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G Protein Regulation of Potassium Ion Channels

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I. Introduction

Upon stimulation of vagal nerves, acetylcholine (ACh)[c] is released from axonal terminals and decelerates the heart beat. This historic discovery by Otto Loewi in the 1920s established the concept of chemical synaptic transmission (Loewi, 1921; Loewi and Navaratil, 1926). Since then, many physiologists have been trying to elucidate the mechanism(s) underlying neurotransmitter (Vagusstoff)-induced bradycardia. Del Castillo and Katz (1955) first described hyperpolarization of the membrane induced by ACh in frog heart. Hutter and Trautwein (1955) measured an increase of K^+ efflux across the cardiac cell membrane with vagal stimulation. Trautwein and Dudel (1958) showed an increase of K^+ conductance under voltage-clamp conditions. Trautwein and colleagues analyzed the kinetics of the ACh-induced K^+ current in the rabbit sinoatrial node and proposed that ACh induces activation of a specific population of K^+ channels, named muscarinic K^+ (K_{ACh}) channels, to decelerate pacemaker activity (Noma and Trautwein, 1978; Osterrieder *et al.*, 1981). The single channel currents of the K_{ACh} channels were recorded for the first time by Sakmann *et al.* (1983), who showed that the channel exhibited kinetic properties that clearly differed from those of the background inwardly rectifying K^+ (I_{K1}) channel in cardiac myocytes.

The next big step was the discovery that pertussis toxin (PTX)-sensitive heterotrimeric G proteins are involved in the activation of the K_{ACh} channel by M_2-muscarinic and A_1 adenosine receptors (Pfaffinger *et al.*, 1985; Breitwieser and Szabo, 1985; Kurachi *et al.*, 1986a and b). Because the K_{ACh} channel could be activated by intracellular guanosine 5′-triphosphate (GTP) (in the presence of agonists) and GTPγS (even in the absence of agonists) in cell-free inside-out patches, the system seemed to be delimited to the cell membrane, which led to the proposal that the channel is directly activated by G proteins (Kurachi *et al.*, 1986a,b,c). The G protein responsible for activation of the K_{ACh} channel was designated G_K according to its function (Breitwieser and Szabo, 1985).

It was quite a surprise that the $\beta\gamma$ subunit ($G_{\beta\gamma}$) but not the α subunit (G_α) of the G_K protein, was proposed to mediate the G_K-induced activation of K_{ACh} channels (Logothetis *et al.*, 1987, 1988; Kurachi et al, 1989a), because it was strongly believed at that time that regulation of different effectors by G proteins was mediated only by G_α, although $G_{\beta\gamma}$ merely served to bind to the GDP-form of G_α ($G_{\alpha\text{-GDP}}$) to anchor the trimeric G protein to the cell membrane (Gilman, 1987). Actually, Brown, Birnbaumer, and their colleagues proposed $G_{K\alpha}$ and not $G_{K\beta\gamma}$ as the physiological activator of K_{ACh} channels (Yatani *et al.*, 1987, 1988; Codina *et al.*, 1987; for review see Brown and Birnbaumer, 1990). The dispute concerning the G protein subunit responsible for the physiological activation of K_{ACh} channels continued for nearly a decade (Ito *et al.*, 1992; Yamada *et al.*, 1993, 1994a,b; Nanavati *et al.*, 1990; Kurachi, 1989, 1990, 1993, 1994, 1995; Kurachi *et al.*, 1992; Clapham and Neer, 1993; Wickman and Clapham, 1995) until the functional interaction between the channel and $G_{\beta\gamma}$ was

[c] Abbreviations: ACh, acetylcholine; K_{ACh} channel, muscarinic K^+ channel; I_{K1}, the background inwardly rectifying K^+ channel in cardiac myocytes; PTX, pertussis toxin; GTP, guanosine 5′-triphosphate; GTPγS, guanosine 5′-O-(3-thiotriphosphate); G_K, the heterotrimeric G protein responsible for the physiological activation of the K_{ACh} channel; $G_{\beta\gamma}$, $\beta\gamma$ subunits of G protein; G_α, α subunits of G protein; $G_{\alpha\text{-GDP}}$, the GDP-bound form of G_α; $G_{K\beta\gamma}$, $\beta\gamma$ subunits of G_K; $G_{K\alpha}$, α subunits of G_K; K_G channel, G protein-gated K^+ channel; G_β, β subunits of G protein; G_γ, γ subunits of G protein; $G_{\alpha\text{-GTP}}$, the GTP-bound form of G_α; RGS, G protein signaling protein; βARK, β-adrenergic receptor kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate; *Vm*, the membrane potential; E_K, the potassium equilibration potential; *i*, the single-channel current amplitude; γ, the single-channel conductance; *Er*, the resting membrane potential of cells; Mg^{2+}i, intracellular Mg^{2+}; Kir channel, the inwardly rectifying K^+ channel; K^+_o, extracellular K^+; $[K^+_o]$, extracellular K^+ concentration; GTP_i, intracellular GTP; *I*, the macroscopic current amplitude; *N*, the number of functional channels; P_o, the single-channel open probability; MWC allosteric model, Monod-Wyman-Changeux's allosteric model; AA, arachidonic acid; LTC_4, leukotriene C_4, K_v channel, the voltage-gated K^+ channel; K_{ATP} channel, the ATP-sensitive K^+ channel; GST, glutathione S-transferase; *gK*, the macroscopic chord conductance; G-V relationship, the relationship between *gK* and *Vm*; *gKmax*, the maximum *gK*; Δ*V*, *Vm* relative to E_K; Δ*Vh*, Δ*V* at which *gK* is the half maximum; *v*, the slope factor in the Boltzman's equation; τ, a time constant; IC_{50}, the half-maximum inhibitory concentration; P_x, the permeability for ion species x; EGL, the external granule cell layer; IGL, the internal granule cell layer; and PND, the postnatal day.

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shown at the molecular level with cloned G protein-gated K^+ (K_G) channel and/or G protein subunits (Kubo *et al.*, 1993b; Dascal *et al.*, 1993; Wickman *et al.*, 1994; Reuveny *et al.*, 1994; Krapivinsky *et al.*, 1995a; Inanobe *et al.*, 1995b). Now it is established that $G_{K\beta\gamma}$ is the physiological activator of K_G channels not only in cardiac myocytes, but also in neurons and endocrine cells. Recently, it was indicated that G protein-inhibition of neuronal Ca^{2+} channels is also mediated by $G_{\beta\gamma}$ and not by G_α (Herlitze *et al.*, 1996; Ikeda, 1996). Efforts are now being made to elucidate the molecular mechanisms underlying $G_{\beta\gamma}$-control of K_G and N-type Ca^{2+} channels.

The importance of the G protein-activation of K_G channel system in receptor-mediated regulation of cell responses is now more widely appreciated than before because a wide variety of membrane receptors, such as M_2-muscarinic, A_1 adenosine, α_2-adrenergic, D_2 dopamine, μ-, δ-, and κ-opioid, 5-HT_{1A} serotonin, somatostatin, galanin, m-Glu, $GABA_B$, and sphingosine-1-phosphate receptors, have been shown to use this system in inhibiting cell excitation in various organs (North *et al.*, 1987; Lacey *et al.*, 1988; Hille, 1992a; Grudt and Williams, 1993; Oh *et al.*, 1995; Saugstad *et al.*, 1996; Sharon *et al.*, 1997; Bünemann *et al.*, 1995; Koppen *et al.*, 1996). In this review, we will first summarize ACh-activation of cardiac K_{ACh} channels, the prototype of this system, and then recent progress in molecular dissection of the K_G channel system.

II. Functional Analysis of G Protein-Mediated Activation of Muscarinic K^+ Channels in Cardiac Atrial Myocytes

A. Time-Dependent Response of the Whole-Cell Muscarinic K^+ Current to Acetylcholine

ACh added to the extracellular solution elicits a K_{ACh} channel current in cardiac atrial myocytes (fig. 1). The activation time-course is sigmoidal and takes several hundred milliseconds to reach a peak (Breitwieser and Szabo, 1988). Thereafter, the evoked current gradually decreases to a quasi-steady-state level within 1 min in the presence of high concentrations of ACh (> 0.3 μM). This reduction of cell K^+ current in the continuous presence of ACh is called "short-term" desensitization (Kurachi *et al.*, 1987b). After wash-out of the agonist, the current disappears within several seconds (deactivation). It is worth noting that in the inside-out patch configuration of the patch-clamp method, one measures K_{ACh} channel activity only in the steady-state phase. Thus, in these experiments, limited information is available regarding the desensitization of the channel.

These three phases of the response involve interactions between an agonist (i.e., ACh), an M_2-muscarinic receptor, a PTX-sensitive G protein, and the K_{ACh} channel. Therefore, to understand the reaction of the K_{ACh} channel to ACh, it is necessary to know how the receptor-generated signal is transferred to the channel

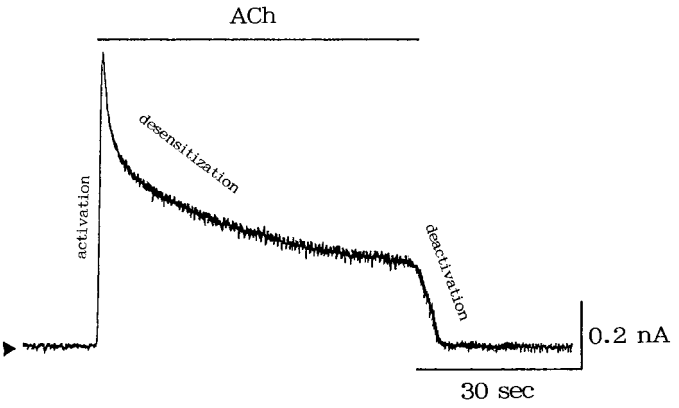

FIG. 1. Time-dependent response of the whole-cell muscarinic K^+ channel current to acetylcholine. By using the whole-cell voltage clamp method of the patch-clamp technique, the response of the whole-cell current of a guinea-pig atrial myocyte to 11 μM acetylcholine (ACh) was measured. In the presence of normal Tyrode solution that contained 5.4 mM external K^+, the cell membrane potential was clamped at −53 mV. The patch pipette contained (in mM): 150 KCl, 2 $MgCl_2$, 5 EGTA, 5 HEPES, and 0.1 GTP (pH = 7.3). ACh was applied to the bath for the period indicated by the horizontal bar above the cell membrane current trace. An arrowhead indicates the zero current level. An upward deflection of the cell current record indicated an outwardly directed cell membrane current that would be carried by the movement of K^+ ions under these circumstances.

through the G protein and how this signal transmission might be modulated by other factors interacting with these different reactions.

