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Persistent Sodium Currents through Brain Sodium Channels Induced by G Protein $\beta\gamma$ Subunits

Jenny Y. Ma, William A. Catterall, and Todd Scheuer*
Department of Pharmacology
Box 357280
University of Washington
Seattle, Washington 98195–7280

Summary

Persistent Na^+ currents are thought to be important for integration of neuronal responses. Here, we show that $\beta\gamma$ subunits of G proteins can induce persistent Na^+ currents. Coexpression of $G\beta_2\gamma_3$, $G\beta_1\gamma3$, or $G\beta_5\gamma_3$, but not $G\beta_1\gamma_1$ subunits with rat brain type IIA Na^+ channel α subunits in tsA-201 cells greatly enhances a component of Na^+ current with a normal voltage dependence of activation but with dramatically slowed and incomplete inactivation and with steady-state inactivation shifted +37 mV. Synthetic peptides containing the proposed $G\beta\gamma$-binding motif, Gln-X-X-Glu-Arg, from either adenylyl cyclase 2 or the Na^+ channel α subunit C-terminal domain reversed the effect of $G\beta_2\gamma_3$ subunits. These results are consistent with direct binding of $G\beta\gamma$ subunits to the C-terminal domain of the Na^+ channel, stabilizing a gating mode responsible for slowed and persistent Na^+ current. Modulation of Na^+ channel gating by $G\beta\gamma$ subunits is expected to have profound effects on neuronal excitability.

Introduction

Voltage-sensitive Na^+ channels determine the threshold for action potential generation and affect the duration and frequency of repetitive neuronal firing. In many central neurons, persistent Na^+ currents through voltage-gated Na^+ channels may play a key role in summation of synaptic inputs and control of frequency of firing (reviewed by Taylor, 1993; Crill, 1996). Persistent Na^+ currents are thought to be generated by the same Na^+ channels that open transiently during action potentials (Alzheimer et al., 1993), but the mechanism by which their activity becomes persistent is unknown.

Na^+ channels from rat brain are heterotrimeric complexes of α (260 kDa), β1 (36 kDa), and β2 (33 kDa) subunits (Catterall, 1995). Expression of the type IIA α subunit alone is sufficient to form functional voltage-gated Na^+ channels in Xenopus oocytes (Goldin et al., 1986; Noda et al., 1986; Auld et al., 1988), Chinese hamster ovary cells (Scheuer et al., 1990; West et al., 1992b), and tsA-201 cells (Eaholtz et al., 1994), but coexpression of β1 and β2 subunits modulates channel expression and gating (Isom et al., 1992, 1995a, 1995b). Brain Na^+ channels are modulated via phosphorylation by protein kinase C (PKC) and protein kinase A (Sigel and Baur, 1988; Dascal and Lotan, 1991; Numann et al., 1991; Li et al., 1992, 1993; Smith and Goldin, 1996). Phosphorylation by PKC slows Na^+ channel inactivation, but does not cause persistent Na^+ currents in hippocampal neurons or transfected cells (Numann et al., 1991; Cantrell et al., 1996).

Neurotransmitters acting at G protein–coupled receptors modulate K^+ channels and Ca^{2+} channels by a direct, membrane-delimited pathway (Hille, 1994; Wickman and Clapham, 1996), and activation of endogenous pertussis toxin–sensitive G proteins shifts the voltage dependence of activation and inactivation of Na^+ channels in hippocampal neurons and stably transfected cell lines (Ma et al., 1994). Activation of heterotrimeric G proteins by GTP binding causes dissociation of the $G\alpha$ from the $G\beta\gamma$ subunits to generate active forms that can interact with effectors. Overexpression of individual G protein subunits has been used to demonstrate direct effects of $G\beta\gamma$ subunits on inward-rectifying K^+ channels (Takao et al., 1994; Huang et al., 1995; Kunkel and Peralta, 1995) and on voltage-gated Ca^{2+} channels (Herlitze et al., 1996; Ikeda, 1996), and it is likely that the $G\beta\gamma$ subunits are the primary mediators of G protein regulation of these channels. Here, we show that coexpression of G protein $\beta\gamma$ subunits with rat brain type IIA Na^+ channel α subunits in tsA-201 cells induces a striking persistent component of Na^+ current with shifted voltage dependence of inactivation, similar to that observed in many central neurons. Peptides containing putative $G\beta\gamma$ binding motifs from adenylyl cyclase 2 (AC2) and the Na^+ channel α subunit inhibit this effect. Thus, $G\beta\gamma$ subunits may act directly on voltage-gated Na^+ channels to generate persistent Na^+ currents in neurons.

Results

Persistent Na^+ Currents Induced by Coexpression of G Protein $\beta\gamma$ Subunits

Expression of the rat brain type IIA Na^+ channel α subunit alone in tsA-201 cells resulted in Na^+ currents that activated rapidly in response to depolarization and then inactivated rapidly and virtually completely (Figure 1A, control). Activation of endogenous pertussis toxin–sensitive G proteins with GTP or GTPγS caused a time-dependent shift in the voltage dependence of channel activation and inactivation toward negative membrane potentials (unpublished data), as observed previously in neurons and in stably transfected Chinese hamster ovary cells (Ma et al., 1994). To examine the effects of individual G protein subunits, the type IIA Na^+ channel α subunit was expressed in combination with either G protein α or $\beta\gamma$ subunits, and Na^+ currents were recorded in the whole-cell voltage clamp configuration. Surprisingly, coexpression of a combination of $G\beta_2$ and $G\gamma_3$ subunits with the Na^+ channel α subunit produced Na^+ currents with dramatically slowed inactivation kinetics in most cells (Figure 1A), an effect not observed with activation of endogenous G proteins using GTPγS.

*To whom correspondence should be addressed.

