 mV, the

standing current was outward, and so its suppression by capsazepine generated an apparent inward current. The current-voltage (I - V) relationship that was suppressed by capsazepine displayed a strong outward rectification (Fig. 2E), comparable to that obtained previously in RBCs (2). These results are consistent with the idea that, capsazepine acts to inhibit the activity of constitutively open TRPM1 channels, although the mechanism for such inhibition is unclear. However, it does not rule out the possibility that capsazepine opens or closes a channel that is distinct from TRPM1. To address this we next expressed TRPM1 in HEK293 cells. Capsazepine elicited a response in HEK293 cells that stably expressed TRPM1 (HEK-TRPM1, Fig. 2D). On the basis of the finding that capsazepine evokes a current with the same properties as TRPM1 in both HEMs and transfected, but not untransfected HEK293 cells, we propose that capsazepine reduces, or completely inhibits TRPM1 current.

Because TRPM1 has been reported to be Ca^{2+} permeable (27, 28), we tested the possibility that changes in intracellular Ca^{2+} concentration could act as a readout for the presence of open TRPM1 channels. HEM or HEK-TRPM1 cells were incubated in Fluo-3 for 30 min and transferred to nominally 0 Ca^{2+} solution

for imaging. Following addition of 5 mM Ca^{2+} to the bath, there was a readily discernible rise in intracellular Ca^{2+} levels. There are two lines of evidence that suggest that influx of Ca^{2+} through TRPM1 channels contributed to this rise. First, the magnitude of the Ca^{2+} signal in HEMs was dramatically reduced when capsazepine was present in the bath (Fig. 2F). Second, capsazepine strongly reduced the Ca^{2+} signal in HEK-TRPM1 cells (Fig. 2G), but had no significant effect in untransfected cells (data summarized in Fig. 2H). The modest effect of capsazepine on Ca^{2+} transients in untransfected cells may be due to blockade of endogenous Ca^{2+} -permeable channels, or perhaps inhibition of Ca^{2+} -activated Ca^{2+} release from stores.

Overexpression or Direct Application of G $\beta\gamma$ Subunits Closes TRPM1 in HEK293 Cells. We further examined the regulation of TRPM1 currents by G-protein subunits using a cell line that does not natively express this channel. Addition of 100 μ M GTP γ S to