1. The G protein cyclic reaction mediating the receptor-to-channel signal transmission. Activation of K_{ACh} channel induced by M_2-muscarinic receptor stimulation is mediated by a heterotrimeric G protein (G_K) (fig. 2). The heterotrimeric G proteins are membrane-bound proteins which transduce signals from receptors to effectors such as adenylyl cyclase, phospholipase C, the K_{ACh} channel, and other ion channels (Gilman, 1987). These proteins are composed of α, β, and γ subunits (G_α, G_β, and $G\gamma$, respectively). Up to now, at least 16 G_α, 5 G_β, and 11 $G\gamma$ genes have been identified (Bourne, 1997). Heterotrimeric G proteins interact with receptors through G_α. It is well known that the interaction between M_2-muscarinic receptors and $G_{K\alpha}$ is blocked by the toxin from *Bordetella* pertussis (PTX) (Ui, 1984; Kurose *et al.*,

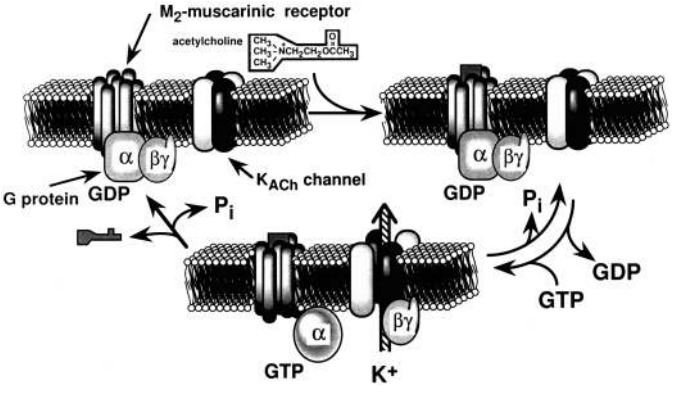

FIG. 2. Schematic representation of the G protein cycle involved in the activation of the muscarinic K^+ channel in response to acetylcholine.

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1986). PTX modifies covalently a cysteine residue at the carboxyl-terminal end of G_α subunits belonging to G_i, G_o, and G_t families by transferring an ADP-ribose group from the nicotinamide adenine dinucleotide moiety to the cysteine residue (Gilman, 1987). Because the receptor-mediated activation of K_G channels in cardiac atrial myocytes and neurons are inhibited by PTX (Pfaffinger *et al.*, 1985; Kurachi *et al.*, 1986a), G_K seems to belong to one of these G protein families. However, its molecular identity has not been fully elucidated, although G_K is proposed to be a member of the G_i class of G proteins in some systems (Kozasa *et al.*, 1996; Takano *et al.*, 1997)

The following is the current understanding of the interaction among receptors, G proteins, and K_{ACh} channels. In the absence of agonists, most of G_α is in the GDP-bound form ($G_{\alpha\text{- GDP}}$) (fig. 2). $G_{\alpha\text{-GDP}}$ has high affinity for $G_{\beta\gamma}$, thereby forming a heterotrimer with $G_{\beta\gamma}$ (Gilman, 1987). A small fraction of G_α does release GDP even in the absence of agonists, and in turn binds GTP (GDP/GTP exchange) and becomes a GTP-bound form ($G_{\alpha\text{-GTP}}$). Receptor stimulation substantially increases the GDP dissociation rate, which results in marked acceleration of the GDP/GTP exchange reaction. Formation of $G_{\alpha\text{-GTP}}$ leads to dissociation of $G_{\beta\gamma}$ from G_α. The dissociated $G_{\beta\gamma}$, which is always a dimer under physiological conditions, interacts with the K_{ACh} channel to activate the channel. Besides the K_{ACh} channel, many effectors of G proteins have been known to be regulated by $G_{\beta\gamma}$ (table 1) (Clapham and Neer, 1993; Iñiguez-Lluhi *et al.*, 1993).

G_α has a slow intrinsic GTPase activity: its K_{cat} value is typically 1 to 5/min (Gilman, 1987). G_α, therefore, hydrolyses the GTP on its own molecule to GDP, thereby returning to the GDP-bound form and re-associating with $G_{\beta\gamma}$. This reaction terminates the effector activation. In the continuous presence of agonists, the heterotrimeric G protein restarts the cyclic reaction by interacting with an agonist-bound receptor.

2. Activation phase. The time to peak of the ACh-induced response of the K_{ACh} channel is dependent on ACh concentration: the higher the concentration of ACh, the faster the activation. In the presence of a maximum effective concentration of ACh, the time to peak is several hundred milliseconds. If the M_2-muscarinic receptor, G_K, and the K_{ACh} channel encountered by simple diffusion in the membrane, the response time requires that all these signaling molecules be within less than 1.5 μm of each other (Hille, 1992a). The molecular mechanism satisfying such a topological requirement has not been clearly identified. However, it was recently suggested that K_{ACh} channel subunits may directly interact with not only $G_{K\beta\gamma}$ but also $G_{K\alpha}$, trimeric G_K, and the receptor and thereby might form a complex with these proteins (Huang *et al.*, 1995; Slesinger *et al.*, 1995) (detailed in the Sections III.F.3. and 4.).

When a recombinant K_G channel corresponding to the K_{ACh} channel is expressed with M_2-muscarinic receptors in *Xenopus* oocytes, the time course of the activation is much slower than in native atrial myocytes (Krapivinsky *et al.*, 1995a). It was recently demonstrated that newly identified molecules known as regulators of G protein signaling proteins (RGS) serve to increase the activation rate of recombinant K_G channels expressed in oocytes and a mammalian cell line (Doupnik *et al.*, 1997; Saitoh *et al.*, 1997). RGS proteins are the members of a multigene family that enhance the intrinsic GTPase activity of certain G proteins (mainly G_i/G_o classes) probably by preferentially binding to and stabilizing G proteins in their transition state for the hydrolysis reaction (Koelle, 1997). Sixteen RGS homologues (RGS1–16) have been identified in mammals. Among them, RGS1, RGS3, RGS4, and RGS8 have been shown to shorten the

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TABLE 1
G protein effectors regulated by G protein $\beta\gamma$ subunits

Effector	G protein type	Effect of G protein $\beta\gamma$ subunits
Ion channels		
Inwardly-rectifying K^+ channels		
Cardiac K_{ACh} channels[a]	PTX-sensitive	Stimulation
Other K_G channels[b]	PTX-sensitive	Stimulation
Voltage-dependent Ca^{2+} channels		
N-type	PTX-sensitive	Inhibition
L-type	PTX-sensitive	Inhibition
Enzymes		
Adenylyl cyclase		
Type I		Inhibition (when activated by $G_{S\alpha}$)
Type II		Stimulation (synergistically with $G_{S\alpha}$)
Type IV		Stimulation (synergistically with $G_{S\alpha}$)
Phospholipase C-β1-3	PTX-insensitive	Stimulation
Phosphatidylinositol 3-kinase	PTX-sensitive	Stimulation
G protein-coupled receptor kinase		Facilitation of membrane translocation
Phospholipase A_2	PTX-sensitive	Stimulation
MAP kinase (ras-dependent pathway)		Stimulation
Unidentified signal transduction pathway		
Pheromon-induced mating (yeast)		Stimulation
Oocytes maturation (starfish)		Stimulation

[a] Muscarinic K^+ channels.
[b] G protein-regulated K^+ channels.

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time to peak of receptor-mediated activation of K_G channels (Doupnik *et al.*, 1997; Saitoh *et al.*, 1997). Enhancement of the GTPase activity by RGS proteins leads to an increase in the off-rate of the G protein-mediated reaction (Koelle, 1997) (fig. 2). This effect, at least in theory, could abbreviate the time to peak when the on-rate of the reaction is not altered by the protein (Doupnik *et al.*, 1997; Saitoh *et al.*, 1997). In this case, the steady state K_G channel activity should be decreased in the presence of a given concentration of an agonist. However, RGS proteins enhance the activation rate without changing the amplitude of the steady-state response in the K_G channel systems (Doupnik *et al.*, 1997; Saitoh *et al.*, 1997). One possible explanation of this phenomenon is that RGS proteins may also enhance the GDP/GTP exchange rate of G_K. However, this could not be confirmed at least in an in vitro system that lacked reconstituted receptor proteins (Saitoh *et al.*, 1997). It is still possible that RGS proteins might increase the on-rate of the G_K-mediated reaction only in the presence of receptors or, alternatively, accelerate the subunit dissociation of G_K. Further studies are necessary to identify the mechanism by which the RGS proteins accelerate the agonist-mediated K_G channel activation without affecting the steady-state response.

3. The phase of short-term desensitization. Short-term desensitization becomes more prominent as the concentration of ACh is increased above 0.3 μM (Kurachi *et al.*, 1987b). This may at least partly arise from the transition of M_2-muscarinic receptors from the high to low affinity-binding state due to dissociation of G_K from receptors after agonist application (Gilman, 1987). Recent studies demonstrated that heterologous coexpression of RGS proteins with M_2-receptors and recombinant K_G channels reestablishes the short-term desensitization, which normally cannot be seen in the absence of RGS proteins in the reconstituted system (Doupnik *et al.*, 1997). Therefore, this protein may also be one of the molecules responsible for the short-term desensitization of the K_G channel system. Other possible candidates for the short-term desensitization include phosphorylation of M_2-muscarinic receptors by β-adrenergic receptor kinase (βARK), dephosphorylation of K_{ACh} channels and functional modulation of G proteins.

It is, however, unlikely that the phosphorylation of M_2-muscarinic receptors by βARK is responsible for the short time desensitization because receptor phosphorylation occurs much slower than the desensitization (Kwatra and Hosey, 1986; Kwatra *et al.*, 1987), and the kinase inhibitor (heparin) does not affect the desensitization time course (Mubagwa *et al.*, 1994). However, the receptor phosphorylation by βARK may underlie the slow desensitization of K_{ACh} channels which occurs in an order of minutes (Shui *et al.*, 1995).

As mentioned in Section II.A., single-channel recording techniques provide only limited information about the time course of channel's response to an extracellular ligand. This is due to the presence of agonists in the pipette solution which is going to be in contact with the cell membrane for a certain amount of time before the "giga-seal" will be formed. In most experiments therefore, short-term desensitization would have been achieved to some extent before single-channel events can be recorded. Under the conditions where a "giga-seal" could form exceptionally very rapidly, Kim (1990 and 1991) showed that the open time of K_{ACh} channels was ~5 msec at the beginning of the cell-attached patch recording and gradually decreased to ~1 msec with time. Such time-dependent reduction of the channel open time might correspond to short-term desensitization. Kim (1990 and 1991) attributed this phenomenon to "dephosphorylation" of the K_{ACh} channels in the presence of high concentrations of ACh although there is no direct evidence for phosphorylation or dephosphorylation of the channel protein. However, the open time of K_{ACh} channels in the presence of low concentrations of ACh or even in the absence of the agonist under steady state conditions is also ~1 msec. A possibility remains that different populations of K_{ACh} channels might be activated by low and high concentrations of ACh. A population with long open times might be less sensitive to G protein-activation due to "phosphorylation" and thus activated only by high concentrations of ACh. The "dephosphorylation" of these channels in the presence of high concentrations of ACh may then cause shortening of the open time, resulting in the decrease of the whole-cell current. The K_{ACh} channels with the short open time of ~1 msec may be dephosphorylated and more sensitive to G protein activation. In the presence of nondesensitizing concentrations of ACh, therefore, the K_{ACh} channels with short open time would be activated preferentially. Consistent with this hypothesis, we have observed that where we had thought to have already maximally activated K_{ACh} channels with exogenously applied $G_{\beta\gamma}$ subunits, the addition of mM intracellular ATP enhanced channel activity by prolonging open time (Yamada M and Kurachi Y, unpublished observation).

