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31 DNA for microinjection was purified by two cycles of CsCl centrifugation and then linearized by Hind III and Eco RI digestion, and the fragments were isolated from a SeaPlague agarose gel (FMC, Rockland, ME) and passed through NACS columns (BRL, Gaithersburg, MD). Microinjections and transfer of fertilized eggs were performed as described (*28*). (C57BL6 × CBA)F_1 mice (Jackson Laboratory, Bar Harbor, ME) were used as stud males, embryo donors, and mature females for breeding. Outbred ICR mice (Harlan Sprague-Dawley, Indianapolis, IL) were used for vasectomized males and pseudopregnant females. Expression of each reporter was observed in three to eight founder animals with similar results. Expression of Myo1565lacZ has been analyzed in F_0, F_1, and F_2 generations with identical results (*20*). Noon of the day that vaginal plugs were detected was counted as day 0.5 p.c., with the diurnal cycle of dark extending from 7:00 p.m. to 5:00 a.m. Embryonic development was also monitored according to Theiler (*29*).
32. Embryos were stored for 30 to 90 min in ice-cold phosphate-buffered saline (PBS) containing 2% paraformaldehyde and 0.2% glutaraldehyde, rinsed twice in PBS, and then stained with X-gal (1 mg/ml), 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, and 5 mM $K_4Fe(CN)_6$ in PBS at room temperature for 12 to 16 hours. They were cleared in PBS for more than 10 hours, fixed, and stored in 4% formaldehyde (*4*).
33. Whole-mount embryos were dehydrated, cleared, and embedded in paraffin. The embryos were oriented such that the limb buds or the thoracic somites were parallel to the sections. Serial sections were cut to a thickness of 5 μm and counterstained with hematoxylin and eosin.
34 Supported by grants from NIH and the Muscular Dystrophy Association to E.N.O. and J.P.M. E.N.O. also obtained support from The Robert A. Welch Foundation. We are grateful to P. Rigby for sharing results before publication.

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Inhibition of Adenylyl Cyclase by $G_{i\alpha}$

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Evidence suggests that both α and βγ subunits of heterotrimeric guanine nucleotide–binding regulatory proteins (G proteins) inhibit adenylyl cyclase. Although type I adenylyl cyclase is inhibited directly by exogenous βγ, inhibition of adenylyl cyclase by $G_{i\alpha}$ has not been convincingly demonstrated in vitro. Concentration-dependent inhibition of adenylyl cyclases by purified $G_{i\alpha}$ subunits is described. Activated $G_{i\alpha}$ but not $G_{o\alpha}$ was effective, and myristoylation of $G_{i\alpha}$ was required. The characteristics of the inhibitory effect were dependent on the type of adenylyl cyclase and the nature of the activator of the enzyme. The concentrations of $G_{i\alpha}$ required to inhibit adenylyl cyclase were substantially higher than those normally thought to be relevant physiologically. However, analysis indicates that these concentrations may be relevant and reasonable.

The protein-protein interactions necessary for activation of adenylyl cyclase are well characterized and first involve the association of an appropriate agonist-receptor complex with the guanosine diphosphate (GDP)–bound form of the heterotrimeric G protein G_s. The receptor catalyzes exchange of GDP for guanosine triphosphate (GTP) on the G protein α subunit. Subsequently, $G_{s\alpha}$-GTP dissociates from a complex of the G protein β and γ subunits and is free to activate adenylyl cyclase (*1*). By contrast, the mechanisms that underlie hormonal inhibition of adenylyl cyclase are less well understood. Although interactions of G_i proteins with hormone-bound receptors also result in guanine nucleotide exchange and subunit dissociation, it is unclear whether the GTP-bound $G_{i\alpha}$ protein, the βγ complex, or both inhibit adenylyl cyclase and by what mechanisms they operate. Furthermore, the heterogeneity of adenylyl cyclases suggests that there may be several mechanisms by which both stimulation and inhibition can be accomplished, depending on the enzyme in question.