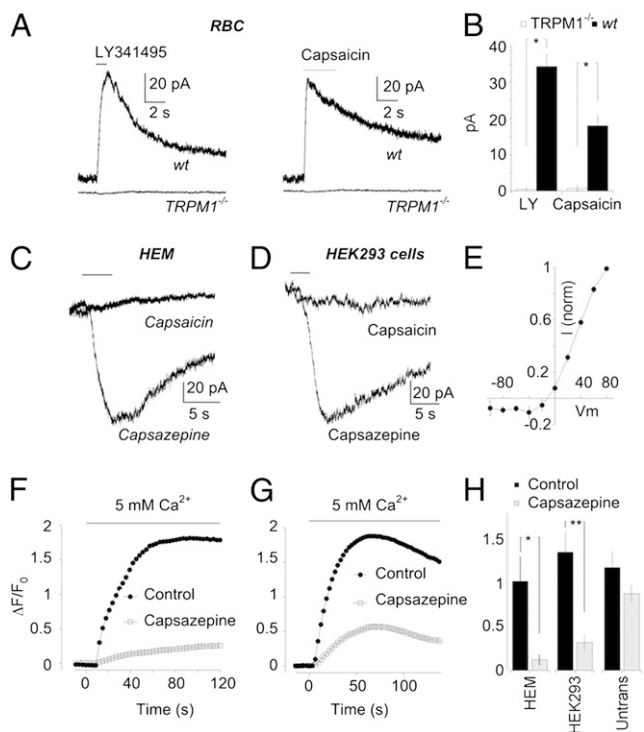


Fig. 2. Functional isolation of TRPM1 using capsazepine as a pharmacological tool. (A) Representative records of RBC responses to LY341495 (Left) and capsaicin (Right) in wild-type and *trpm1*^{tm1Lex} mice. Holding potential for all cells was +40 mV. (B) Summary of data in A. LY341495 response in wild type ($n = 64$) vs. *trpm1*^{tm1Lex} ($n = 6$). Capsaicin responses in wild type ($n = 46$) vs. *trpm1*^{tm1Lex} ($n = 11$), $*P < 0.0001$. (C) Representative responses to capsaicin and capsazepine (as indicated) in an HEM. Both traces were obtained from the same cell. (D) Similar experiment as in C using a HEK293 cell stably transfected with TRPM1 (HEK-TRPM1). (E) Averaged I - V relation obtained from three HEMs. (F) Typical example of Ca^{2+} imaging experiments showing the response of HEMs to increasing bath Ca^{2+} from 0 to 5 mM. Each trace represents changes in fluorescence intensity in an average of 5–10 cells in a single field of view in control solution (filled symbols) and in a solution containing 100 μ M capsazepine (open symbols). The same cells were used for both conditions. (G) As in F except with HEK-TRPM1 cells. (H) Summary of Ca^{2+} imaging experiments in the absence and presence of capsazepine performed on HEMs ($n = 6$ coverslips; $*P = 0.01$) and both stably transfected ($n = 7$ coverslips; $**P = 0.006$) and untransfected ($n = 8$ coverslips) HEK293 cells.

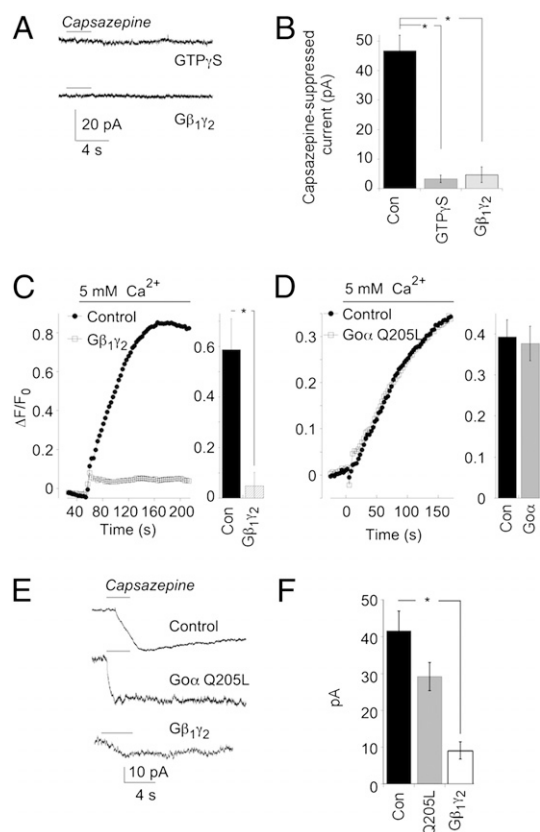


Fig. 3. Application of exogenous G $\beta\gamma$ closes TRPM1 channels. (A) Representative responses to capsazepine in an HEK-TRPM1 cell dialyzed with 100 μ M GTP γ S (Upper), and an HEM dialyzed with 100 nM G $\beta\gamma$ (Lower). (B) Summary of experiments in A. Control data are pooled from both HEK-TRPM1 and HEMs ($n = 24$ cells). Experiments with dialysis of GTP γ S ($n = 7$) were carried out on HEK-TRPM1 cells. Dialysis of G $\beta\gamma$ ($n = 3$) was carried out on HEMs. $*P < 0.0001$. (C, Left) example of a Ca^{2+} imaging experiment performed on control (filled symbols) and G $\beta\gamma$ -transfected (open symbols) HEK-TRPM1 cells. (Right) Summary of the maximum $\Delta F/F_0$ for each group (control, $n = 6$ coverslips; G $\beta\gamma$, $n = 9$ coverslips; $*P = 0.005$). (D) As in C, except that cells were transfected with G α_o Q205L. (Right) Summary of the maximum $\Delta F/F_0$ for each group (control, $n = 7$ coverslips; G α_o Q205L-transfected cells, $n = 9$ coverslips). (E) Representative responses to capsazepine from an HEK-TRPM1 cell transfected with GFP alone (Top), G α_o Q205L and GFP (Middle), and G $\beta\gamma$ and GFP (Bottom). (F) Mean \pm SEM of responses to capsazepine recorded in each condition (GFP only, $n = 18$; G α_o Q205L-transfected cells, $n = 8$; G $\beta\gamma$ -transfected cells, $n = 12$). The difference between control and G $\beta\gamma$ ($P < 0.0001$), but not G α_o Q205L ($P = 0.13$), was highly significant.