Huang *et al.* (1998) recently reported that exogenously applied phosphatidylinositol 4,5-bisphosphate (PIP_2) increased the sensitivity of recombinant K_G channels to $G_{\beta\gamma}$ in inside-out patch membranes of *Xenopus* oocytes. Because activation of M_2-muscarinic receptors in atrial cardiac myocytes induces the phosphoinositide turnover (Quist, 1982), the resultant decrease in PIP_2 content in the membrane might cause the short-term desensitization. Huang *et al.* (1998) also showed that intracellular ATP activated the recombinant K_G channels by increasing PIP_2 contents in the membrane. Therefore, the ATP-induced elongation of the open time of K_{ACh} channels might be caused by an increase in PIP_2 contents in the membrane.

4. Deactivation of the response of the muscarinic K^+ channel. The ACh-induced K^+ current disappears quickly when the agonist is washed out from the extra-

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cellular solution (fig. 1). The rate of deactivation of the whole-cell K_{ACh} channel current was estimated as ~30 to 200/min, which is much more rapid than either the GTP hydrolysis rate of G proteins (~1 to 5/min) or the rate of dissociation of $G_{\beta\gamma}$ from K_G channel subunits (~0.01/min) estimated in vitro (Breitwieser and Szabo, 1988; Nakajima *et al.*, 1992; Gilman, 1987; Doupnik *et al.*, 1997; Krapivinsky *et al.*, 1995c). This discrepancy might in part be attributed to positive cooperativity in the interaction between the channel and $G_{K\beta\gamma}$ that will be described in the Section II.B.2., where even a slight decrease in free $G_{K\beta\gamma}$ concentration in the membrane should cause a larger reduction of the channel activity.

The deactivation of K_G channels heterologously expressed in *Xenopus* oocytes occurs much more slowly than that of the native channel (Dascal *et al.*, 1993; Slesinger *et al.*, 1995). Again, RGS proteins have been found to enhance the deactivation rate of recombinant K_G channels approximately to the value of the native K_{ACh} channel (Doupnik *et al.*, 1997; Saitoh *et al.*, 1997). This effect of RGS proteins can be explained in terms of their increasing the GTPase activity of G_i/G_o proteins (Koelle, 1997). Therefore, RGS proteins accelerate both activation and deactivation rates of K_G channel systems and thus enable the systems to faithfully follow such a train of brief increases in agonist concentration as occurs in synaptic signal transmission (Doupnik *et al.*, 1997).

In the presence of the same concentration of ACh, the apparent potency of GTP in activating the K_{ACh} channel in excised membrane patches differ depending on the intracellular anion species (Nakajima *et al.*, 1992). The apparent potency of GTP decreases in the order: $Cl^- > Br^- > I^- > SO^{4-}$ or aspartate. Because the potency of the nonhydrolyzable GTP analogue, GTPγS is not affected by intracellular anion species, the GTPase activity of G_K seems to be modulated by intracellular anions. These effects of intracellular anions need to be taken into consideration because in most studies the internal side of the inside-out patch membrane is perfused with solution containing a much higher concentration of Cl^- than that in the cytosol of most cells.

One related issue to be discussed here is the basal activity of the K_G channel system that is observed in the absence of agonists. The native K_{ACh} channel exhibits much smaller basal activity relative to the agonist-induced maximum activity than heterologously expressed recombinant K_G channels (Kurachi, 1990; Kubo *et al.*, 1993b; Dascal *et al.*, 1993). RGS proteins significantly reduce the basal activity of recombinant K_G channels probably by activating GTPase of G_K (Doupnik *et al.*, 1997).

B. Quantitative Analysis of G Protein-Mediated Activation of the Muscarinic K^+ Channel

The unique feature of the K_G channel is the increase in channel activity in response to G_K activation. This response is mediated by interaction between $G_{K\beta\gamma}$ and a K_G channel. How they interact with each other and how the interaction leads to channel activation are intriguing questions.

The mechanism of $G_{K\beta\gamma}/K_G$ channel interaction has been mainly investigated in the K_{ACh} channel with inside-out patch membranes of cardiac atrial myocytes because in this system it is relatively easy to obtain many K_G channels that will respond to guanine nucleotides and G protein subunits applied to the internal side of the patch membranes. One can then directly analyze the membrane-delimited activation of the K_G channel by G_K in detail. In the following, we discuss the results obtained from such studies. We first describe the single-channel characteristics of the K_{ACh} channel and then go into the detail of the quantitative analysis of the G_K/K_{ACh} channel interaction.

1. Single-channel characteristics of the muscarinic K^+ channel. Fig. 3A shows single-channel recording of the K_{ACh} channel obtained from a cell-attached membrane of a guinea-pig atrial myocyte (Kurachi *et al.*, 1986a). In general, K^+ ions flow through K^+ channels depending on the electrochemical gradient for K^+ ions across the plasma membrane. This gradient is the difference between the membrane potential (V_m) and the K^+ equilibrium potential (E_K): $V_m - E_K$. The single-channel cur-

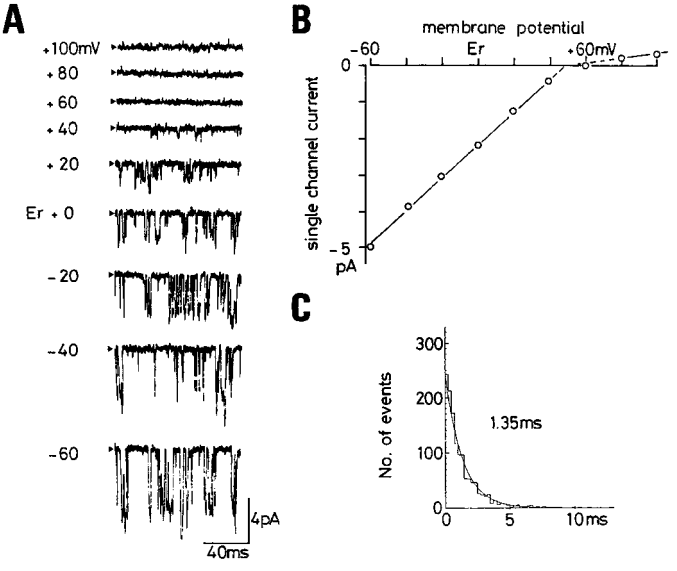

FIG. 3. Single-channel properties of the muscarinic K^+ channel. A: Cell-attached recordings of muscarinic K^+ channel currents from a single guinea pig atrial myocyte at different membrane potentials. The patch pipette contained 150 mM K^+ and 5.5 μM acetylcholine, although K^+ concentration in the bath was 5.4 mM. The resting membrane potential (Er) of the cell was −52 mV. Membrane potentials are indicated to the left of each trace as the difference from Er. Arrowheads indicate the zero current level. Downward reflection of the current record represent ion current passing inwards into the cell from the pipette. Upward deflections represent passing from the cell outwards into the pipette. B: The single-channel current-voltage relationship of the muscarinic K^+ channel shown in A. The line was fitted to the data by eye, and the single channel conductance was 46 pS at potentials between Er −60 and Er +40 mV. C: Open time histogram of the muscarinic K^+ channel at Er. The line is the fit of the data with a single exponential curve with a time constant of 1.35 msec. [Modified from Kurachi *et al.* (1986a)].

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rent flowing through a K^+-selective channel can be described as follows:

$$i = \gamma * (V_m - E_K) \quad [1]$$

where i is the single-channel current amplitude, and γ is the single-channel conductance of the channel. The current is positive (outwardly flowing across the membrane) at V_m positive to E_K, although it is negative (inwardly flowing) at V_m negative to E_K.

Under the conditions of the experiment shown in fig. 3A, the cell had a resting membrane potential (E_r) of ~−60 mV, although E_K across the patch membrane was ~0 mV. Therefore, the ACh-activated K_{ACh} channel elicited inward K^+ currents at potentials negative to E_r + *60* mV (i.e., $V_m < E_K$) and outward currents at potentials positive to E_r + *60* mV (fig. 3A). The outward currents were, however, very small compared with the inward currents at the corresponding potential relative to E_K (compare the data at E_r + *100* mV and E_r + *20* mV). Thus, the K_{ACh} channel current readily flowed in the inward but not the outward direction. This occurs because intracellular Mg^{2+} ($Mg^{2+}{}_i$) blocks the channel at the depolarized potentials (Horie et al, 1987 and 1989). Such a property is called "inward rectification," and the K^+ channels with this property are collectively termed as "inwardly rectifying" K^+ (Kir) channels. All known K_G channels including the K_{ACh} channel belong to this category.

The γ of the K_{ACh} channel estimated at V_m negative to E_K is ~40 pS in the presence of 145 mM extracellular K^+ ($K^+{}_o$) (fig. 3B). Based on the constant field theory, the permeability of K^+ through a single K_{ACh} channel has been estimated to be of the order of 10^{-13} cm^3 sec^{-1}, a value comparable to that of the I_{K1} channel or the axonal delayed rectifier K^+ channel (Sakmann *et al.*, 1983; Sakmann and Trube, 1984a; Conti and Neher, 1980). Because γ increases approximately in proportion to the square root of the concentration of $K^+{}_o$ ($[K^+]_o$) (Sakmann *et al.*, 1983), as is the case for the other types of Kir channels (Sakmann and Trube, 1984a), the γ is estimated as ~8 pS at physiological $[K^+]_o$.

The mean open time of the K_{ACh} channel at potentials negative to E_K is ~1 msec (fig. 3C), which is several orders of magnitude shorter than that of the I_{K1} channel (Sakmann and Trube, 1984b). The open time histogram sometimes reveals less frequent opening with a longer open time. This component has been reported to appear more frequently when the internal side of inside-out patch membranes is treated with MgATP (Kim, 1990, 1991). The closed time distribution is composed of at least two distinct components with mean closed times of ~1 and 100 msec (Sakmann *et al.*, 1983). There are also distinct, very long closed events that cannot be reliably analyzed in single-channel recordings (Sakmann *et al.*, 1983; Hosoya *et al.*, 1996). Analysis of burst behavior indicates that the K_{ACh} channel opens in bursts with a mean duration of 11 msec and consisting on average of ~5 channel openings separated by short closed events (Sakmann *et al.*, 1983). However, the majority of the K_{ACh} channel opening is solitary events separated by very short intervals of ~1 msec on average (Sakmann *et al.*, 1983). Overall, the burst behavior of K_{ACh} channel is not as evident as that of the I_{K1} channel.