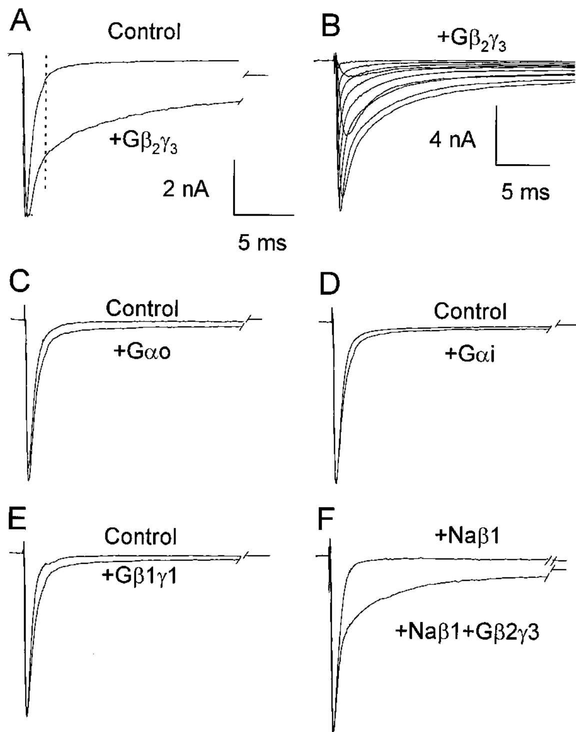

Figure 1. Na^+ Currents from tsA-201 Cells Transfected with Na^+ Channels Alone or Cotransfected with G Protein α or G Protein $\beta\gamma$ Subunits

The normalized current traces shown were evoked by depolarizations to +10 or +15 mV (A, C–F) from cells transfected with the Na^+ channel α subunit alone; cotransfected with G protein β_2 and γ_3 cDNAs (A,B), $G\alpha_{oA}$ (C), $G\alpha_{i1,3}$ (D), $G\beta_{1\gamma 1}$ (E); or cotransfected with $G\beta_{2\gamma 3}$ and the Na^+ channel β1 subunit (F). The currents in (B) were recorded in response to depolarizations ranging from −20 to +70 mV in 10 mV steps. The holding potential was −90 mV. The dotted line in (A) marks 2 ms of depolarization, the time at which inactivation-resistant current $P(I_r)$ was quantified in subsequent figures. The current after the break in the G protein subunit traces is the current at the end of the 108 ms pulse.

Inactivation was incomplete, even after a 108 ms depolarization, and Na^+ channels in tsA-201 cells cotransfected with $G\beta_2\gamma_3$ subunits inactivated slowly at all potentials (Figure 1B). The fraction of slowly inactivating Na^+ current was similar at all test potentials in the individual cells studied. This persistent Na^+ current was conducted by Na^+ channels since tetrodotoxin (50 nM) completely blocked the transient, slowly inactivating, and persistent components of current. In contrast, coexpression of plasmids encoding the pertussis toxin–sensitive G protein α subunits $G\alpha_o$ or a mixture of $G\alpha_{i1}$ and $G\alpha_{i3}$ ($G\alpha_{i1,3}$) yielded Na^+ currents, which were indistinguishable from those observed after expression of the Na^+ channel α subunit alone (Figures 1C and 1D). Thus, excess $G\beta\gamma$ subunits have a unique effect on the time course of Na^+ channel inactivation, generating persistent Na^+ currents that are not observed with other forms of Na^+ channel modulation.

Type IIA Na^+ channels in brain neurons contain β1 and β2 auxiliary subunits. The β1 subunits have important effects on Na^+ channel gating when coexpressed with type IIA α subunits in Xenopus oocytes, but only minor electrophysiological effects when coexpressed in Chinese hamster ovary cells, 1610 cells (Isom et al., 1995b), or tsA-201 cells (Scheuer and Catterall, unpublished data). To test whether the effects of coexpression of $G\beta_2\gamma_3$ subunits with Na^+ channels composed of both α and β subunits were similar to effects due to coexpression of the α subunit alone, we examined the effects of $G\beta\gamma$ subunits on Na^+ channels formed by coexpression of Na^+ channel α and β1 subunits. Such cells showed large non-inactivating Na^+ currents (Figure 1F). Thus, effects of $G\beta\gamma$ coexpression are similar whether the Na^+ channel is composed of the α subunit alone or a complex of α and β1 subunits.

Fraction of Transfected Cells with Persistent Na^+ Currents

To quantitate the effect of $G\beta_2\gamma_3$ subunits in individual cells, we measured the percentage of peak current remaining after 2 ms of depolarization during pulses to the peak of the current–voltage relationship (+10 to +20 mV; Figure 1A, dotted line). In the cells with the most rapid inactivation, the Na^+ current is nearly completely inactivated (>90%) by 2 ms at these potentials (Figures 1A–1D). Therefore, the Na^+ current remaining at this time (I_r) can be attributed primarily to slowly inactivating channels.

The percentage of Na^+ current remaining at 2 ms, $P(I_r)$, for each population of cells is presented in histogram format in Figure 2. Most cells in all experimental groups exhibited some inactivation-resistant current. However, of 42 cells recorded in the absence of coinjected G-protein subunits, 40 cells had $P(I_r)$ values of 0%–30%, and only 2 cells had $P(I_r) > 30\%$. Similarly, no cells in which $G\alpha_{oA}$ had been coexpressed and only 3 of 24 cells in which G protein $\alpha_{i1,3}$ subunits had been coexpressed had $P(I_r)$ values >30%. In contrast, 46 of 75 cells transfected with $G\beta_2\gamma_3$ had $P(I_r) > 30\%$. Thus, the percentage of cells having >30% of the Na^+ current remaining 2 ms after depolarization increased from 4.8% for control cells to 61.3% for $G\beta_2\gamma_3$-expressing cells (Figure 2F). Even if we pool the data for channels in rapidly and slowly inactivating groups of cells, the mean $P(I_r)$ was 39.0 ± 2.5% in $G\beta_2\gamma_3$-expressing cells but only 17.0 ± 1.5% in control cells, 20.0 ± 3.4% in cells expressing $G\alpha_{i1,3}$, and 14.2 ± 2.2% in cells expressing $G\alpha_{oA}$. The pooled data for $G\beta_2\gamma_3$-expressing cells are significantly different than control ($P < 0.01$, Mann-Whitney U test).

Variation in the effects of cotransfection of $G\beta\gamma$ in different cells is expected because of the uncertainty of equal cotransfection of individual cells by all of the transfected cDNAs. Nevertheless, G protein $\beta\gamma$ subunits produced a dramatic increase in slowly inactivating Na^+ current in most transfected cells, whereas no effect of coexpression of $G\alpha$ subunits was detected. Similar results for coexpression of $G\alpha$ subunits were obtained using solutions containing a high GTP/GDP ratio or containing GTPγS. Thus, these $G\alpha$ subunits do not induce persistent Na^+ currents, although the effects of other $G\alpha$ subunits remain to be tested.