HEK293 cells that were transfected with TRPM1 mRNA resulted in the closure of TRPM1 channels, indicated by the lack of a response to capsazepine (Fig. 3*A*, *Upper*), implying that the mobilization of endogenous G protein subunits was sufficient to close TRPM1. Dialysis of G $\beta\gamma$ subunits (100 nM) also resulted in complete suppression of TRPM1 current, as there was no response to capsazepine (Fig. 3*A*, *Lower*). These results are summarized in Fig. 3*B*. Thus, dialysis of G $\beta\gamma$ subunits into both HEMs and RBCs inhibits TRPM1 function.

To examine further the role of G $\beta\gamma$ in the regulation of TRPM1, we transfected HEK-TRPM1 cells with G $\beta\gamma$ subunits. Cells were cotransfected with DsRed for the identification of G $\beta\gamma^+$ cells. In the presence of the G-protein dimer, Ca $^{2+}$ signals triggered by switching from a 0–5 mM Ca $^{2+}$ solution were markedly reduced compared with those in untransfected cells or cells transfected with DsRed alone (Fig. 3*C*). This finding is consistent with the idea that the G $\beta\gamma$ subunit closes TRPM1 and reduces Ca $^{2+}$ influx. We also tested the possibility that the G α_o subunit plays a direct role in closing TRPM1. HEK-TRPM1 cells were transfected with a constitutively activated form of G α_o (G α_o Q205L) along with DsRed. However, transfection of G α_o Q205L had no significant effect on the amplitude of Ca $^{2+}$ transients compared with cells that were untransfected, or transfected with DsRed alone (Fig. 3*D*). Interpretation of the Ca $^{2+}$ imaging experiments was supported by experiments with patch clamp recordings. TRPM1 currents were smaller in G $\beta\gamma$ -transfected cells (identified by cotransfection with EGFP) than in control cells, and this difference was highly significant ($P < 0.001$), whereas the magnitude of currents recorded in cells transfected with G α_o Q205L were not significantly different from control ($P = 0.13$; Fig. 3*E* and *F*).

Activation of an Endogenous G α_o -Independent Pathway Closes TRPM1. HEK293 cells express a variety of endogenous receptors, including the P2Y metabotropic purinergic receptors (29, 30), which couple predominantly to the Gq category of G protein (31). This class of G protein couples poorly to the mGluR6 receptor and is therefore unlikely to play a role in mGluR6 transduction cascade (10, 32). Activation of P2Y receptors should result in the liberation of endogenous G $\beta\gamma$ dimers from Gq α . We reasoned that if the G $\beta\gamma$ subunit is necessary to close TRPM1, then activation of the P2Y pathway should result in TRPM1 closure via a pathway that is independent of G α_o . To test this possibility, we first activated endogenous P2Y receptors in HEK-TRPM1 cells with 10 μ M ATP, resulting in a rise in Ca $^{2+}$ attributable to Gq α -mediated release from stores (Fig. 4*A*). This rise in Ca $^{2+}$ was prevented by 100 μ M suramin, a broad-spectrum P2 purinergic receptor antagonist, and also when stores were depleted by preincubation in 5 μ M thapsigargin. Because store released Ca $^{2+}$ would obscure the Ca $^{2+}$ transient that resulted from influx through TRPM1 channels, experiments were performed in the presence of thapsigargin. Switching from nominally Ca $^{2+}$ -free solution to a solution containing 5 mM Ca $^{2+}$ induced a rise in intracellular Ca $^{2+}$ as expected. However, when cells were exposed to 10 μ M ATP before increasing extracellular Ca $^{2+}$, the Ca $^{2+}$ transient was sharply reduced (Fig. 4*B*). When P2Y receptors were blocked with suramin, ATP had little effect on the transient response (Fig. 4*C*). Furthermore, ATP had almost no effect on Ca $^{2+}$ transients elicited in untransfected HEK293 cells (data summarized in Fig. 3*D*). These results are consistent with the idea that free G $\beta\gamma$ dimers generated by activation of Gq α are capable of closing TRPM1, thereby reducing Ca $^{2+}$ influx. Because this pathway does not couple to G α_o activation, this experiment provides further evidence that the G α_o subunit is not necessary to close TRPM1.