2. Positive cooperative effect of GTP on muscarinic K^+ channel activity. Activation of K_{ACh} channels by intracellular GTP (GTP_i) can be reproduced in inside-out patch membranes of atrial myocytes in the presence of ACh in the pipette (Kurachi *et al.*, 1986a, 1990; Ito *et al.*, 1991). Fig. 4 shows the concentration-dependent effect of GTP_i in the presence of different concentrations of ACh.

Both in the presence and the absence of ACh, GTP_i increases the channel activity in a concentration-

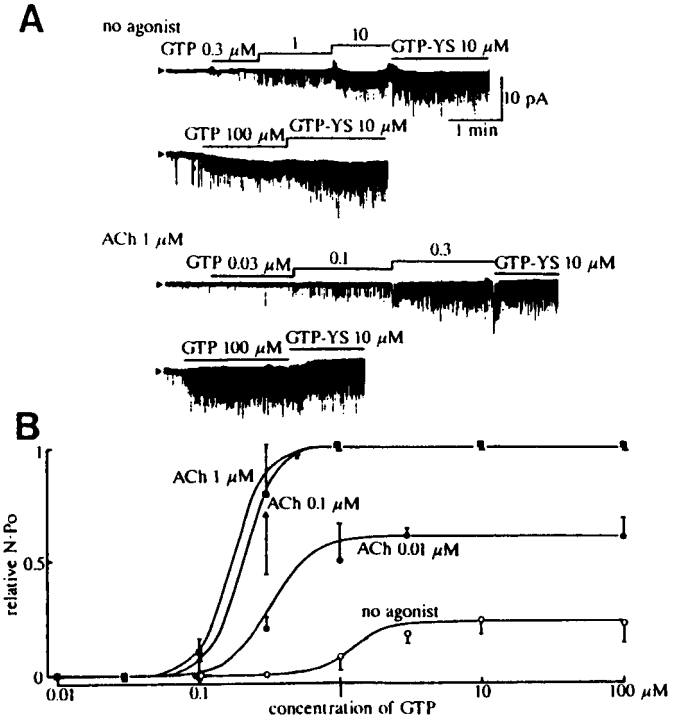

FIG. 4. Concentration-dependent effect of intracellular GTP on the muscarinic K^+ channel in the absence and presence of acetylcholine. A: Examples of inside-out patch experiments obtained from guinea-pig atrial myocytes. The channel currents were recorded at −80 mV with the symmetrical 145 mM K^+ solutions. The concentration of acetylcholine (ACh) in the pipette was 0 or 1 μM as indicated. The bars above each trace indicates the protocol of application of different concentrations of GTP or 10 μM GTPγS to the internal side of the patch membrane. The 3- to 10-fold increase in GTP concentration resulted in a dramatic increase of $N{\cdot}P_o$ of muscarinic K^+ channels, indicating the existence of a highly cooperative process. B: The relation between the concentration of GTP and the $N{\cdot}P_o$ of muscarinic K^+ channels normalized to the maximum $N{\cdot}P_o$ induced by 10 μM GTPγS in each patch. Symbols and bars are mean ± SD. The continuous lines indicate the fit of the relationship between GTP and channel activity in the presence of each concentration of ACh with the following Hill equation:

$$f = V_{max}/\{1 + (K_d/[GTP])^n\}$$

where f is the relative $N{\cdot}P_o$; V_{max}, the maximum $N{\cdot}P_o$ available in the presence of 10 μM GTPγS; K_d, the apparent dissociation constant of GTP; and n, the Hill coefficient. [Reproduced with permission from Ito *et al.* (1991)].

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dependent manner (Ito *et al.*, 1991). Channel currents in a patch membrane containing multiple K_{ACh} channels can be quantified as follows:

$$I = N * P_o * i = N * P_o * \gamma * (V_m - E_K) \qquad [2]$$

where I is the total channel current, N is the number of functional K_{ACh} channels in the patch, and P_o is the open probability of each channel. The increase in channel currents in response to GTP_i resulted from an increase in the $N*P_o$ value because V_m was fixed at −60 mV and γ is independent of G protein activity (Hosoya *et al.*, 1996). As the concentration of ACh was increased, both the apparent potency and efficacy of GTP_i were increased. Presumably, this is because a given concentration of GTP_i induced a higher steady state concentration of free $G_{K\beta\gamma}$ in the presence of higher concentrations of the agonist.

The Hill coefficient for the response was almost constant at ~3 irrespective of ACh concentration (fig. 4B). Therefore, the receptor/G_K/K_{ACh} channel interaction includes a certain positive cooperative process at step(s) distal to the receptor/G_K interaction. Because dissociation of G protein subunits induced by GTP is a one to one reaction (Gilman, 1987), the cooperativity probably results from the $G_{K\beta\gamma}$/K_{ACh} channel interaction (Kurachi *et al.*, 1990). Two pieces of evidence support this hypothesis. First, transducin $\beta\gamma$ subunits applied to the internal side of inside-out patch membranes activate K_{ACh} channels reversibly (Yamada *et al.*, 1994a). The concentration-response relationship of this reaction is also fitted by a Hill coefficient of ~3. Second, the K_{ACh} channel partially and irreversibly preactivated by brain $G_{\beta\gamma}$ exhibited apparently higher sensitivity to GTP_i than the control (Yamada *et al.* 1993). This potentiation can be explained only by assuming that the same cooperative mechanism mediates $G_{\beta\gamma}$- and GTP_i-induced channel activation. We might be able to understand how $G_{K\beta\gamma}$ activates the K_{ACh} channel when we can determine which kinetic parameter(s) of the K_{ACh} channel is modulated by GTP_i in a positive cooperative manner.

3. Spectral analysis of the muscarinic K^+ channel currents in the presence of different concentrations of intracellular GTP. Precise and reliable analysis of the single-channel kinetics of the K_{ACh} channel is difficult because multiple K_{ACh} channels are usually included in a single membrane patch of atrial myocytes (fig. 4A). In these cases, the spectral analysis of the channel currents (an analysis based on a frequency domain) is one of the most reliable and powerful ways to assess the channel kinetics (fig. 5). The power spectrum constructed from inside-out patch recordings of the K_{ACh} channel currents is always well fitted with the sum of two Lorenzian curves irrespective of GTP_i concentration (Hosoya *et al.*, 1996). These observations indicate that the K_{ACh} channel possesses three distinct open/closed states. Because the channel possesses a single open state (Sakmann *et al.*, 1983), the equilibrium of the states can be described as *C2*↔*C1*↔*O*, where O represents the open state although *C1* and *C2* are closed states. It is likely that the transition among these three states is responsible for the open and closure of K_{ACh} channel currents observed at the single-channel level (figs. 3A, 4A and 5A). The corner frequencies of the two Lorenzian functions (the frequencies at which the power of the each component is the half-maximum) were constant irrespective of GTP_i concentration (fig, 5B). The ratio of the powers of the two Lorenzians at 0 Hz was also unaffected by GTP_i concentration. These results indicate that the kinetics of the fast open-close transition of the channel is not a function of G_K activity. In other words, G_K activates the K_{ACh} channel without altering the channel's fast open-close kinetics.

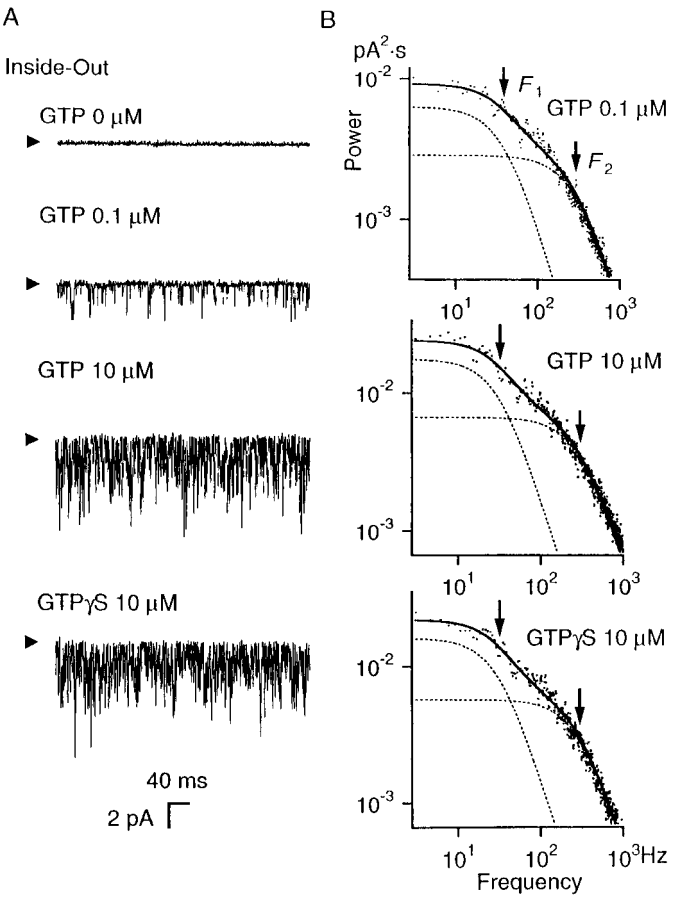

FIG. 5. Spectral analysis of the muscarinic K^+ channel currents in an inside-out patch. A: The muscarinic K^+ channel currents in the inside-out patch membrane of a guinea pig atrial myocyte. The channel currents were recorded at −60 mV with the symmetrical 150 mM K^+ solutions. The patch pipette also contained 0.5 μM acetylcholine. Different concentrations of GTP were applied to the internal side of the patch membrane as indicated above each current trace. B: Power density spectra calculated from the data shown in A. Each spectrum could be fitted with the sum of two Lorentzian functions. F_1 and F_2 indicated by arrows indicate the corner frequencies of the slow and the fast Lorentzian components, respectively. [Reproduced with permission from Hosoya *et al.* (1996)].

How then does G_K activate the K_{ACh} channel? As GTP_i concentration was raised, the powers of both the Lorenzian components at 0 Hz became progressively larger

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(fig. 5B) (Hosoya *et al.*, 1996), implying that G_K increases K_{ACh} channel activity through a process too slow to be detected by spectral analysis. For reasons of simplicity, we a priori assume the presence of another transition with slow kinetics between two channel states $U \leftrightarrow A$, where U and A, respectively, represent "unavailable" and "available" states of the channel. In this framework, the $U \leftrightarrow A$ transition is independent of the fast transition $C2 \leftrightarrow C1 \leftrightarrow O$ and the A but not the U state allows the channel to be conducting when the channel passes into the O state. Furthermore, it is hypothesized that G_K causes a shift of the equilibrium toward A to increase channel activity.