To determine whether the ability to produce increased persistent Na^+ currents was specific to $G\beta_2\gamma_3$, we expressed other $G\beta\gamma$ combinations. Coexpression of $G\beta_1\gamma_3$

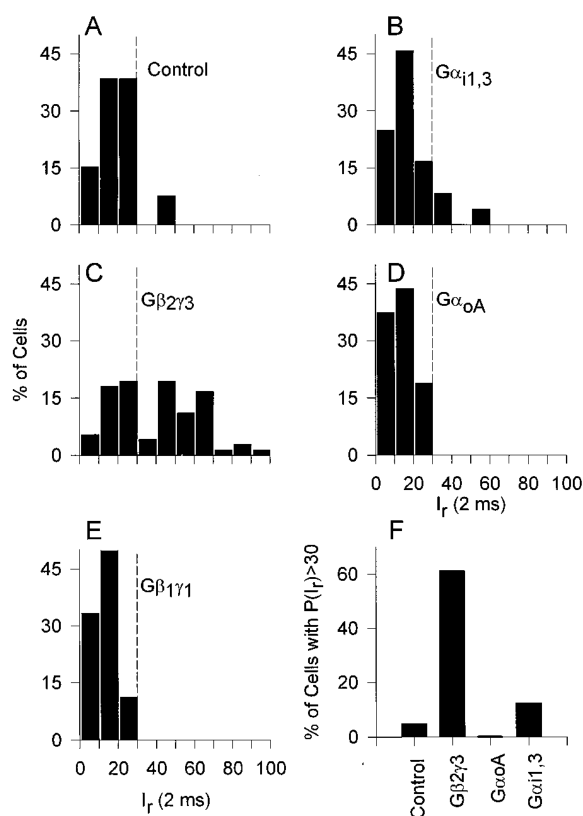

Figure 2. Effect of G Protein Subunits on Persistent Na^+ Current

(A–E) Histograms of the amount of persistent Na^+ current in each cell measured as $P(I_r)$ in control (A) or cotransfected with $G\alpha_{i1,3}$ (B), $G\beta_2\gamma_3$ (C), $G\alpha_o$ (D), or $G\beta_1\gamma_1$ (E). The dotted lines indicate $P(I_r) = 30\%$. (F) Percentage of cells in each transfection condition with $P(I_r) > 30$.

and $G\beta_5\gamma_3$ both produced cells with slowly inactivating currents like $G\beta_2\gamma_3$ ($G\beta_1\gamma_3$: $P[I_r] > 30\%$ in two of five cells; $G\beta_5\gamma_3$: $P[I_r] > 30\%$ in two of four cells). In contrast to these $G\beta\gamma$ subunit combinations, coexpression of the transducin G protein $\beta\gamma$ subunit combination, $G\beta_1\gamma_1$, had little effect on Na^+ channel kinetics (Figure 1D; $P[I_r] > 30\%$ in zero of six cells). Overall, our results indicate that multiple $G\beta\gamma$ subunit combinations (but not $G\beta_1\gamma_1$) specifically induce persistent Na^+ currents through brain Na^+ channels.

$G\beta\gamma$-Induced Persistent Na^+ Current Does Not Require Phosphorylation or Increased Intracellular Calcium

In addition to acting directly, G proteins modulate ion channels via diffusible second messengers, which cause activation of protein kinases (Hille, 1994). To prevent phosphorylation, ATP was omitted from the pipette solution in all of our experiments. The lack of requirement for ATP argues against effects of phosphorylation. Of the effects of phosphorylation reported to date, modulation by PKC most closely resembles our results, but maximum activation of PKC does not slow inactivation nearly as effectively as overexpression of $G\beta\gamma$ and does not induce persistent Na^+ currents (Numann et al., 1991; Cantrell et al., 1996). Moreover, including the specific inhibitor of PKC, $PKCI_{19-36}$ (House and Kemp, 1987) in the pipette at 10 μM had little effect on the persistent current after 15 min of intracellular dialysis (n = 6), a time at which PKC-dependent effects are blocked in whole-cell recording experiments (Cantrell et al., 1996). Effects of phosphorylation on Na^+ currents are rapidly reversible during whole-cell recording in these cells (Cantrell et al., unpublished data), indicating that endogenous phosphatases are highly active. The long-term stability of $G\beta\gamma$-induced effects that we have observed in the absence of exogenous ATP would be unlikely in the presence of active phosphatases if phosphorylation were required for the effect of $G\beta\gamma$. Thus, activation of PKC or of other protein kinases is not likely to be required for the maintenance of the persistent Na^+ current induced by $G\beta_2\gamma_3$. Increased intracellular Ca^{2+} was also not required since Ca^{2+} was chelated with 10 mM EGTA in all experiments. These results suggest that $G\beta\gamma$ does not act via phosphorylation or require maintained changes in intracellular Ca^{2+} concentration, consistent with a direct action of $G\beta\gamma$ subunits on Na^+ channel α subunits.

Characterization of the Na^+ Current Modified by G Protein $\beta\gamma$ Subunits

The unusual properties of this persistent Na^+ current were particularly evident from experiments on the voltage dependence of steady-state inactivation (Figure 3). In most control cells expressing only the Na^+ channel α subunit, Na^+ currents had a relatively negative voltage dependence of inactivation (Figure 3A, left). They were inactivated virtually completely by prepulses positive to −30 mV, and the voltage dependence of inactivation could be well described by a single Boltzmann function (Figure 3B, closed circles) with a mean half inactivation voltage, $V_{1/2}$, of −47.7 ± 1.9 mV (n = 16). In contrast, much more Na^+ current was retained after prepulses to −30 mV when $G\beta\gamma$ was coexpressed (Figure 3A, right), and steady-state inactivation curves measured in cells expressing $G\beta\gamma$ subunits had a distinctly biphasic voltage dependence (Figure 3B, closed squares), requiring the sum of two Boltzmann distributions for an adequate fit. The $V_{1/2}$ for the more negative component was −47.6 ± 0.74 mV, similar to that observed for control cells, while the $V_{1/2}$ for the more positive component was −10.9 ± 1.44 mV (n = 10) and was most prominent in $G\beta\gamma$-expressing cells. These curve fits indicate that >95% of the negatively inactivating component of Na^+ current was inactivated by prepulses to −25 mV, whereas >80% of the positively inactivating component of Na^+ current remained available. Thus, the fraction of current not inactivated by a prepulse to −25 mV (see dotted line in Figure 3B) is a good indication of the size of the positively inactivating component of current. The size of this fraction was different in each cell but was well correlated with the fraction of slowly inactivating Na^+ current in any individual cell as indicated by $P(I_r)$ (Figure 3C), suggesting that the channels inactivating at positive potentials were also responsible for the slowly inactivating, persistent Na^+ current.