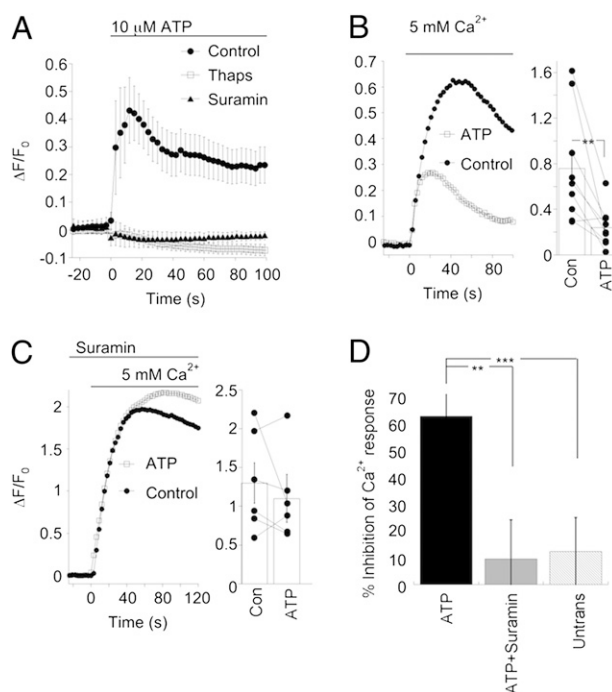


Fig. 4. Stimulation of receptors that do not signal via G α_o can modulate TRPM1 function. (*A*) Time course of the change in intracellular Ca $^{2+}$ concentration following bath application of the P2Y receptor agonist ATP alone (10 μ M, filled circles) or in the presence of the broad-spectrum P2 receptor antagonist suramin (open circles), or 5 μ M thapsigargin (filled triangles). The same cells were imaged in the absence and presence of 100 μ M suramin ($n = 6$ coverslips). Responses to ATP after treatment with thapsigargin were measured in cells from separate coverslips ($n = 9$). (*B*, *Left*) Example of an experiment comparing Ca $^{2+}$ transients evoked by increasing extracellular Ca $^{2+}$ in the absence and presence of ATP. (*Right*) Summary of individual paired experiments as well as the mean \pm SEM ($P = 0.005$, $n = 9$ coverslips). (*C*) Same experiment as in *B* except that suramin was added to the bath 2 min before the start of the experiment. In the presence of suramin, ATP did not significantly change the amplitude of the Ca $^{2+}$ transient ($P = 0.35$, $n = 6$ coverslips). Thapsigargin was added to the bath to block release from Ca $^{2+}$ stores in both *B* and *C*. (*D*) Summary of the effect of ATP on Ca $^{2+}$ influx through TRPM1 channels, expressed as percent inhibition. Ca $^{2+}$ influx was strongly reduced by ATP, but not in the presence of suramin (control; $n = 9$ coverslips, suramin; $n = 6$ coverslips, $**P = 0.01$) or in untransfected HEK293 cells ($n = 12$ coverslips, $***P = 0.006$).