Based on these assumptions, one should be able to calculate the fraction of the A state (i.e., $A/(A + U)$) in the presence of a given concentration of GTP_i by extracting some parameters from the spectral analysis (the corner frequencies and the ration of the powers at 0 Hz) and the single-channel analysis (the single-channel open time and the N^*P_o value). Fig. 6A shows the calculated fraction of the A state, which increased as the concentration of GTP_i was raised in such a way that the concentration-response relationship could be well fitted by a Hill coefficient of ~3. From this result we conclude that G_K modulates a slow process in the K_{ACh} channel that corresponds to an increase in the number of operational ion channels in the membrane. The fast open-close kinetics of the channels seem not to be influenced by G_K. Thus, N, but not P_o, in equation 2 is affected by G_K.

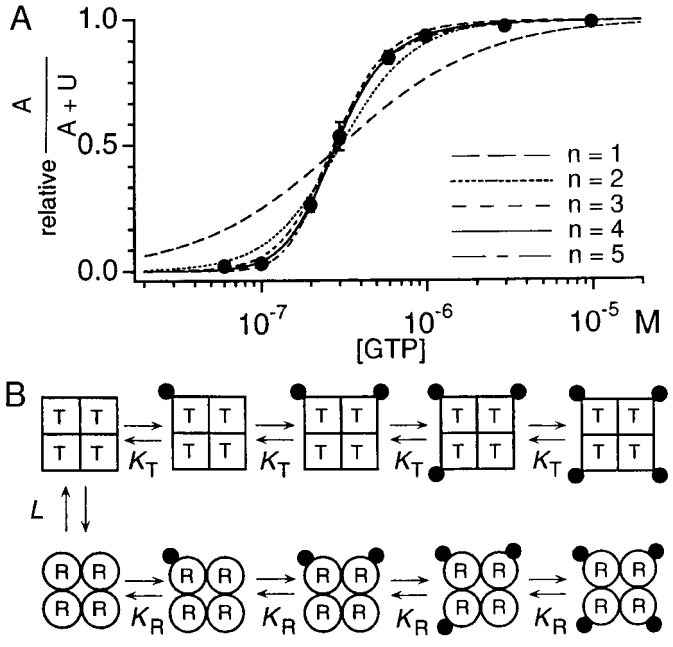

FIG. 6. The relationship between GTP concentration and the fraction of the "available" state of the muscarinic K^+ channel, and the concerted allosteric model of Monod, Wyman, and Changeux. A: The fraction of the "available" state ($A/(A + U)$) was calculated from inside-out membrane patch experiments such as shown in fig. 5. Symbols indicate the relationship between GTP concentration and the calculated fraction of the "available" state. Lines indicate the fit of the data with the Monod, Wyman, and Changeux allosteric model with different assumed numbers of n (see Section II.B.4.). B: Schematic representation of the Monod, Wyman, and Changeux allosteric model. In this scheme, each muscarinic K^+ channel is assumed to be an oligomer composed of four identical subunits (i.e., $n = 4$). Each subunit is in either the tense (T) or the relaxed (R) state, which is represented by squares and circles, respectively. Each subunit in the T or R state binds with one dissociated G protein $\beta\gamma$ subunit (solid circles) independently of each other with the microscopic dissociation constant of K_T or K_R, respectively. In this model, all subunits in the same oligomer must change their conformations simultaneously. Therefore, the channel can be either T_4 or R_4. T_4 and R_4 are in the equilibrium through an allosteric constant L. [Reproduced with permission from Hosoya *et al.* (1996)].

4. A possible mechanism for the G protein-mediated increase in the functional numbers of muscarinic K^+ channels. Recent studies have revealed that Kir channels, including K_{ACh} channel, have an oligomeric structure (Yang *et al.*, 1995b; Krapivinsky *et al.*, 1995a) that may underlie the positive cooperativity of the $G_{K\beta\gamma}$ protein/K_{ACh} channel interaction (Monod *et al.*, 1965).

In the presence of a supermaximum concentration of ACh (≥1 μM), $G_{\beta\gamma}$ exogenously applied to the internal side of inside-out patch membranes does not further increase the channel activity once the channel is preactivated with more than 1 μM of GTP_i (Ito *et al.*, 1992; Yamada *et al.*, 1993). In this case, the maximum channel activity is determined by the number of K_{ACh} channels and not the $G_{K\beta\gamma}$ available in a patch membrane. Under these conditions, the interaction between $G_{K\beta\gamma}$ and K_{ACh} channel subunits can be quantitatively assessed by analyzing the relationship between GTP_i concentration and the fraction of the A state with Monod-Wyman-Changeux's (MWC) allosteric model (fig. 6B) (Monod *et al.*, 1965; Hosoya *et al.*, 1996).

This model is based on the following assumptions: (*a*) a single K_{ACh} channel is composed of a finite number (n) of functionally identical subunits: fig. 6B illustrates the case of $n = 4$; (*b*) each subunit independently binds only one $G_{K\beta\gamma}$; (*c*) each subunit has two distinct conformations: relaxed (R) and tense (T); (*d*) R and T bind $G_{K\beta\gamma}$ with microscopic dissociation constant K_R and K_T, respectively. R has higher affinity for $G_{K\beta\gamma}$ than T (i.e., $K_R < K_T$); (*e*) all subunits in an oligomer must change the conformation simultaneously. As a result, any oligomer is either R_n or T_n; (*f*) R_n and T_n are in the equilibrium through an allosteric constant L.

According to this model, an increase in $G_{K\beta\gamma}$ concentration leads to an increase in the fraction of R_n [i.e., $R_n/(R_n + T_n)$]. When one replaces R_n and T_n of the MWC model with the A and U states of the K_{ACh} channel, the data shown in fig. 6A can be fitted with this model by changing the assumed number of n. Such analysis indicates that n must be greater than 3 to account for the data (fig. 6A) (Hosoya *et al.*, 1996). This result is consistent with the view that Kir channels including K_G possess a tetrameric structure as described in Section III.E. (Krapivinsky *et al.*, 1995a; Yang *et al.*, 1995b).

Therefore, we may summarize our current understanding of the interaction between G_K and the K_{ACh} channel as follows. G_K activates the K_{ACh} channel by increasing the functional number of channels without modulating the fast open-close transition of the channel

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gate. The positive cooperativity observed in the GTP_i-induced activation of the K_{ACh} channel arises from the intrinsic property of the $G_{K\beta\gamma}/K_{ACh}$ channel interaction. This property can be explained in terms of the oligomeric structure of the K_{ACh} channel that is composed of more than three functionally identical subunits, each of which independently binds one $G_{K\beta\gamma}$ molecule. As we shall see later in Section IV., K_{ACh} channel activity is controlled not only by G_K but by V_m. However, ACh does not modulate the relationship between channel activity and V_m (Kurachi, 1990). Therefore, the model described here is applicable to the G_K-mediated activation of the K_{ACh} channel at any potential.

C. Modulation of G Protein-Mediated Activation of the Muscarinic K^+ Channel

Although the $G_{K\beta\gamma}/K_{ACh}$ channel interaction is the essential step of G protein-mediated activation of the K_{ACh} channel, this reaction is modulated by many factors such as intracellular ATP, Na^+ ions, and arachidonic acid metabolites. Intracellular ATP has been shown to activate native and recombinant K_G channels in an $Mg^{2+}{}_i$-dependent manner (Otero *et al.*, 1988; Heidbüchel *et al.*, 1990; Kaibara *et al.*, 1991; Kim, 1991; Lesage *et al.*, 1995; Sui *et al.*, 1996). Although the molecular mechanism underlying this phenomenon has not been unequivocally identified, PIP_2 may be involved in this phenomenon (Huang *et al.*, 1998).

The activity of K_G channels pretreated with intracellular MgATP could be further enhanced by intracellular Na^+ (Lesage *et al.*, 1995; Sui *et al.*, 1996). The site of action of Na^+ is unknown. Sui *et al.* (1996) showed that intracellular Na^+ increased the activity of the K_{ACh} channel (and also the corresponding recombinant K_G channel) with an EC_{50} of ~40 mM mainly by increasing the frequency of the channel's opening. They found that priming of channels with MgATP was a prerequisite for the action of Na^+. Lesage *et al.* (1995), however, found that 20 mM intracellular Na^+ activated recombinant K_G channel whether or not they had been pretreated with MgATP. This discrepancy might have occurred due to the different subunit composition of the K_G channels used in these two studies. Interestingly, Sui *et al.* (1996) showed that a cardiac glycoside ouabain, an inhibitor of the Na^+/K^+ pump, induced the opening of the K_{ACh} channel. They found that the N^*P_o value of the channel increased although the mean open time was unchanged, indicating that the activating effect of ouabain was probably mediated by accumulation of intracellular Na^+ but not a possible local increase in ATP concentration. However, they did not directly measure intracellular Na^+ concentration nor reported the apparent change in the reversal potential of the K_{ACh} channel that might be expected when intracellular K^+ concentration decreased due to blockade of Na^+/K^+ pump. Therefore, further studies may be necessary to conclude that cardiac glycosides activate the K_{ACh} channel through accumulation of intracellular Na^+. This phenomenon might, at least in part, underlie the "direct" negative chrono- and dromotropic effects of the agent on the heart.

Arachidonic acid (AA) metabolites are known to modulate K_{ACh} channels (Kurachi *et al.*, 1989c; Kim *et al.*, 1989; Yamada *et al.*, 1994b). The effect of AA is mimicked by leukotriene C_4 (LTC_4) and specifically blocked by AA861, a 5-lipoxygenase inhibitor (Kurachi *et al.*, 1989c). Therefore, the effect of AA may be mediated by LTC_4 or its metabolites. Although the site of action of LTC_4 has not been clearly identified, the complete dependency of the LTC_4 effect on the presence of GTP_i indicates that LTC_4 does not directly act on the K_{ACh} channel (Kurachi *et al.*, 1989c). In the absence of receptor agonists, GTP_i usually induces only 20% of the maximum K_{ACh} channel activity in the inside-out patch membranes even when Cl^- is used as an intracellular anion. However, GTP_i fully activated the channel in an agonist-independent manner when the patches were pretreated with AA before patch excision (Kurachi *et al.*, 1989c). Thus, AA metabolites may stimulate the basal turn-on reaction of G_K. Stimulation of K_{ACh} channels by platelet-activating factor or α_1-adrenergic receptors may be mediated by this second-messenger pathway (Nakajima *et al.*, 1991, Kurachi *et al.*, 1989b).