Exogenously added βγ inhibits adenylyl cyclase activity in platelet and S49 cell membranes, whereas activated $G_{i\alpha}$ has only a modest effect at what have been considered high concentrations (*2*). The researchers hypothesized that the mechanism of inhibition by βγ was indirect, resulting from the deactivation of stimulatory $G_{s\alpha}$. The βγ subunit complex also has a direct inhibitory effect on calmodulin- or $G_{s\alpha}$-activated type I adenylyl cyclase (*3*, *4*). (This form of adenylyl cyclase is not the type that is present in platelets or S49 cells.) However, this phenomenon is not general. For example, βγ activates type II and type IV adenylyl cyclases directly but only in the presence of activated $G_{s\alpha}$ (*5*, *6*).

Direct inhibition of adenylyl cyclase by activated $G_{i\alpha}$ proteins is the most obvious potential mechanism. However, biochemical evidence for this interaction is lacking. Brain $G_{i\alpha}$ inhibits adenylyl cyclase only modestly when this protein is tested at concentrations between 10 and 50 nM (*2*). By contrast, these proteins affect K^+ channels in the picomolar range (*7*). Recombinant $G_{i\alpha}$ proteins (from *Escherichia coli*) have no effect on adenylyl cyclase activity at 2.5 μM concentrations (*8*). Unlike their natural counterparts, these proteins are neither myristoylated nor palmitoylated (9). Nevertheless, the expression of constitutively activated $G_{i\alpha}$ proteins, but not $G_{o\alpha}$, does cause substantial inhibition of adenylyl cyclase activity (*10*). The methods used to reach this conclusion involved long-term overexpression of α, which meant that mechanisms could not be assessed and compensatory cellular reactions were difficult to exclude.

Given the availability of several newly discovered isoforms of adenylyl cyclase (including some that are expressed in nonneural tissues) and the capacity to produce myristoylated recombinant G_α proteins in *E. coli* (*11*), we have again assessed the possibility of direct interactions between $G_{i\alpha}$ and adenylyl cyclase. Type V adenylyl cyclase was expressed in Sf9 cells with recombinant baculovirus, and the enzyme in Sf9 cell membranes was activated with either half-maximally effective concentrations of recombinant GTP-γ-S–$G_{s\alpha}$ (Fig. 1) or forskolin (Fig. 2). Concentration-dependent inhibition of adenylyl cyclase activity was observed on addition of either activated $G_{i\alpha}$ from bovine brain (a mixture of isoforms, predominantly $G_{i\alpha1}$) (Fig. 1) or activated, myristoylated recombinant $G_{i\alpha1}$ (from *E. coli*) (Figs. 1 and 2). In the latter case the inhibition was saturable; the concentration required for 50% inhibition (IC_{50}) was approximately 100 nM. Boiled protein did not elicit the inhibitory response. Myristoylated recombinant $G_{o\alpha}$ and nonmyristoylated recombinant $G_{i\alpha1}$ were ineffective. The GDP-bound form of myristoylated recombinant $G_{i\alpha1}$ was similarly inactive (*12*). The inhibitory effects of myristoylated recombinant $G_{i\alpha2}$ and $G_{i\alpha3}$ were indistinguishable from those of myristoylated recombinant $G_{i\alpha1}$ (*12*). The inhibition by activated myristoylated $G_{i\alpha1}$ of both $G_{s\alpha}$- and forskolin-stimulated adenylyl cyclase indicates that this inhibition does not result only from competition with $G_{s\alpha}$

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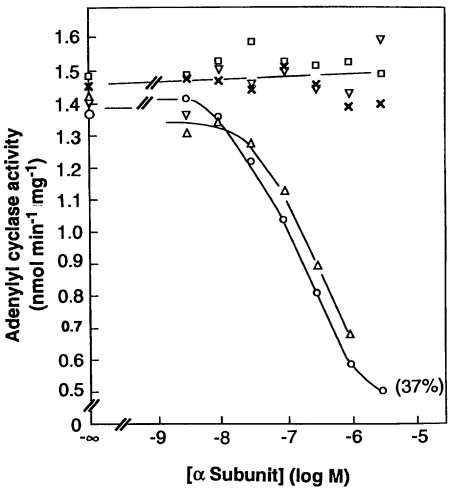