Discussion

Here we provide evidence that the G $\beta\gamma$ dimer plays a critical role in coupling mGluR6 activation with closure of the TRPM1 channel downstream. We favor a role for the G $\beta\gamma$ dimer rather than its counterpart G α_o for several reasons. First, the response to the mGluR antagonist LY341495, which is believed to be mediated largely by TRPM1 (2, 3), is robustly inhibited by delivery of exogenous G $\beta\gamma$ through the recording pipette. In principle, G $\beta\gamma$ can reduce the LY341495 by inhibiting TRPM1 or by constitutively opening the channel. The latter would also reduce the response to LY341495, but through an occlusion mechanism rather than channel inhibition. However, analysis of the baseline current indicated that the inhibition was due to closure of the channel by G $\beta\gamma$, rather than as a result of the constitutive opening of TRPM1. On the other hand, we failed to see a significant effect of activated G α_o when it was delivered to RBCs under the same conditions. Second, we were able to observe clear evidence of TRPM1 channel closing in response to stimulation of an endogenous G-protein-coupled pathway that does not involve G α_o . A critical observation is that in unstimulated cells, the TRPM1 channel is open, indicating that under these

conditions, endogenous G-protein activity is too low to close TRPM1. TRPM1 closed only when G-protein activity was mobilized by the addition of GTP γ S, or by activation of the endogenous P2Y receptor. P2Y receptors are not known to couple to Go (31), providing further evidence that TRPM1 can be closed independently of G α_o .

Importantly, our findings differ from recent studies (4, 17) in which the authors concluded that it is G α_o rather than G $\beta\gamma$ that is responsible for closing TRPM1. They observed that application of G α_o to inside-out excised patches obtained from CHO cells reduced the open time of a channel believed to be TRPM1. These authors also show that glutamate suppresses a standing current in CHO cells coexpressing mGluR6, TRPM1, and G α_o , consistent with mGluR6-mediated suppression of TRPM1 current. At the present time, it is difficult to resolve the discrepancy between those studies and the findings described here. For example, the differences could result from the use of CHO cells as a model system, rather than bipolar cells. This explanation seems unlikely, however, as we observed nearly identical effects of G $\beta\gamma$ on TRPM1-L expressed in HEK293 cells and on the endogenous channel found in HEMs, as well as RBCs. There are also differences in the experimental approaches of those studies and the one described here. Most of the experiments with G $\beta\gamma$ described here made use of recombinant subunits, whereas the other studies were carried out using purified protein from brain. However, this is also unlikely to explain the difference in results, as we observed a robust effect of purified brain G $\beta\gamma$ on the light responses of RBCs.

We have previously suggested that capsazepine also antagonizes TRPM1 current in RBCs, and that capsaicin acts as an agonist (2). Recently Morgans et al. (3) reported a 50% reduction in the average response to capsaicin in ON bipolar cells of *trpm1*^{tm1Lex} mice compared with wild type, but not complete elimination of the response. In the same study, it was reported that a subset of cone-driven ON bipolar cells may express other mGluR6-coupled channels in addition to TRPM1. If these channels are also sensitive to capsaicin, this could account for a residual capsaicin response in mice lacking functional TRPM1 receptors. We have chosen to target RBCs in the present study, and were unable to observe significant responses to capsaicin in *trpm1*^{tm1Lex} mice. It is possible that the responses to capsaicin observed by Morgans et al. in *trpm1*^{tm1Lex} mice were from cone-driven bipolar cells rather than RBCs, as this issue was not specifically addressed in their study.

Our results are consistent with known mechanisms of channel modulation by transmitters that couple to the Go pathway, specifically high-voltage-activated Ca²⁺ channel (14, 15) and GIRK channels (33, 34). In both examples, modulation of channel function is mediated directly by the $\beta\gamma$ dimer, with the α subunit playing a supporting role (35–37). There are also several important differences that set G-protein regulation of the TRPM1 channel apart from other channels. GIRK channels are opened by G-protein activation, whereas the G-protein cascade activated by mGluR6 clearly closes a TRPM1. High-voltage-activated Ca²⁺ channels can be closed by G $\beta\gamma$, but this effect is voltage dependent and can be reversed by strong depolarization (35, 36), whereas G-protein inhibition of TRPM1 appears to be independent of voltage.