III. Molecular Analysis of G Protein-Gated K^+ Channels

A. Cloning of Inwardly Rectifying K^+ Channels

In 1993, the molecular structure of inwardly rectifying K^+ channels (Kir) was disclosed. The cDNAs encoding an ATP-dependent Kir channel, ROMK1 (Ho *et al.*, 1993), and a classical Kir channel, IRK1 (Kubo *et al.*, 1993a), were isolated by expression cloning from the outer medulla of rat kidney and a mouse macrophage cell line, respectively (fig. 7). The primary structure of these channels were similar with two putative membrane-spanning regions (M1 and M2) and one potential

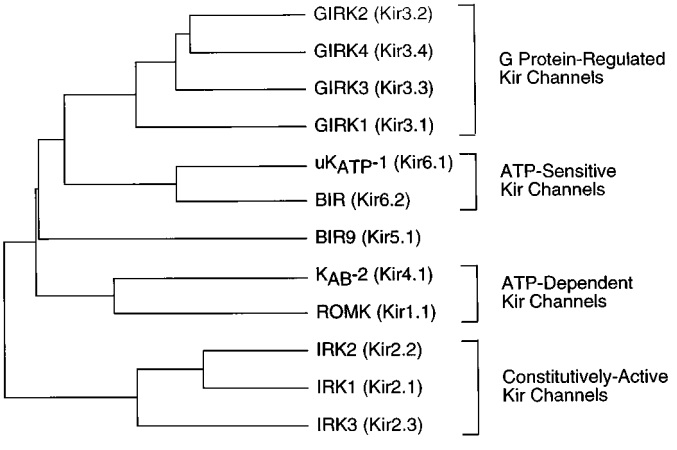

FIG. 7. Evolutionary tree of Kir subunits. The tree was made using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) Tree Window in Geneworks (IntelliGenetics, Inc., Mountain View, CA).

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pore-forming region (H5). This structure resembles that of the S5, H5, and S6 segments of the voltage-gated K^+ (K_v) channels. Because the voltage-sensor of the K_v channel subunit exists in the S4 segment that possesses repeated positively-charged amino acid residues, Kir channel subunits lack an obvious voltage-sensor region. This is consistent with electrophysiological studies that show the kinetics of Kir channels apparently depends on the difference of *Vm* from E_K and not on *Vm* itself.

After the cloning of ROMK1 and IRK1, the cDNAs encoding the main subunits of K_G and K_{ATP} channels (GIRK1 and BIR) were also cloned (Kubo *et al.*, 1993b; Dascal *et al.*, 1993; Inagaki *et al.*, 1995a). All of these Kir channel subunits exhibit basically the same primary structure. So far, at least 11 cDNAs encoding Kir channel subunits have been isolated. The evolutionary tree of this family is depicted in fig. 7.

These cloned Kir subunit cDNAs encode proteins composed of 327 to 501 amino acids. The identity of the predicted amino acid sequences is ~30 to 40% among the members of the different Kir subfamilies and more than 60% among those in the same subfamilies. The highest level of sequence identity (50 to 60%) is found in the H5 region and the proximal part of the C-terminal cytosolic domain. The cloned Kir channel subunits have been classified at least into four groups (Doupnik *et al.*, 1995a): (*a*) IRK (Kir2.x) subfamily made of the classical constitutively active "inward rectifier" Kir channels: IRK1 (Kubo *et al.*, 1993a; Morishige *et al.*, 1993), IRK2 (Koyama *et al.*, 1994; Takahashi *et al.*, 1994) and IRK3 (Morishige *et al.*, 1994; Makhina *et al.*, 1994; Pärier *et al.*, 1994); (*b*) GIRK (Kir3.x) subfamily, corresponding to G protein-regulated K^+ channels: GIRK1 (Kubo *et al.*, 1993b; Dascal *et al.*, 1993), GIRK2 (Lesage *et al.*, 1994, 1995; Isomoto *et al.*, 1996; Tsaur *et al.*, 1995; Stoffel *et al.*, 1995; Bond et al, 1995; Ferrer *et al.*, 1995), GIRK3 (Lesage *et al.*, 1994), GIRK4 (Ashford *et al.*, 1994; Krapivinsky *et al.*, 1995a; Chan *et al.*, 1996), and GIRK5 (Hedin *et al.*, 1996); (*c*) K_{AB} subfamily of ATP-dependent K^+ channels (Kir1.1 and Kir4.1): ROMKs (Ho *et al.*, 1993; Zhou *et al.*, 1994; Yano *et al.*, 1994; Shuck *et al.*, 1994; Boim *et al.*, 1995; Kondo *et al.*, 1996) and K_{AB}-2 (Bond *et al.*, 1994; Takumi *et al.*, 1995); and (*d*) K_{ATP} subfamily (Kir6.x), the ATP-sensitive K^+ channels: uK_{ATP}-1 and BIR (Inagaki *et al.*, 1995a,b; Sakura *et al.*, 1995).

Recent progress in the molecular biology of Kir channels has enabled us to study the structure-function relationship of biophysics, physiological regulation, and pharmacology of these channels at the molecular level.

B. Subunits of G Protein-Gated K^+ Channels

GIRK1 was first isolated from the rat atrium (Kubo *et al.*, 1993b; Dascal *et al.*, 1993). From a mouse brain cDNA library, two additional homologues of GIRK1 were isolated and designated GIRK2 and GIRK3 (table 2) (Lesage *et al.*, 1994). Furthermore, it has been shown that at least three different isoforms of mouse GIRK2 are generated by alternative splicing of transcripts from a single gene, and we designated them GIRK2A, GIRK2B, and GIRK2C in the order of identification (Isomoto *et al.*, 1997). These alternatively spliced transcripts share an N-terminal end and a central core, and differ at their C-terminal ends. GIRK2B was isolated from mouse brain cDNA library and shown to be ubiquitously expressed in various tissues (Isomoto *et al.*, 1996). Its amino acid sequence is shorter than that of GIRK2A by 87 amino acids. The eight amino acid residues in the C-terminal end of GIRK2B are different from those of GIRK2A. GIRK2C has a C-terminus which is longer than that of GIRK2A by 11 amino acids. GIRK2C was isolated from cDNA libraries of insulinoma cells and brain (Lesage *et al.*, 1994, 1995; Tsaur *et al.*, 1995; Stoffel *et al.*, 1995; Bond *et al.*, 1995; Ferrer *et al.*, 1995).

GIRK2C was originally termed K_{ATP}-2 because it was thought to be a subunit of the K_{ATP} channel (Stoffel *et al.*, 1995; Tsaur *et al.*, 1995) due to its sequence similarity to cK_{ATP}-1, which was isolated by Ashford *et al.* (1994). However, GIRK4, which is virtually identical with rat cK_{ATP}-1, reconstitutes cardiac K_{ACh} channel with GIRK1 and does not contribute to the K_{ATP} channel as described in the Section III.D. (Krapivinsky *et al.*, 1995a,b). Thus, it is now clear that both cK_{ATP}-1 and K_{ATP}-2 belong to the GIRK subfamily. GIRK5 was cloned from *Xenopus* oocytes (Hedin *et al.*, 1996). Although its mammalian homologue has not been reported, the amino acid sequence of GIRK5 is most homologous to that of GIRK4 among mammalian GIRKs.

The GIRK clones contain various known functional motifs in their amino acid sequences that may be important for the physiological functions of the subunits in K_G channels (fig. 8). GIRK1 possesses an amino acid sequence homologous to the $G_{\beta\gamma}$-binding domain of βARK1 in its C-terminus, which is therefore the candidate for the site of $G_{\beta\gamma}$-binding to the K_G channel (Reuveny *et al.*, 1994). As with all the other Kir channel subunits, GIRKs possess conserved cationic residues adjacent to the C-terminal end of the M2 domain. One of these positively charged residues, arginine (R) at position 188 of ROMK1, was shown to be critically involved in PIP_2-induced activation of rundown ROMK1 channels (Huang *et al.*, 1998). Thus, it is conceivable that the corresponding residues in GIRK subunits (R190 for GIRK1; R201 for GIRK2s; R167 for GIRK3; and R196 for GIRK4) also participate in the PIP_2-induced activation of K_G channels. All of the GIRK clones have an arginine-glycine-aspartate (RGD) motif in their linker region between M1 and H5. This motif could be an integrin receptor-site (Hynes *et al.*, 1992), whose role in K_G channels has not been examined yet. The characteristic feature of GIRK2C is the serine/threonine-X-valine/isoleucine (S/T-X-V/I) motif at its C-terminus end (Gomparts, 1996). This motif has been shown to be important for interactions with the PSD-95/SAP90 family

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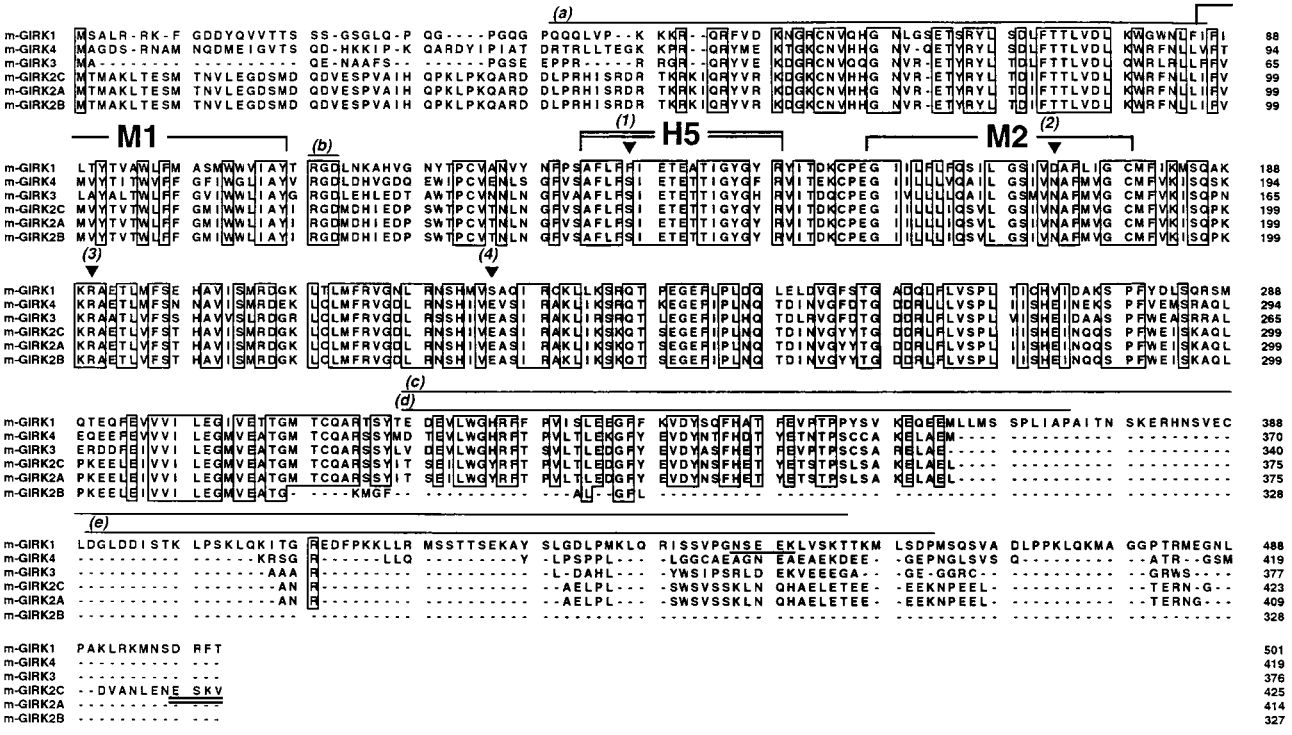