Fig. 1. Inhibition of $G_{s\alpha}$-stimulated adenylyl cyclase type V by $G_{i\alpha}$. Membranes were prepared from Sf9 cells (*4*) infected with baculovirus-encoding type V adenylyl cyclase (*21*) and were incubated for 3 min with activated $G_{s\alpha}$ (derived from *E. coli*) and various concentrations of brain $G_{i\alpha}$ (triangles), myristoylated recombinant $G_{i\alpha 1}$ (circles), myristoylated recombinant $G_{o\alpha}$ (squares), nonmyristoylated recombinant $G_{i\alpha 1}$ (inverted triangles), or boiled myristoylated recombinant $G_{i\alpha 1}$ (X's). Adenylyl cyclase assays were then done for 5 min as described by Smigel (*22*), except that the concentration of $MgCl_2$ was 4 mM and assays contained 10 μg of membranes, 50 nM $G_{s\alpha}$, and 0.01% Lubrol ($C_{12}E_{10}$). All G protein α subunits were activated by incubation with 200 μM GTP-γ-S as described in (*8, 23*). Procedures for purification of brain $G_{i\alpha}$ and α subunits derived from *E. coli* have been described (*8, 11, 23, 24*). All determinations were performed in duplicate and are representative of at least two experiments. The concentration of α subunits refers to the final value in the assay. The number in parentheses is the maximal inhibition observed, expressed as a percent of the control value in the absence of myristoylated recombinant $G_{i\alpha 1}$.

(Fig. 2). It was not possible to test the effect of brain $G_{i\alpha}$ on forskolin-activated type V adenylyl cyclase because of small amounts of $G_{s\alpha}$ in the preparation; $G_{s\alpha}$ (at picomolar concentrations) and forskolin activate type V adenylyl cyclase synergistically.

We cannot explain why $G_{i\alpha 1}$ must be myristoylated for its inhibitory effect on adenylyl cyclase to be observed. However, myristoylation of members of the $G_{i\alpha}$-$G_{o\alpha}$ family increases their affinities for G protein βγ subunits in membranes or in detergent-containing solutions (*11*). Binding studies will be necessary to see if this modification also affects the interaction between $G_{i\alpha}$ and adenylyl cyclase.

We tested whether inhibition of adenylyl cyclase by $G_{i\alpha 1}$ was specific for particular isoforms of the enzyme. Three different membrane preparations were used: Sf9 cell membranes containing either type I or type V adenylyl cyclase and membranes from cyc^- ($G_{s\alpha}$-deficient) S49 cells. Although S49 cells probably contain a mixture of adenylyl cyclases, they were studied because much work has been done with this system. Myristoylated recombinant $G_{i\alpha 1}$ inhibited $G_{s\alpha}$-activated, adenylyl cyclase activity in Sf9 cell membranes containing type V adenylyl cyclase and in cyc^- membranes (Fig. 3). Although saturating concentrations were not achieved, it appears that the inhibitory G protein α subunit was a more effective inhibitor of the S49 cell adenylyl cyclases than was the type I or type V enzyme.

Although myristoylated $G_{i\alpha 1}$ inhibited $G_{s\alpha}$-activated type V and S49 cell adenylyl cyclases by more than 60%, the $G_{s\alpha}$-activated type I enzyme was inhibited by only 10%. The inhibition of type I adenylyl cyclase was more prominent when calmodulin or forskolin was the activator (Fig. 4). This effect of $G_{i\alpha}$ was observed at lower concentrations (IC_{50} = 20 to 30 nM) than was the inhibition of the type V enzyme. Thus, calmodulin-stimulated type I adenylyl cyclase is inhibited by both $G_{i\alpha}$ and βγ, whereas the $G_{s\alpha}$-stimulated enzyme is inhibited predominantly by βγ and not by $G_{i\alpha}$ (*3*). The inhibition of adenylyl cyclase activity by $G_{i\alpha}$ seems dependent on both the type of adenylyl cyclase and the activator. This dependence enhances the complexity of modulation of adenylyl cyclase activity in cells that are exposed to convergent regulatory inputs.