This conclusion is also supported by the shape of the current traces recorded during test pulses following different conditioning depolarizations. The time course

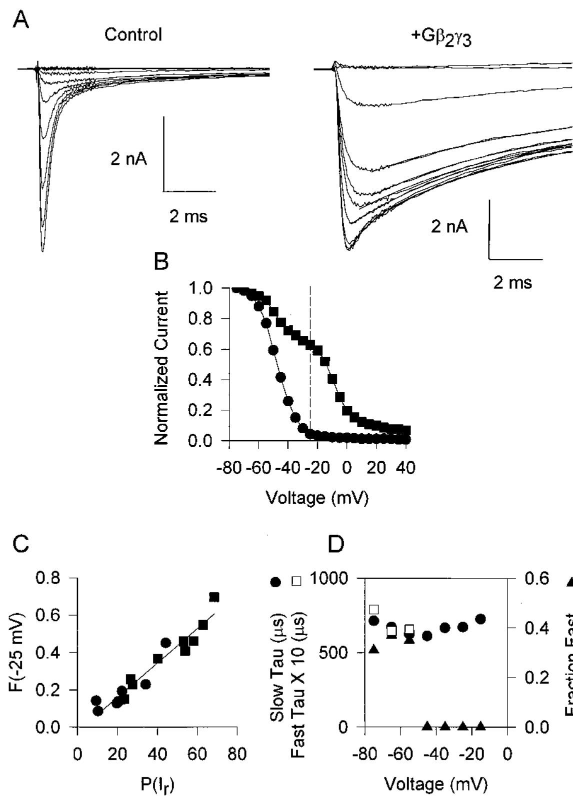

Figure 3. Inactivation Properties of Na^+ Current in Control Cells and Cells Expressing Gβγ

(A) Current traces from a control cell (left) and a $G\beta_2\gamma_3$-expressing cell (right) generated by test pulses to +15 mV following 108 ms prepulses ranging from −75 mV (largest current) to +15 mV (smallest current) in 10 mV steps. The solid lines are fits of two (−75 mV trace) or one (−45, −35, −25, and −15 mV traces) exponential(s) to the time course of inactivation of the current.

(B) Normalized steady-state inactivation curves for the control cell (closed circles) and for the $G\beta_2\gamma_3$ cotransfected cell (closed squares). Peak test pulse current is plotted as a function of prepulse potential. The curve from the control cell was well fit by a single Boltzmann distribution while the biphasic curves for the $G\beta_2\gamma_3$ cells required the sum of two Boltzmann distributions. The dotted line indicates −25 mV.

(C) Correlation of slowed inactivation with positive voltage dependence of steady-state inactivation. The fraction of current not inactivated by a prepulse to −25 mV (F [−25 mV]; see dotted line in [B]) is plotted versus $P(I_r)$ for a series of control (closed circles) and $G\beta_2\gamma_3$-coexpressing cells (closed squares). The solid line is a regression with r^2 = .93.

(D) Voltage dependence of time constants for fast and slow inactivating components and the fraction of fast inactivating component. Fits of one or two exponential(s) and a baseline to the decaying phase were made to each of the current traces in the right hand of (A). For fits to test pulses following prepulses to −75, −65, and −55 mV, the fast (open squares) and slow (closed circles) time constants (τ) are plotted as well as the fraction of the decaying current represented by the fast time constant (closed triangles). For more positive test pulses, the time constants were derived from the single exponential fits to the data because fits of two exponentials gave a poorly defined fast time constant representing <10% of the current and a well-defined slow time constant for >90% of the current. For these potentials, the residuals for fits of one and two exponentials were virtually identical, indicating that the fast component had become insignificant.

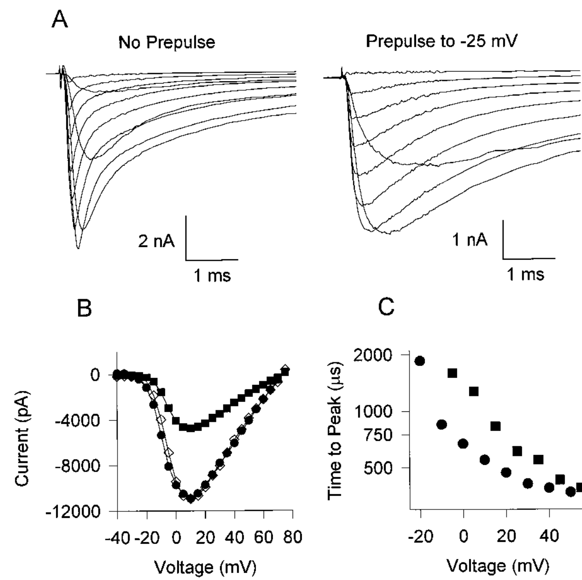

Figure 4. Activation Properties of the Total and Inactivation-Resistant Current in a $G\beta_2\gamma_3$-Expressing Cell

(A) Current traces from a $G\beta_2\gamma_3$-expressing cell elicited by test pulses ranging from −20 mV to +70 mV in 10 mV steps without a prepulse (left) and elicited by test pulses ranging from −15 mV to +75 mV in 10 mV steps preceded by 108 ms prepulses to −25 mV (right).

(B) Peak current versus voltage relationship of Na^+ currents without (closed circles) and with (closed squares) the prepulse. The open diamonds are the currents obtained with the prepulse normalized to the same magnitude as the total (no prepulse) current.

(C) Time to peak current for total current (no prepulse, closed circles) and inactivation-resistant current (−25 mV prepulse, closed squares).

of inactivation during test pulses strongly depended on prepulse potential (Figure 3A, right). The rapidly decaying component of the Na^+ current observed with the most negative prepulses was preferentially inactivated by weak depolarizations to relatively negative potentials. Much stronger depolarizations were required to inactivate the persistent component of Na^+ current. The decaying phase of the current traces following different prepulses were well fit by the sum of two exponentials (Figure 3A, right). For any individual cell, the two time constants remained relatively constant as the prepulse potential was varied (Figure 3D, closed circles, open squares). The slower rate of overall current decay with increasing prepulse potential was primarily due to a reduced fraction of current inactivating with the fast time constant (Figure 3D, closed triangles). For the particular $G\beta_2\gamma_3$-expressing cell shown in Figure 3, fits of two exponentials to the current traces following the most negative prepulses indicated that the fast time constant represented between 31% and 37% of the total current. This rapidly inactivating component disappeared when the prepulse was increased from −55 mV to −45 mV. This voltage range corresponds to the midpoint of the negative component of the two-Boltzmann fit to the inactivation curve (Figure 3B). Thus, the Na^+ current in Gβγ-expressing cells consists of two components: one that inactivates at negative potentials with voltage-dependent and kinetic properties, which resemble the major