ON bipolar cells do not fire action potentials, and are thought to be nearly isopotential. Thus, the dendrites can be considered as an electrical extension of the axon terminal. We propose that in darkness, G $\beta\gamma$ inhibits TRPM1, which hyperpolarizes the entire ON bipolar cell, including the axon terminal. Viewed in this way, the unique action of G $\beta\gamma$ in ON bipolar cells is therefore functionally analogous to the presynaptic regulation of excitability and transmitter release observed in a number of other central synapses.

Methods

Electrophysiological Recording and Data Analysis. For recording from mouse RBCs, retinal slices were prepared from wild-type 4- to 6-wk-old C57BL/6 mice (Charles River) or *trpm1*^{tm1Lex} mice from the same background (a gift from Ronald Gregg, University of Louisville, Louisville, KY, which was generated by Lexicon Genetics and obtained from the European Mouse Mutant Archive. More information is available at <http://www.emmanet.org/>). Experiments were conducted in accordance with the National Institutes of Health Guidelines, and the protocols were approved by the institutional animal care and use committee of Albert Einstein College of Medicine.

After euthanization, whole retinas were isolated and placed on a 0.65- μ m cellulose acetate/nitrate membrane filter (Millipore), which was secured with vacuum grease to a glass slide adjacent to the recording chamber. Slices were cut to a thickness of 100 μ m using a tissue slicer (Stoelting), transferred to the recording chamber, and viewed through a Nikon E600FN upright microscope with a water-immersion 40 \times objective and differential interference contrast optics (DIC), and were continuously perfused with Ames' media bubbled with 95% O₂/5% CO₂.

Patch pipettes of resistance 7–9 M Ω were fabricated from borosilicate glass (WPI) using a two-stage vertical puller (Narishige), and filled with solution that contained 145 mM CsCl, 0.5 mM EGTA, 10 mM Hepes, 4 mM ATP, and 1 mM GTP (pH 7.4 by CsOH). The metabotropic receptor antagonist LY341495, or TRP channel reagents, were delivered to the retina from a pipette using positive pressure (2–4 psi) with a computer-controlled solenoid valve (Picospritzer; General Valve), and the mGluR6 agonist L-AP4 (4 μ M) was added to the bath. Drugs and chemicals were purchased from Sigma-Aldrich, with the exceptions of L-AP4 and LY341495 (Tocris Bioscience).

In experiments where light responses were evoked from RBCs, the dissection was carried out under infrared illumination with an infrared converter mounted to the dissecting microscope (B. E. Meyers). Flashes of light were delivered to the slice from a 100-W mercury lamp through the objective lens. The duration, intensity, and wavelength of illumination were controlled by a computer-controlled shutter, neutral density, and interference filters in the light path. The internal solution contained 100 mM Cs-gluconate, 40 mM Hepes, 2.5 mM EGTA, 4 mM ATP, and 1 mM GTP; and L-AP4 was omitted from the bath solution.

For HEK293 or HEM recordings, the internal solution was composed of 145 mM KCl, 5.5 mM EGTA, 10 mM Hepes, and 4 mM MgATP (pH 7.2, KOH). The bath solutions used for recording were 145 mM NaCl, 5.4 mM KCl, 2 mM CaCl₂, 11 mM glucose, and 10 mM Hepes (pH 7.4 with NaOH). Stocks of capsazepine and capsaicin (Sigma-Aldrich) were freshly diluted to the final concentration (50–100 μ M) and then sonicated immediately before each experiment. Recordings were obtained with an Axopatch 1D amplifier, and an ITC-18 analog-to-digital converter. Data were filtered at 2 kHz and digitized at 5 kHz. To minimize voltage errors, 70–80% of the series resistance was compensated. I–V relations were obtained by step protocols from –80 mV to +80 mV. Data were analyzed offline with Excel (Microsoft), Axograph X, and KaleidaGraph (Synergy Software).