Three factors appear to have prevented the observation of substantial inhibition of adenylyl cyclase by $G_{i\alpha}$: the concentrations of G protein tested, the G protein preparation, and the nature and activator of the adenylyl cyclase. The concentrations of brain $G_{i\alpha}$ that were used previously (*2*) were limited by the availability of protein, minor contamination by $G_{s\alpha}$ (which is active at picomolar concentrations), and the expectation that the relevant concentration of $G_{i\alpha}$ would approximate that of $G_{s\alpha}$ (although cellular concentrations of $G_{i\alpha}$ are much higher than those of $G_{s\alpha}$). Furthermore, indirectly elicited inhibitory effects of βγ were more prominent than those of $G_{i\alpha}$ (*2*). Recombinant $G_{i\alpha}$ protein was used to achieve higher concentrations of defined isoforms of $G_{i\alpha}$ (2.5 μM) and to obviate the problem of contamination by $G_{s\alpha}$ (*8*). However, these proteins were not myristoylated, an important modification (*13*). Finally, many experiments were done with $G_{s\alpha}$-activated type I adenylyl cyclase, which is largely unresponsive to inhibition by $G_{i\alpha}$.

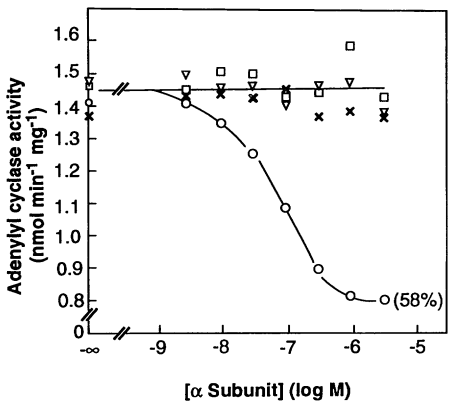

Fig. 2. Inhibition of forskolin-stimulated adenylyl cyclase (type V) by $G_{i\alpha}$. Adenylyl cyclase activity was measured in the presence of myristoylated recombinant $G_{i\alpha 1}$, myristoylated recombinant $G_{o\alpha}$, nonmyristoylated recombinant $G_{i\alpha 1}$, or boiled myristoylated recombinant $G_{i\alpha 1}$; symbols as in Fig. 1. All assays were done as described for Fig. 1 except that GTP-γ-S–$G_{s\alpha}$ was omitted and 50 μM forskolin was added at the time the assay was initiated.

Why are such high concentrations of $G_{i\alpha}$ required to observe these effects? How can the inhibition of adenylyl cyclase by nanomolar concentrations of $G_{i\alpha}$ be relevant if K^+ channels in the same cell (for example, atrial cardiomyocytes) are activated by picomolar concentrations of the G protein and if similar concentrations of acetylcholine elicit both responses (*14, 15*)? We hypothesize that high nanomolar concentrations of $G_{i\alpha}$ are both relevant and reasonable for several reasons. First, the concentration of G_i at the plasma mem-

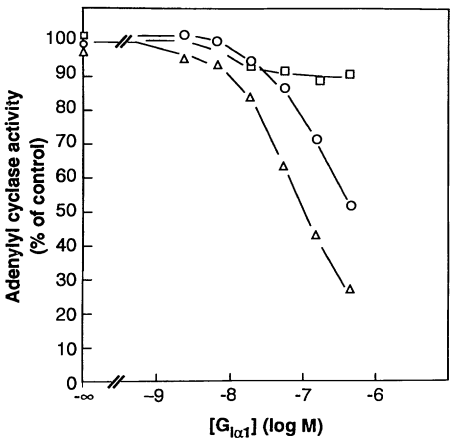

Fig. 3. Type-specific inhibition of adenylyl cyclase by myristoylated recombinant $G_{i\alpha 1}$. Adenylyl cyclase activity of membranes prepared from Sf9 cells expressing the type I (squares) (*3*) or type V (circles) enzyme or membranes prepared from cyc^- S49 cells (triangles) (*25*) was measured in the presence of 50 nM GTP-γ-S–$G_{s\alpha}$. All assays were performed as in Fig. 1 and contained 10 μg of Sf9 cell membranes or 50 μg of cyc^- cell membranes. The activities are expressed as a percent of the activity measured in the absence of myristoylated recombinant $G_{i\alpha 1}$. Control values for type I, type V, and cyc^- membranes were 2.8, 1.4, and 0.67 nmol min^{-1} mg^{-1}, respectively.

brane may exceed 1 mM (*16*), and immunohistochemical studies suggest that $G_{i\alpha}$ localizes nonhomogeneously to patches within the plasma membrane (*17*). Thus, local concentrations of the protein may be high, and distinct effectors could be exposed to different concentrations of $G_{i\alpha}$, depending on their position relative to such patches.