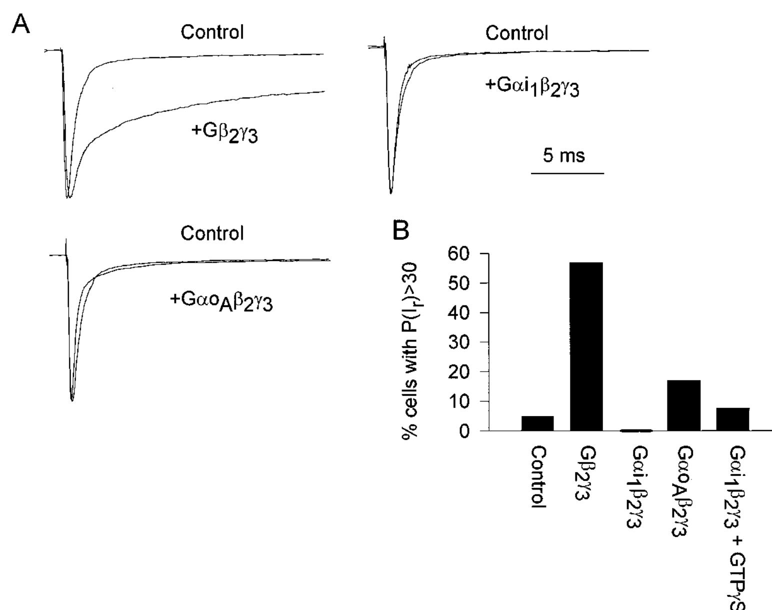

Figure 5. Coexpression of $G\alpha_i$ or $G\alpha_o$ with $G\beta_2\gamma_3$ Subunits Restores Fast Inactivation

(A) Scaled Na^+ current traces recorded from control cells and cells cotransfected with $G\beta_2\gamma_3$, $G\alpha_{i1}$ + $G\beta_2\gamma_3$, or $G\alpha_o$ + $G\beta_2\gamma_3$ without inclusion of exogenous guanine nucleotides in the pipette.

(B) Percentage of cells in each group with $P(I_r) > 30$ for experiments without or with 1 mM GTP or 0.5 mM GTPγS added to the intracellular solution. All conditions were indistinguishable from control ($P > 0.05$, Mann-Whitney U test) except for cells coexpressing $G\beta_2\gamma_3$ alone ($P < .01$, Mann-Whitney U test).

fraction of current in control cells, and one inactivating at more positive potentials characterized by slow and incomplete inactivation.

Since the slowly decaying component of Na^+ current required stronger depolarizations to inactivate it, we used prepulses 108 ms long to −25 mV to inactivate the rapidly decaying current and study the properties of the slowly inactivating Gβγ-induced current that remained in isolation (Figure 4). Such prepulses inactivate >95% of the rapidly inactivating component of Na^+ current and effectively isolate the persistent Na^+ current. The persistent current activated during a test depolarization applied following the prepulse to −25 mV had a slow, voltage-dependent inactivation time course (Figure 4A, right). The voltage dependence of activation was similar to that of the total current (Figure 4B). The time course of macroscopic activation was slowed relative to activation of the total current across a wide voltage range (Figures 4A and 4C). This slowing could be caused directly by slower activation of single channels, but could also be caused indirectly by disruption of rapid inactivation that truncates the normal activation time course, thus resulting in slower apparent activation of macroscopic currents (Gonoi and Hille, 1987). Whatever the mechanism for the effects on the macroscopic kinetics of activation, the expression of Gβγ results in a component of Na^+ current with an unaltered voltage dependence of activation but with slowed inactivation and more positive voltage dependence of inactivation.

Coexpression of Gα Subunits Blocks the Effects of Gβγ Subunits

The experiments above show that overexpression of $G\beta_2\gamma_3$ subunits greatly increases the slow component of Na^+ currents while coexpression of $G\alpha_{i1,3}$ or $G\alpha_{oA}$ subunits had no detectable effect. Gα subunits form an inactive trimer with Gβγ subunits in the absence of activation (Hepler and Gilman, 1992). Thus, coexpression of Gα subunits with Gβγ under conditions promoting their association would be expected to reduce the concentration of free Gβγ subunits and block their effects (Takao et al., 1994; Slepak et al., 1995; Ikeda, 1996; Herlitze et al., 1996). Coexpression of $G\alpha_o$ and $G\beta_2\gamma_3$ with the Na^+ channel resulted in one of six cells (17%) having $P(I_r) > 30$ compared to 61% of cells with $P(I_r) > 30$ for coexpression of $G\beta_2\gamma_3$ alone (Figure 5). Coexpression of $G\alpha_{i1}$ yielded zero of eight cells with $P(I_r) > 30$ (Figure 5). Thus, under conditions where little free Gβγ is expected because of formation of complexes with Gα subunits, no effect of Gβγ subunits was observed.

In cells coexpressing both Gα and Gβγ, inclusion of GTPγS in the pipette solution would be expected to activate G proteins and cause dissociation of the heterotrimer into its Gα and Gβγ components. Surprisingly, when $G\alpha_i$, $G\beta_2$, and $G\gamma_3$ subunits were coexpressed together with Na^+ channel α subunits and studied in the presence of GTPγS, only Na^+ currents with fast inactivation were observed (Figure 5B). The number of cells with >30% non-inactivated current at 2 ms decreased from 61% for transfection with $G\beta_2\gamma_3$ to 7.7% in cells expressing both $G\alpha_i$ and $G\beta_2\gamma_3$ studied in the presence of GTPγS. These data demonstrate that coexpression of $G\alpha_i$ subunits with Gβγ subunits prevents the effect of Gβγ, even under conditions of G-protein activation. Evidently, expression of an excess of Gβγ subunits is required to induce persistent Na^+ currents.

Block of G Protein Modulation of Na^+ Channels by Gβγ Binding Peptides

Comparison of the sequences of adenylyl cyclases having differences in regulation by Gβγ subunits identified a putative Gβγ-binding consensus sequence, Gln-X-X-Glu-Arg (Chen et al., 1995). This sequence motif is also present in the β-adrenergic receptor kinases, βARK-1 and βARK-2, phospholipase Cβ, and the G protein–gated K^+ channel GIRK-1, which all interact with Gβγ

subunits directly (Koch et al., 1994; Reuveny et al., 1994; Touhara et al., 1994; Kunkel and Peralta, 1995; Krapivinsky et al., 1995). A synthetic 27 amino acid peptide containing this motif from adenylyl cyclase 2 (peptide AC2) bound to Gβγ, and disruption of this consensus sequence in the peptides reduced that binding (Chen et al., 1995). In addition, peptide AC2 blocked Gβγ stimulation of K^+ channels, βARK, AC2, and phospholipase C-β3, evidently because the peptide bound to free Gβγ subunits at the site that would normally interact with these target proteins (Chen et al., 1995). A similar consensus sequence is present in the α_{1A} and α_{1B} subunits of brain Ca^{2+} channels, which are also modulated by Gβγ (Ikeda, 1996; Herlitze et al., 1996). Peptides containing this consensus sequence blocked modulation by Gβγ, and mutations of single amino acid residues within and adjacent to this motif in the α_{1A} subunits had strong effects on the efficiency of modulation by Gβγ (De Waard et al., 1997; Herlitze et al., 1997; Zamponi et al., 1997). Evidently, the Gln-X-X-Glu-Arg motif is broadly involved in the actions of Gβγ in multiple effector proteins including voltage-gated ion channels.