G α_o and G $\beta_1\gamma_2$ recombinant protein were purchased from Calbiochem/EMD Chemicals and Genway Biotech (10-783-79554) and diluted from frozen stocks on the day of recording. G α_o was preactivated by incubation in 100 nM GMP-PNP for 30 min at 4 °C. G $\beta_1\gamma_2$ recombinant protein was used in experiments with HEM cells and RBCs stimulated with LY341495. For examples on light-evoked responses in RBCs, G $\beta\gamma$ protein purified from brain was used (Calbiochem/EMD Chemicals). Identical results were observed for protein obtained from both sources, so they are collectively referred to as G $\beta\gamma$ in the text and figures.

Calcium Imaging. TRPM1 activity was assessed in HEK293 and HEM cells by measuring changes in intracellular calcium ([Ca²⁺]_i). Relative changes in [Ca²⁺]_i were monitored by digital fluorescence imaging in cells loaded with Fluo-3. Cells were plated on 24-well chamber cover glass slides and studied 1–3 d after plating. Cells were loaded with 10 μ M Fluo-3, AM (Invitrogen, Molecular Probes) in extracellular solution (120 mM NaCl, 2 mM KCl, 0.5 mM CaCl₂, 1.0 mM MgCl₂, 5 mM glucose, 23 mM NaHCO₃, and 15 mM Hepes, adjusted to pH 7.4 with NaOH) for 30 min at 37 °C followed by washing in extracellular solution. During recordings extracellular Ca²⁺ was switched between 0 mM and 5 mM with or without 100 μ M capsazepine. In some experiments, 5 μ M thapsigargin was included during Fluo-3 loading to deplete calcium stores. Cells were imaged with a Retiga 2000 \times CCD camera (Qimaging) attached to an inverted Nikon fluorescent microscope equipped with a 20 \times lens. Hardware control, data acquisition, and analysis were through MetaMorph software (Molecular Devices). Calcium responses were determined by averaging the change in Fluo-3 fluorescence ($\Delta F/F_0$) in each

experiment. In a typical experiment, responses were measured in 5–8 randomly selected cells per field of view.

Transfection. HEK293 cells (ATCC) were cultured in DMEM (Gibco/Invitrogen) and supplemented with 10% FBS. Neonatal HEMs were purchased from Cascade Biologics and cultured in Medium 254 (Gibco/Invitrogen) with human melanocyte growth supplement (Cascade Biologics). Cells were cultured on glass coverslips for imaging, and patch clamp recording experiments. HEK293 cells were transiently transfected with either a full-length mouse TRPM1 cDNA plasmid fused to an HA tag (a gift from Ronald Gregg, University of Louisville, Louisville, KY) using the CalPhos Mammalian Transfection kit (Clontech) according to the manufacturer's instructions, or human TRPM1 cDNA plasmid (hTRPM1, a gift from Noga Vardi, University of Pennsylvania, Philadelphia, PA). Cells were plated at least 24 h before the transfection and grown to 50% confluence and were replaced with complete growth medium and cultured for another 24–48 h before experiments. A mouse TRPM1-expressing stable cell line (HEK-TRPM1) was established by selection

in G418-containing media (500 $\mu\text{g}/\text{mL}$ to 1 mg/mL) (Sigma). Individual colonies were isolated and TRPM1 expression was confirmed by electrophysiology and HA antibody (Covance; MMS-101P) labeling. In some experiments, HEK-TRPM1 cells were transfected with a constitutively activated $\text{G}\alpha_o$ mutant Q205 plasmid (Missouri S&T cDNA Resource Center) or a $\text{G}\beta_1$ and $\text{G}\gamma_2$ plasmid (a gift from Stephen Ikeda, National Institutes of Health, Bethesda, MD). Transfected cells were identified by cotransfection with an EGFP plasmid for electrophysiology experiments or a DsRed plasmid for imaging experiments.

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