Estimates of the concentrations of G protein α subunits necessary for interaction with effectors are almost always made with GTP-γ-S–activated protein. Under this condition, the G protein α subunit cannot deactivate itself by its intrinsic guanosine triphosphatase (GTPase); the rate constant for catalysis of GTP hydrolysis (k_{cat}) is 0. We assume that the concentration of G_α substantially exceeds that of the effector; this is almost certainly true for $G_{i\alpha}$, adenylyl cyclase, and the relevant K^+ channels. Because k_{cat} is greater than 0 when GTP is the activating ligand, the concentration of activated G protein is reduced by deactivation and more G_α-GTP is required to activate the effector. Thus, the concentration curve for interaction of GTP-$G_{i\alpha}$ with the effector would be shifted to the right compared to that for GTP-γ-S–$G_{i\alpha}$. The concentration of G protein necessary to activate an effector half-maximally ($K_{app,50}$) is a function of both the actual dissociation constant (K_d) for the interaction and k_{cat}. Furthermore, phospholipase C-β1 serves as a GTPase-activating protein (GAP) on $G_{q\alpha}$ (*18*). If two effectors are controlled by the same G protein, one might act as a GAP but the other need not (*18*). Thus, it seems possible that values of k_{cat} differ, if one considers the interactions of the same G protein α subunit with two different effectors. The rapid kinetics of deactivation of cardiac K^+ channels after activation by acetylcholine suggests that the channel may have GAP activity, accelerating the GTPase activity of $G_{i\alpha}$ by 75- to 100-fold (*14*).

A speculative model describes the interaction of GTP-G_α with an effector, *E*

$$G_\alpha - GTP + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} G_\alpha - GTP - E \xrightarrow{k_{cat}} G_\alpha - GDP + E$$

which is described by the equation

$$K_{app,50} = K_d + \frac{k_{cat}}{k_1}$$

It is assumed that the concentration of G_α substantially exceeds that of the effector and that the effector may stimulate the hydrolysis of GTP to GDP by G_α. On the basis of this model, computer simulations examining the effect of k_{cat} on $K_{app,50}$ for high- and low-affinity effectors were performed and are shown in Fig. 5. The only other factor to be considered is k_1, the rate constant for association of G_α with effector. A large value of k_1 (10^9 M^{-1} min^{-1}, consistent with diffusion) has been assumed; smaller values magnify the effect. The effect of k_{cat} is substantial for high-affinity (low-K_d) interactions between G_α and effector and becomes smaller as the affinity of G_α for effector decreases. The $K_{app,50}$ is 10 nM (a 100-fold shift) if K_d is 0.1 nM, k_{cat} is 10 min^{-1}, and k_1 is 10^9 M^{-1} min^{-1}; however, $K_{app,50}$ is 110 nM (a 10% change) if K_d is 100 nM and k_{cat} and k_1 are unchanged. Thus, most of the apparent discrepancy among concentrations of GTP-γ-S–$G_{i\alpha}$ required to activate K^+ channels and inhibit adenylyl cyclases disappears when one considers the case of GTP-$G_{i\alpha}$, the physiological regulator. If the K^+ channel has a significant GAP activity on GTP-$G_{i\alpha}$ but adenylyl cyclase does not, the remaining difference will also disappear. Adenylyl cyclase does not appear to have GAP activity on $G_{s\alpha}$ (*19*), and it seems reasonable that $G_{i\alpha}$ would also be immune to this effect.

Some of the corollaries of this hypothesis can be summarized as follows: (i) Relevant (intracellular) concentrations of G_α subunits are underestimated during examination of the effects of GTP-γ-S–G_α on effectors. The underestimation is substantial for high-affinity interactions and becomes less significant as affinity decreases (because the system can then deactivate by dissociation of GTP-G_α-E rather than by hydrolysis of GTP to GDP). (ii) If the system is designed to regulate two effectors with a single G_α (at similar concentrations) and only one of these effectors is deactivated rapidly because of GAP activity, then the intrinsic affinity of the GAP effector for G_α must be high relative to the affinity between G_α and the nonGAP effector. In other words, the affinity of adenylyl cyclase for $G_{i\alpha}$ would have to be low if the K^+ channel is a GAP. (iii) The same considerations apply to concentration requirements for simultaneous modulation of an effector by G_α proteins and other regulators