FIG. 8. Alignment of amino acid sequences of GIRK1, GIRK2A, B, C, GIRK3, and GIRK4. Positions at which all six amino acid sequences are identical are boxed. The putative transmembrane segments (M1 and M2) and pore-forming region (H5) are indicated above the sequences. Overlined sequences of GIRK1, (a), the domain in the N terminus that was shown to bind G protein $\beta\gamma$ subunits, the GDP-form of G protein α subunits and heterotrimeric G proteins (Huang *et al.*, 1995); (b), RGD sequence included in the integrin-binding site of fibronectin, vitronectin, and a variety of other adhesive proteins (Hynes, 1992), which is also found in other GIRK subunits; (c), the region whose amino acid sequence is similar to the G protein $\beta\gamma$ subunit-binding domain of the β-adrenergic receptor kinase 1 (Reuveny *et al.*, 1994); and (d) and (e), the domains in the C terminus that was shown to interact with G protein $\beta\gamma$ subunits (Huang *et al.*, 1995). An underlined sequence of GIRK1: the sequence similar to that of the region of adenylyl cyclase 2 which is critical for activation of the enzyme by G protein $\beta\gamma$ subunits (N-X-X-E-R) (Chen *et al.*, 1995; Huang *et al.*, 1995). Numbered amino acids, (1) the phenylalanine (F137) of GIRK1 was shown to be responsible for the slow relaxation of GIRK1-containing K_G channels (Kofuji *et al.*, 1996a), although the interaction between F137 and the corresponding serine of other GIRK subunits may be important for the larger macroscopic current amplitude yielded with coexpression of GIRK1 and other GIRK subunits than the sum of those obtained with expression of each subunit alone (Chan *et al.*, 1996; Wischmeyer *et al.*, 1997); (2) the amino acid residue corresponding to aspartate at position 172 of the IRK subunit which was shown to be critically involved in the Mg^{2+}/polyamine block of IRK1 channels (Stanfield *et al.*, 1994; Taglialatela *et al.*, 1994; Lu and MacKinnon, 1994; Wible *et al.*, 1994; Lopatin *et al.*, 1994; Ficker *et al.*, 1994; Yang *et al.*, 1995a); (3) the residue corresponding to the arginine of ROMK1 which was shown to be involved in interaction of ROMK1 channels with phosphatidilinositol 4,5-bisphosphate (PIP_2) (Huang *et al.*, 1998); and (4) the residues corresponding to glutamate at position 225 of the IRK1 subunit that was shown to be critically involved in the Mg^{2+}/polyamine block of IRK1 channels (Yang *et al.*, 1995a). The double-underlined sequence of GIRK2C: the sequence including the consensus sequence (S/T-X-V/I) for interaction with PSD-95/SAP90 anchoring protein (Gomparts, 1996; and Cohen *et al.*, 1996), although the glutamate (E) preceding the sequence was also proposed to be important for the interaction of Shaker-type K^+ channels with these anchoring proteins (Kim *et al.*, 1995).

of anchoring proteins, not only for K_v and NMDA receptor channels (Kim *et al.*, 1995; Kornau *et al.*, 1995), but also for Kir channels such as IRK3 and K_{AB}-2 (Cohen *et al.*, 1996; Horio *et al.*, 1997).

C. Tissue Distribution of GIRK Subunits

1. Peripheral tissues. Tissue distribution of mRNAs for GIRK subunits is summarized in table 3 (Kubo *et al.*, 1993b; Dascal *et al.*, 1993; Lesage *et al.*, 1994; Stoffel *et al.*, 1995; Bond *et al.*, 1995; Dixon *et al.*, 1995; Iizuka *et al.*, 1995). In tissues other than brain, the atrium of the heart most abundantly expresses GIRK1 and GIRK4, both of which constitute the K_{ACh} channel. Both GIRK1 and GIRK4 proteins are diffusely immunostained in the atrium by antibodies specific for individual subunits (cf., fig. 15) (Iizuka *et al.*, 1995). GIRK1 may be moderately expressed in the ventricle (Kubo *et al.*, 1993b; Dascal *et al.*, 1993; Karschin *et al.*, 1994), although there seems to be a significant species-to-species difference in the level of expression of GIRK4 protein in the ventricle (Iizuka *et al.*, 1995; Krapivinsky *et al.*, 1995b). Iizuka *et al.* (1995) showed that GIRK4 immunoreactivities exist in subendocardial myocytes and also in dorsal atrial ganglia of rat. GIRK1 is also moderately expressed in other peripheral tissues except for spleen (table 3). GIRK2 and GIRK3 are rather brain-specific and barely found in peripheral tissues. However, GIRK2 (probably GIRK2C) exists in pancreatic islets (Stoffel *et al.*, 1995), although GIRK2B mRNA is expressed ubiquitously in peripheral tissues (Isomoto *et al.*, 1996). GIRK4 is also found in some other peripheral tissues.

2. Central nervous system. Detailed distribution of GIRK mRNAs in rat brain was analyzed with in situ hybridization and tabulated by Karschin *et al.* (1994, 1996) and DePaoli *et al.* (1994). Expression pattern of GIRK transcripts in the mouse brain is similar to that in

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TABLE 2
Subunits of G protein-regulated inward-rectifying K^+ channels

Name used in text	Kir classification[a]	References (original name)
GIRK1	Kir3.1	Kubo *et al.*, 1993b (GIRK1)
		Dascal *et al.*, 1993 (KGA)
GIRK2A	Kir3.2a	Lesage *et al.*, 1994 (GIRK2)
GIRK2B	Kir3.2b	Isomoto *et al.*, 1996 (GIRK2B)
GIRK2C	Kir3.2c	Lesage *et al.*, 1995 (GIRK2A)
		Tsauer *et al.*, 1995 (K_{ATP}-2)
		Stoffel *et al.*, 1995 (K_{ATP}-2)
		Bond *et al.*, 1995 (BIR1)
		Ferrer *et al.*, 1995 (GIRK2)
GIRK3	Kir3.3	Lesage *et al.*, 1994 (GIRK3)
GIRK4	Kir3.4	Ashford *et al.*, 1994 (cK_{ATP}-1)
		Krapivinsky *et al.*, 1995a (CIR)
		Chan *et al.*, 1996 (KGP)
GIRK5	Kir3.5	Hedin *et al.*, 1996 (XIR)

[a] The naming based on Doupnik *et al.*, 1995a.

TABLE 3
Tissue expression of mRNAs for GIRK subunits

	GIRK1	GIRK2	GIRK3	GIRK4
Brain	++[a,b,i]	++[c,f,g,i]	++[c]	+[i,j]
Eye	+[i]	+[i]		+[i]
Submaxillary Gland		−[h]		
Heart				
Atrium	++[a,b,i,d]	−[c,f,g,h,i]	−[c]	++[e,i,j,k]
Ventricle	±[a,b,d]	−[c,g,h]	−[c]	±[e,k] or ++[j]
Aorta	+[i]	−[i]		−[i,j]
Thymus		−[g]		−[i]
Lung	+[b,i] or −[d]	−[c,f,h,i]	−[c]	+[i]
Liver	±[a,i]	−[c,f,h,i]	−[c]	−[i,j]
Spleen	−[i]	−[f,h,i]		−[i,j]
Pancreas		+[f]		±[j]
Kidney	−[d] or +[i]	−[c,f,h,i]	−[c]	−[i] or +[j]
Stomach	+[i]	−[i]		−[i]
Small Intestine	+[i]	−[h,i]		−[i]
Colon	+[i]	−[i]		−[i]
Adrenal Gland		−[h]		
Urinary Bladder	+[i]	−[i]		+[i]
Testis	+[i]	+[f,h,i]		−[i]
Ovary		−[h]		
Uterus	+[i]	−[h,i]		+[i]
Skin	+[i]	−[i]		−[i]
Skeletal Muscle	±[a,b,i]	−[c,f,h,i]	±[c]	+[i] or −[i]

++, strong expression; +, distinct expression; ±, marginal expression; and −, no expression.

References and experimental conditions: [a] Kubo *et al.*, 1993: Northern blot analysis of rat tissues; [b] Dascal *et al.*, 1993: Northern, rat; [c] Lesage *et al.*, 1994: Northern, mouse; [d] Karschin *et al.*, 1994: In situ hybridization, rat; [e] Krapivinski *et al.*, 1995a: Northern, rat; [f] Stoffel *et al.*, 1995: Northern, rat; [g] Bond *et al.*, 1995: Northern, rat; [h] Bond *et al.*, 1995: RT-PCR, rat; [i] Dixon *et al.*, 1995: RT-PCR, rat; [j] Iizuka *et al.*, 1995: Northern, pig; and [k] Spayschus *et al.*, 1996, In situ hybridization, rat.

the rat brain (Kobayashi *et al.*, 1995). In general, GIRK1–3 mRNAs are abundantly expressed throughout the brain with overall similar distribution, although GIRK4 mRNA is expressed in the brain to a much lesser extent than other GIRK transcripts (Karschin *et al.*, 1996; Iizuka *et al.*, 1997).

In the rat main olfactory bulb, all GIRK1–4 mRNAs are expressed in the granular cell layer and mitral cell layer, although only GIRK3 mRNA is abundant in glomerular cells (Karschin *et al.*, 1994, 1996; Dixon *et al.*, 1995; Ponce *et al.*, 1996; Iizuka *et al.*, 1997). All GIRK1–3 mRNAs are strongly expressed in every area of neocortex and by virtually all pyramidal neurons in the cortex (Karschin *et al.*, 1994, 1996; DePaoli *et al.*, 1994; Dixon *et al.*, 1995; Ponce *et al.*, 1996). GIRK4 mRNA may be expressed in the pyramidal neurons (Iizuka *et al.*, 1997). Basal ganglia exhibit basically very poor expression of GIRK family members (Karschin *et al.*, 1994, 1996; Ponce *et al.*, 1996) except for amygdala nuclei where all GIRK1–3 mRNAs are abundantly expressed (DePaoli *et al.*, 1994; Dixon *et al.*, 1995; Karschin *et al.*, 1996). The septum expresses all GIRK1–3 mRNAs although GIRK1 and GIRK4 mRNAs are especially abundant in lateral septal nuclei (Karschin *et al.*, 1994, 1996; DePaoli *et al.*, 1994). In the rat hippocampus, all GIRK mRNAs are strongly expressed by dentate gyrus granule cells and CA1-CA3 pyramidal neurons (Karschin *et al.*, 1994 and 1996; DePaoli *et al.*, 1994; Dixon *et al.*, 1995; Iizuka *et al.*, 1997). Mouse hippocampus is, however, reported not to express GIRK3 mRNA (Kobayashi *et al.*, 1995). In the rat proximal hilus of the dentate gyrus, GIRK2 and GIRK4 mRNAs are more strongly expressed than the GIRK1 transcript (Karschin *et al.*, 1996). Expression of GIRK4 mRNA is most prominent in CA3 pyramidal neurons (Spauschus *et al.*, 1996). Many cells in the entorhinal cortex and the subiculum of the hippocampal formation also express GIRK4 mRNA (Spauschus *et al.*, 1996).