We tested the same peptide derived from AC2 for its ability to inhibit $G\beta_2\gamma_3$ effects on the rat brain Na^+ channel. The peptide was included in the recording pipette at 100 μM during analysis of Na^+ currents in cells coexpressing Na^+ channels and $G\beta_2\gamma_3$. Na^+ current traces in response to depolarization were monitored as a function of time after achieving the whole-cell configuration and beginning dialysis of the cell with the peptide. In control cells without AC2 in the pipette, the Na^+ current time course was stable during recordings up to 50 min, consistent with the stability of currents due to Na^+ channels expressed in Chinese hamster ovary cells using the same solutions (Ma et al., 1994). With peptide AC2 in the pipette, the inactivation rate of Gβγ-modified Na^+ currents became faster during the experiment, and the slowly inactivating fraction of current was reduced by 38.3 ± 2.3% (n = 6) 20 min after breaking the membrane (Figures 6A and 6B). This experiment is consistent with binding of peptide AC2 to Gβγ and resultant reversal of the slowed inactivation of the Na^+ current.

The C terminus of the Na^+ channel α subunit also contains the motif Gln-X-X-Glu-Arg (Gln-Met-Glu-Glu-Arg; amino acid residues 1878–1882 in the rat brain type IIA α subunit; [Auld et al., 1988]). We synthesized a peptide corresponding to a 25 amino acid segment of the rat brain type IIA Na^+ channel α subunit containing this motif (SPβγ) with the idea that this peptide might also bind Gβγ and inhibit its effects on Na^+ channel inactivation. When 100 μM SPβγ was included in the pipette during recording from cells expressing the Na^+ channel and $G\beta_2\gamma_3$, the slow component of current was reduced by 46.2 ± 6.5% (n = 7) during the first 20 min of recording (Figures 6A and 6B). Two control peptides were also studied. The first (SP59) was derived from a different intracellular portion of the Na^+ channel, a 28 amino acid stretch of the loop connecting homologous domains III and IV of the α subunit containing the F1489Q mutation (West et al., 1992a). When the same concentration of this peptide was included in the recording pipette, there was little effect on current time course (Figures 6A and 6B). Another control peptide, FVIII, was negatively charged like SPβγ and had similar amino acid composition (77% identical) but different primary sequence and did not contain the Gln-X-X-Glu-Arg motif. This peptide (100 μM) also had little effect (Figures 6A and 6B). The absence of effect of these control peptides is consistent with specific actions of the AC2 and SPβγ peptides by binding and reducing the concentration of free Gβγ subunits. Because the SPβγ peptide was derived from the Na^+ channel α subunit sequence and had effects consistent with binding and blocking the action of Gβγ, these experiments suggest that the Na^+ channel may be modulated by direct binding of Gβγ to the channel and that the binding site may include the C-terminal region corresponding to the SPβγ peptide.

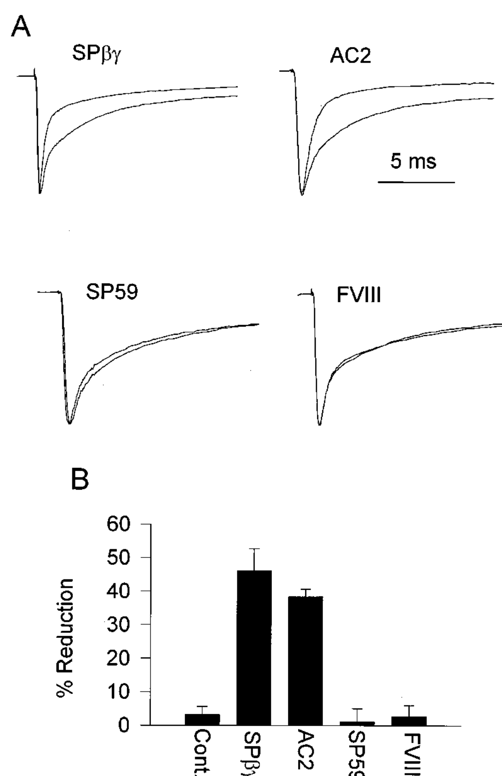

Figure 6. Effects of Intracellular Peptides Predicted to Bind Gβγ on Cells Coexpressing Na^+ Channels and $G\beta_2\gamma_3$ Subunits

(A) The normalized traces shown were recorded either immediately after breaking the cell membrane to achieve the whole-cell configuration or 20 min later with the indicated peptide in the pipette (intracellular) solution. Pulses were from a holding potential of −90 mV to test potentials of +10 or +15 mV. The peptide sequences were (in single letter amino acid code): SPβγ: DALRIQMEERFMASNPSKV SYEPIT; AC2: QEHAQEPERQYMHIGTMVEFAYALVGK; SP59: GGQD QQQTEEQKKYYNAMKKLGDKKPQK; FVIII: YEDSYEDISAYLLSKNN AIEPR. The underlined residues indicate the QXXER motif.

(B) The $P(I_r)$ at 2 ms relative to peak current was used as an indicator of Gβγ-modified channels. The percent reduction in $P(I_r)$ after 20 min of exposure to intracellular peptide is plotted for each peptide. In this series of experiments, six cells were studied with control intracellular solution; and 7, 6, 5, and 6 with intracellular solutions containing SPβγ, AC2, SP59, and FVIII, respectively.