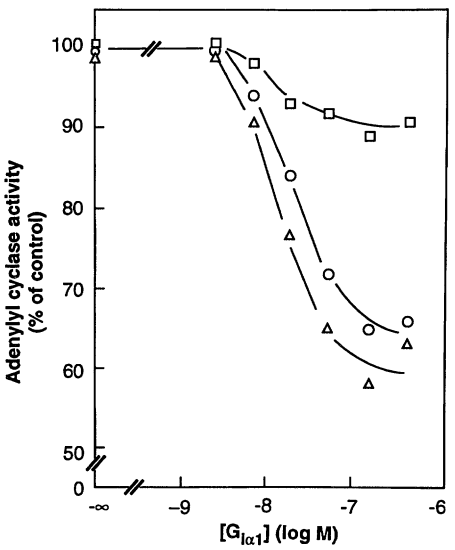

Fig. 4. Inhibition of type I adenylyl cyclase by myristoylated recombinant $G_{i\alpha1}$. Adenylyl cyclase activity of membranes from Sf9 cells expressing type I adenylyl cyclase was measured in the presence of increasing concentrations of myristoylated recombinant $G_{i\alpha1}$ and 50 nM GTP-γ-S–$G_{s\alpha}$ (squares), 50 μM forskolin (circles), or 50 nM calmodulin (triangles). Assays were performed as in Fig. 1 except that 2.5 mM $CaCl_2$ and 2.5 mM EGTA were included during the first 3-min incubation when activation by calmodulin was assessed. Activities are expressed as the percent of the activity measured in the absence of myristoylated recombinant $G_{i\alpha1}$ for each of the activators tested. Control values for type I adenylyl cyclase activity in membranes activated with GTP-γ-S–$G_{s\alpha}$, forskolin, and calmodulin were 2.8, 3.5, and 3.2 nmol min^{-1} mg^{-1}, respectively.

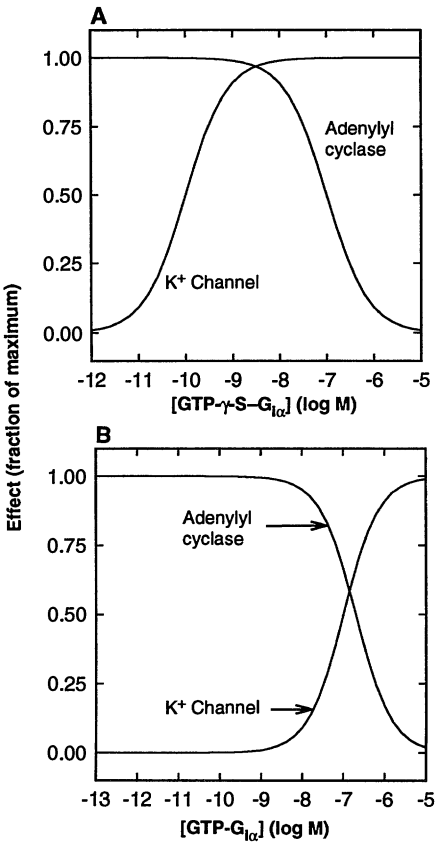

Fig. 5. Effect of GTPase activity of G proteins on concentration requirements for GTP-$G_{i\alpha}$. (**A**) Dose-response curves for two effects of GTP-γ-S–$G_{i\alpha}$. The apparent affinity of GTP-γ-S–$G_{i\alpha}$ for K^+ channels is approximately 10 to 100 pM, whereas inhibition of adenylyl cyclase requires 20 to 100 nM concentrations of the α subunit. (**B**) Simulated dose-response curves for GTP-$G_{i\alpha}$. Curves were calculated with the following values: for interaction of GTP-$G_{i\alpha}$ with K^+ channels, K_d = 0.1 nM, k_{cat} = 100 min^{-1}, and k_1 = 10^9 M^{-1} · min^{-1}; for interaction of GTP-$G_{i\alpha}$ with adenylyl cyclase, K_d = 100 nM, k_{cat} = 10 min^{-1}, and k_1 = 10^9 M^{-1} · min^{-1}.

that cannot deactivate themselves, such as G protein βγ subunits. It has been argued that the effects of βγ on cardiac K^+ channels (*20*) and phospholipase C-β occur at high, physiologically irrelevant concentrations when compared with the effects of GTP-γ-S–G_α. Half-maximal stimulation of phospholipase C-β by GTP-γ-S–$G_{q\alpha}$ occurs at concentrations of approximately 300 pM. It is estimated that phospholipase C-β increases the k_{cat} for hydrolysis of GTP by $G_{q\alpha}$ approximately 100-fold to a value greater than 80 min^{-1} (*18*). These values (on the assumption that $k_1 = 10^9$ M^{-1} min^{-1}) yield a $K_{app,50}$ greater than 80 nM, which is similar to the concentration of βγ required to activate phospholipase C-β. Thus, the necessary concentrations of GTP-G_α and βγ may be comparable. (iv) High values of $K_{app,50}$ (compared to the low values of K_d obtained with GTP-γ-S–G_α) are consistent with the high concentrations of $G_{i\alpha}$, and in particular $G_{o\alpha}$, found in many cells. We suggest that the affinities of activated $G_{o\alpha}$ for its effectors are low, that these effectors are GAPs, or that both of these conditions exist.

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mSlo, a Complex Mouse Gene Encoding "Maxi" Calcium-Activated Potassium Channels

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Complementary DNAs (cDNAs) from *mSlo*, a gene encoding calcium-activated potassium channels, were isolated from mouse brain and skeletal muscle, sequenced, and expressed in *Xenopus* oocytes. The *mSlo*-encoded channel resembled "maxi" or BK (high conductance) channel types; single channel conductance was 272 picosiemens with symmetrical potassium concentrations. Whole cell and single channel currents were blocked by charybdotoxin, iberiotoxin, and tetraethylammonium ion. A large number of variant *mSlo* cDNAs were isolated, indicating that several diverse mammalian BK channel types are produced by a single gene.

Calcium-dependent K^+ channels [K(Ca) channels] are associated with a broad spectrum of cell physiology, including bursting in neurons (*1*), secretion in endocrine and exocrine cells (*2*), contraction in muscle cells (*3*), activation of T cells (*4*), and the regulation of myogenic tone in arterial smooth muscle (*5*). Although all K(Ca) channels are dependent on Ca^{2+} for their activation, some are synergistically activated by voltage and Ca^{2+}, whereas others are insensitive to voltage (*6*). K(Ca) channels from different cells vary widely in their conductance, regulation, and sensitivity to Ca^{2+} and voltage. This diversity may reflect the existence of a multigene family encoding homologous channel proteins or a mechanism such as alternative RNA splicing that produces variant channels from a single gene. Both mechanisms generate the diversity of purely voltage-dependent K^+ channels (*7*). We show here that in the mouse, many variant K(Ca) channel peptides are produced by a single gene.

The *Drosophila slo* gene (*8–10*) encodes K(Ca) channels present in both neurons (*11*) and muscle (*8, 12*). To isolate a mammalian homolog of *slo*, we first isolated a *Drosophila slo* cDNA. Polymerase chain reaction primers based on published sequence (*9*) were used to amplify *slo* gene fragments directly from genomic DNA. Two ~200–base pair (bp) fragments representing either end of hydrophobic segments S1 to S6 (Fig. 1A) were generated and used as hybridization probes to isolate a *slo* cDNA containing a large portion of the coding region. A 1300-bp fragment spanning the S1 to S6 region was then used as a probe for hybridization to a mouse brain cDNA library (Clontech, Palo Alto, California) under low stringency conditions (*13*). Clone mbrl was found to contain a single open reading frame encoding a peptide homologous to the *Drosophila* slo protein from amino acid 96 to 448 (Fig. 1C). The mbrl clone was then used as a hybridization probe for the isolation of *mSlo* cDNAs from both mouse brain and skeletal muscle cDNA libraries.

The deduced translation product of *mSlo* (mSlo) displays extensive sequence conservation with the *Drosophila* slo protein (Fig. 1). Similar to slo, as well as the extended family of voltage-dependent K^+ channels (*7*), mSlo contains six hydrophobic segments (S1 to S6; Fig. 1) that are presumed to span the membrane and surround the pore

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