Discussion

Gβγ Subunits Cause a Component of Persistent Na^+ Current

We show that coexpression of $G\beta_2\gamma_3$, $G\beta_1\gamma_3$, or $G\beta_5\gamma_3$ subunit combinations with rat brain type IIA Na^+ channel α subunits results in slowly inactivating, persistent Na^+ currents in tsA-201 cells. Transducin $G\beta_1\gamma_1$ was without effect as in other non-retinal tissues (Ueda et al., 1994; Wickman et al., 1994). Coexpression of $G\alpha_i$ or $G\alpha_o$ with the Na^+ channel in the presence of GTP or GTPγS had no detectable effect. However, coexpression of $G\alpha_i$ or $G\alpha_o$ subunits with $G\beta_2\gamma_3$ prevented the effects of the βγ subunits, confirming that Gα subunits were expressed at effective concentrations. Thus, Gβγ subunit combinations can uniquely induce slowly inactivating, persistent Na^+ current through brain Na^+ channels.

The effects of Gβγ subunits probably occur by modulating the properties of a single type of Na^+ channel α subunit since only one was introduced into the cells. Two functionally distinct channel populations were evident from the current time course and the voltage dependence of inactivation. However, modulation by Gβγ was reversed by the Gβγ-binding peptide, AC2, without significant change in the peak Na^+ current, arguing that Gβγ effects result from modulation of a single population of Na^+ channels.

The major effects of Gβγ are on the inactivation process. Inactivation of the Na^+ current during depolarizations is slowed and incomplete, leaving a significant persistent Na^+ current at the end of long depolarizations (Figure 1). Inactivation from closed states is also impaired, as revealed by the positively shifted voltage dependence of steady-state inactivation. Inactivation was disrupted to varying degrees in individual cells, as expected for varying levels of Gβγ subunit overexpression. It is most likely that the cells with the largest effect of Gβγ subunits reflect most closely the fully modulated state of individual Na^+ channels with bound Gβγ and that cells with smaller effects on Na^+ currents reflect a smaller fraction of Na^+ channels interacting with Gβγ. Thus, individual modulated Na^+ channels probably have highly disrupted inactivation with up to 70% persistent Na^+ current as observed in the most strongly modulated cells studied in this work.

Mechanism of Gβγ Modulation

One mechanism that may cause the observed changes in Na^+ channel function in response to Gβγ is a shift from a fast to a slow gating mode. Na^+ channels in intact cells (Patlak and Ortiz, 1986; Nilius, 1988) and cloned Na^+ channels expressed in cell lines (Ukomadu et al., 1992) exhibit multiple gating modes. Such modal behavior has been studied most extensively for Na^+ channels expressed in Xenopus oocytes, which exhibit two distinct gating modes: a predominant fast gating mode in which inactivation is rapid and complete within a few milliseconds and an infrequent slow gating mode in which activation is slightly slowed, inactivation is dramatically slowed, and steady-state inactivation is shifted to more positive membrane potentials (Krafte et al., 1990; Moorman et al., 1990; Zhou et al., 1991; Ji et al., 1994). In Xenopus oocytes, coexpression of Na^+ channel β1 subunits causes a shift from the slow to the fast gating mode (Isom et al., 1992, 1995a; Patton et al., 1994), but the effects of the β1 subunits are much less prominent in mammalian cells (Isom et al., 1995b). Because the effects of coexpression of Gβγ subunits closely resemble the effects of a shift from fast to slow gating mode, the level of free Gβγ subunits may be an important regulator of gating mode for Na^+ channels expressed in mammalian cells.

The gating mode hypothesis makes three quantitative predictions about our data. (1) The different inactivation time courses and steady-state inactivation curves observed in individual cells should be fit by sums of two exponentials and two Boltzmann curves, respectively. (2) The fraction of slowly inactivating Na^+ current should be quantitatively correlated with the fraction of positively inactivating Na^+ current in different cells. (3) Only the fraction of these two components of Na^+ current should vary among cells while the other parameters of the exponential and Boltzmann fits should remain constant. Our results fulfill all three of these predictions. First, current traces measured during depolarizations were well fit by a sum of two exponentials (Figures 3A and 7A). Second, steady-state inactivation was a biphasic function of voltage, and the fraction of current inactivating at positive potentials had a slower time course of inactivation. Inactivation curves measured in Gβγ-coexpressing cells required the sum of two Boltzmann relationships to be fit adequately (Figure 3B), and there was a strong correlation between the slowly inactivating, persistent current and the current inactivating at positive potentials (Figure 3C). Third, the fraction of the total current fit by each exponential component varied widely in 10 different cells selected for analysis (Figure 7B). In contrast, for the same 10 cells, the time constants of each of the two exponential components were similar at a given membrane potential (Figure 7C). These results show that the change in inactivation time course is entirely caused by a shift of channels from a rapidly inactivating to a slowly inactivating pool. Thus, all of our data are consistent with the total Na^+ current resulting from two underlying gating modes. One gating mode has rapid activation and inactivation as well as a negative voltage dependence of inactivation; the other has slower activation and inactivation and a more positive voltage dependence of inactivation. Expression of Gβγ increases the number of Na^+ channels in the slow gating mode.

Our results suggest that Gα and Gβγ subunits interact in an unexpected manner in regulating persistent Na^+ current. First, activation of endogenous G proteins in stably transfected Chinese hamster ovary cells and in acutely dissociated hippocampal neurons negatively shifts the voltage dependence of activation and inactivation but does not induce persistent Na^+ currents (Ma et al., 1994). Second, activation of endogenous G proteins with GTPγS did not increase persistent Na^+ currents in transiently transfected tsA-201 cells in these experiments (unpublished data), even though overexpression of Gβγ induces persistent currents. Third, coexpression of $G\alpha_i$ subunits with Gβγ subunits blocks the effect of

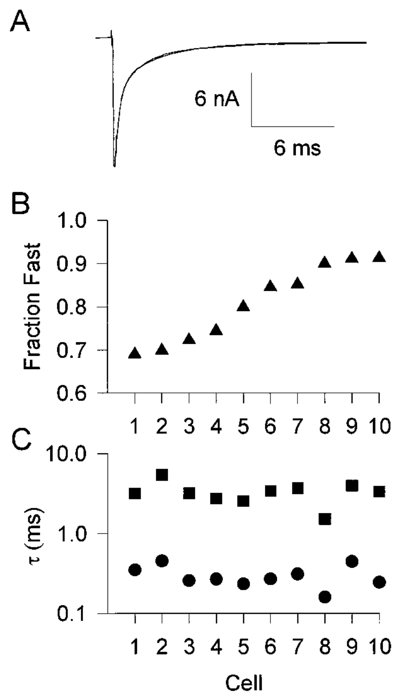

Figure 7. Least-Squares Fits to Current Time Courses in $G\beta_2\gamma_3$-Expressing Cells

Current traces during depolarizations to +30 mV were fit with two exponential components and a baseline.

(A) Example of a two exponential fit to the current after the peak in an individual cell. For this cell, the fast time constant was 349 μs, representing 69% of the current, and the slow time constant was 314 ms.

(B and C) Cells have been ranked by the fraction of current with the faster time constant (B), and the time constants of the fast (closed circles) and slow (closed squares) exponential components for the corresponding cells are plotted in (C).

Gβγ, even in the presence of GTPγS. Fourth, in contrast to these results with Na^+ channels, activation of endogenous G proteins with GTPγS in tsA-201 cells is sufficient to substantially modulate transfected P/Q-type Ca^{2+} channels containing α_{1A} subunits under essentially identical conditions (Herlitze et al., 1996). Each of these manipulations is expected to release equal numbers of Gα and Gβγ subunits. Therefore, one conceivable explanation for these data is that persistent Na^+ currents are produced by Gβγ only when there is an excess of free Gβγ subunits over free Gα subunits. Such an excess would not be produced by simply activating heterotrimeric G proteins unless Gα subunits are preferentially bound by effectors other than the Na^+ channel, leaving Gβγ subunits free. Further experiments will be required to define the interrelationship between Gα and Gβγ subunits in induction of persistent Na^+ currents.

A Peptide Derived from the Intracellular C-Terminal Domain of the Na^+ Channel α Subunit Binds Gβγ

The reversal of Gβγ modulation by the SPβγ peptide is an important clue to the molecular basis of the modulatory process. This 25 amino acid peptide was derived from a segment of the C terminus of the Na^+ channel α subunit containing the putative Gβγ binding motif Gln-X-X-Glu-Arg. Reversal of Gβγ-induced effects by peptides is thought to be caused by their binding of Gβγ subunits, thereby preventing their regulatory effects (Koch et al., 1994). Thus, the simplest interpretation of our data concerning SPβγ reversal of Gβγ effects on Na^+ channels is that this peptide also binds Gβγ. Since SPβγ is derived from the Na^+ channel C terminus, this finding suggests that this portion of the Na^+ channel C terminus can bind Gβγ and is a target for modulation of the channel by Gβγ.

Physiological Relevance of Gβγ Modulation of Brain Na^+ Channels

Slowly inactivating, persistent Na^+ currents of the type induced by Gβγ are proposed to have important physiological effects in central neurons. Non-inactivating components of Na^+ current are common features of electrophysiological recordings from brain neurons where they typically are on the order of 1%–5% of the peak Na^+ current (reviewed by Taylor, 1993; Crill, 1996). Single channel analysis of these persistent Na^+ currents indicates that they are caused by a gating mode shift of the same Na^+ channels, which conduct the transient Na^+ current (Alzheimer et al., 1993), just as we have observed for transfected type IIA brain Na^+ channels coexpressed with Gβγ in these experiments. Furthermore, the persistent Na^+ currents recorded in central neurons resemble those in transfected tsA-201 cells in having a positively shifted voltage dependence of steady-state inactivation (French et al., 1990) as well as slow and incomplete inactivation during test pulses (Taylor, 1993; Crill, 1996). Because of the large size of Na^+ currents relative to other ionic currents, a persistent Na^+ current representing only a few percent of the peak would still be substantial compared to the other ionic currents that determine the membrane potential during repolarization and between action potentials. Such persistent Na^+ currents in neuronal cell bodies would be excitatory and support bursting behavior and trains of high frequency action potentials (Alzheimer et al., 1993). Persistent Na^+ currents are also thought to be important for summation and boosting of synaptic inputs in dendrites and in this context may play a key role in determining the input–output relationships of neurons (Schwindt and Crill, 1995; Stuart and Sakmann, 1995). Examination of effects of Gβγ on persistent Na^+ currents in central neurons will require development of methods for transfecting Gβγ subunits into neurons that can subsequently be analyzed by voltage clamp as well as methods for blocking the expression of endogenous Gβγ subunits in such neurons.

Experimental Procedures

Expression of Na^+ Channels and G Protein Subunits

The tsA-201 cell subclone of HEK293 cells were maintained as described (Herlitze et al., 1996). cDNA encoding the rat brain type IIA α subunit was in pCDM8, $G\alpha_{i1}$ and $G\alpha_{oA}$ in pCD-PS, $G\alpha_{i2}$ and $G\alpha_{i3}$ in pNUT, and $G\beta_2$ and $G\gamma_3$ in pcDNA-I. Plasmids containing cDNA encoding the appropriate G-protein constructs and the rat brain type IIA Na^+ channel α subunit were cotransfected with a cDNA encoding the CD8 antigen (EBO-pCD-Leu2, American Type Culture Collection) into tsA-201 cells by $CaPO_4$ precipitation as described

previously (Margolskee et al., 1993). Cells were grown to 75% confluence in 35 mm tissue culture dishes and transfected with 4 μg of DNA containing the Na^+ channel α subunit and different G-protein subunits in a mass ratio of ~1:1. After addition of DNA, cells were incubated at 37°C in 5% CO_2. Twelve hours after transfection, the cells were removed from the dishes using 2 mM EDTA in phosphate-buffered saline, and replated at low density for electrophysiological recordings. Positive transfectants were selected visually by their binding of anti-CD8-coated beads (Dynabeads M-450 CD8; Dynal, Great Neck, NY) (Jurman et al., 1994). There was no systematic difference with transfection mixture in the percentage of cells labeled with Dynabeads.

Electrophysiological Recording

Whole-cell voltage-clamp recording was performed as described previously (Ma et al., 1994). Conductance-voltage (g–V) relationships were calculated according to $g = I/(V - V_r)$, where I was the peak current at voltage V, and V_r was the reversal potential. Normalized g–V relationships and inactivation curves were fit with a Boltzmann distribution, $1/(1 + \exp[(V - V_{1/2})/k])$, where $V_{1/2}$ was the voltage of half activation or inactivation, and *k* was a slope factor. Pooled data were reported as means ± SEM. Statistical comparisons were made using Student's *t*-test or the Mann-Whitney U test (Statistica, Statsoft, Tulsa, OK).

The standard intracellular solution contained (in mM) 120 Aspartate, 5 NaCl, 2 $MgCl_2$, 10 HEPES, 10 EGTA, and 4 MgATP, (pH 7.3) with CsOH. The external solution contained (in mM) 140 NaCl, 2 $CaCl_2$, 2 $MgCl_2$, and 10 HEPES, (pH 7.4) with NaOH. Concentrated stocks of PKCI (PKC19–36) (Peninsula Labs, Belmont, CA) and synthetic peptides were diluted immediately prior to recording. The tips of recording pipettes were filled with peptide-free internal solution to enhance seal quality and then back filled with a 100 μM solution of the desired peptide.

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