In the thalamus, GIRK1 and GIRK3 mRNAs are abundantly expressed in all nuclei and especially in anterior nuclei, although GIRK2 mRNA is present at high levels in the lateral and geniculate nuclei (Karschin *et al.*, 1994, 1996; Ponce *et al.*, 1996). All large neurons in the rat thalamus seem to coexpress GIRKs1–4 (Karschin *et al.*, 1996), although expression of GIRK2 mRNA was not found in mouse thalamus (Kobayashi *et al.* 1995). In the hypothalamus, GIRK mRNAs are not abundantly expressed (Karschin *et al.*, 1994, 1996; Ponce *et al.*, 1996) although GIRK1 mRNA is expressed only in the ventral medial hypothalamus (DePaoli *et al.*, 1994). The anterior pituitary strongly expresses GIRK1 mRNA (Karschin *et al.*, 1994).

In the midbrain, the superior colliculus contains a very high level of GIRK2 mRNA and a distinct level of expression of GIRK4 transcripts (Spauschus *et al.*, 1996; Karschin *et al.*, 1996; Ponce *et al.*, 1996). The inferior colliculus contains high levels of GIRK1 and GIRK3 but no GIRK2 mRNAs (Karschin *et al.*, 1996; Ponce *et al.*, 1996). Red nuclei abundantly express GIRK1 and GIRK3 mRNAs and also possess GIRK4 transcripts (DePaoli *et al.*, 1994; Karschin *et al.*, 1996; Ponce *et al.*, 1996; Iizuka *et al.*, 1997). In dopaminergic neurons of the substantia nigra pars compacta and the ventral tegmental area, GIRK2 mRNA is expressed at extremely high levels (Karschin *et al.*, 1996; Dixon *et al.*, 1995). GIRK3 mRNA is found throughout the substantia nigra and ventral tegmental area but at significantly lower levels than GIRK2 mRNA. GIRK1 is virtually absent (Karschin *et al.*, 1996; Ponce *et al.*, 1996), although GIRK4 transcripts may exist in these regions (Iizuka *et al.*, 1997).

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Cerebellar granule cell layer has abundant mRNAs for all GIRK1–4 (Karschin *et al.*, 1994 and 1996; Dixon *et al.*, 1995; Iizuka *et al.*, 1997). Purkinje cells express mRNAs for GIRK3 strongly and GIRK4 to a certain extent, but for GIRK1 or GIRK2 only moderately (Spauschus *et al.*, 1996; Karschin *et al.*, 1996; Iizuka *et al.*, 1997). Basket cells have a moderate level of GIRK4 mRNA (Iizuka *et al.*, 1994). The large neurons in the deep cerebellar nuclei contain high levels of GIRK1 and GIRK3 but not GIRK2 transcripts (Karschin *et al.*, 1996; Ponce *et al.*, 1996).

All GIRK transcripts are abundantly expressed in the brain stem (Karschin *et al.*, 1996). Especially a high level of expression of GIRK1 mRNA is observed in pontine nucleus, trapezoid body, pontine reticular formation, superior olivary nuclei, cochlear nuclei, hypoglossal nucleus, and principal and spinal trigeminal nuclei; GIRK2, in vestibular and cochlear nuclei; and GIRK3, in lateral parabrachial nucleus (Karschin *et al.*, 1994, 1996; DePaoli *et al.*, 1994; Ponce *et al.*, 1996). GIRK4 mRNA is also found in hypoglossal, trigeminal, and oculomotor nuclei (Iizuka *et al.*, 1997). GIRK1 mRNA is absent in inferior olivary and solitary nuclei, whereas GIRK2 transcripts are not found in the trapezoid body, inferior olivary nuclei and raphä nuclei (Karschin *et al.*, 1996). Iizuka *et al.* (1997) found that GIRK4 mRNA is relatively strongly expressed in the choroid plexus in the lateral, third, and fourth ventricles. It is uncertain whether other GIRK transcripts exist in this tissue.

Immunohistochemical approaches revealed that the overall distribution pattern of GIRK1, 2, and 4 immunoreactivities in the rat brain is similar to that of the mRNAs for these subunits (Karschin *et al.*, 1994 and 1996; DePaoli *et al.*, 1994; Ponce *et al.*, 1996; Liao *et al.*, 1996; Iizuka *et al.*, 1997). No studies have been done on the distribution of GIRK3 proteins in the brain to our knowledge. It is also known that there are certain discrepancies in distribution between mRNAs and proteins of GIRKs in the brain, indicating that GIRK subunits are translocated into nerve fibers, terminals, and dendrites after synthesized in the somata. The precise subcellular localization of GIRK proteins in neurons in the central nervous system shall be discussed in the section VI.B.

D. Expression of G Protein-Gated K^+ Channels

When cRNAs for GIRK1 and M_2-muscarinic receptor are coinjected into *Xenopus* oocytes, a Kir current induced by ACh is expressed (Kubo *et al.*, 1993b; Dascal *et al.*, 1993). This current mimics at least some characteristics of the K_{ACh} channel current. GIRK1 expressed in *Xenopus* oocytes has been, therefore, successfully used to investigate the structure-function relationship of K_G channels (Reuveny *et al.*, 1994; Slesinger *et al.*, 1995; Kofuji *et al.*, 1996a). However, Krapivinsky *et al.* (1995a) proposed that the K_{ACh} channel in cardiac atria is a heteromultimer of GIRK1 and GIRK4 rather than a homomultimer of GIRK1 because GIRK1 and GIRK4 proteins are immunocoprecipitated by both specific anti-GIRK4 and anti-GIRK1 antibodies from atrial membrane preparation (fig. 9A). Furthermore, coexpression of GIRK1 and GIRK4 in *Xenopus* oocytes yields greatly enhanced K_G channel currents compared with expression of either of the subunits alone (fig. 9B). Now, it is generally believed that GIRK1 is inactive by itself because expression of GIRK1 alone fails to give rise to K_G channel currents in different mammalian cell lines including CHO, COS, and HEK cells (Krapivinsky *et al.*, 1995a; Philipson *et al.*, 1995; Spauschus *et al.*, 1996; Wischmeyer *et al.*, 1997). It is likely that functional expression of GIRK1 alone is possible in *Xenopus* oocytes because oocytes endogenously express GIRK5 whose amino acid sequence is 78% identical with that of GIRK4 (Hedin *et al.*, 1996). However, GIRK2 and GIRK4, but not GIRK3, may be able to form functional homomeric K_G channels, although not very efficiently (Lesage *et al.*,

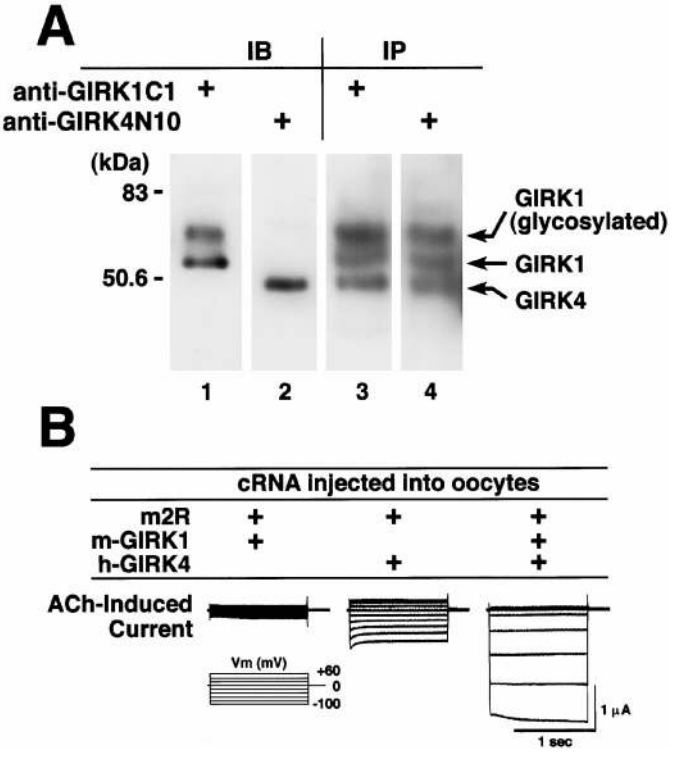

FIG. 9. Heteromultimeric structure of the muscarinic K^+ channel. A: Immunological analysis for mouse atrial muscarinic K^+ channel. Atrial membrane proteins were immunoblotted (IB) with anti-GIRK1C1 (lane 1) and anti-GIRK4N10 (lane 2) antibodies specific to GIRK1 and GIRK4 proteins, respectively. Some part of the GIRK1 protein, but not GIRK4 protein, seems to be glycosylated in the atrium. Lanes 3 and 4, the imunoprecipiants (IP) obtained from biotinylated mouse atrial membranes with the anti-GIRK1C1 and anti-GIRK4N10 antibodies, respectively. Both immunocomplexes of anti-GIRK1C1 and anti-GIRK4N10 seemed to be composed of three proteins that had molecular weights identical with GIRK1 and GIRK4 proteins on the gel. B: Acetylcholine-(Ach) induced K^+ currents observed in *Xenopus* oocytes expressing M_2-muscarinic receptors (m2R) plus mouse GIRK1 (m-GIRK1) and/or human GIRK4 (h-GIRK4). Acetylcholine- (1 μM) induced K^+ currents at different membrane potentials were measured in the presence of 96 mM external K^+ and are shown under the table. The voltage-clamp protocol is depicted at the left lower corner.

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1994 and 1995; Bond *et al.*, 1995, Krapivinsky *et al.*, 1995a; Duprat *et al.*, 1995; Kofuji *et al.*, 1995; Velimirovic *et al.*, 1996; Wischmeyer *et al.*, 1997).

Heteromultimerization of GIRK subunits occurs not only between GIRK1 and GIRK4 but within any pairs of GIRK1-4 subunits (Kofuji *et al.*, 1995; Iizuka *et al.*, 1995; Duprat *et al.*, 1995; Lesage *et al.*, 1995; Spauschus *et al.*, 1996; Velimirovic *et al.*, 1996; Isomoto *et al.*, 1996; Wischmeyer *et al.*, 1997). Coexpression of GIRK1 with either of GIRKs2-4 generally yields several- to many-fold larger macroscopic currents than the sum of those obtained with each subunit alone. However, it has not been unequivocally answered whether similar synergistic interaction also occurs between GIRKs2-4. The synergistic enhancement of current expression was reported to occur with GIRK2/GIRK4 (Duprat *et al.*, 1995; Ferrer *et al.*, 1995) and GIRK3/GIRK4 combinations (Spauschus *et al.*, 1996). However, these results were not necessarily confirmed by the others (Lesage *et al.*, 1995; Velimirovic *et al.*, 1996; Wischmeyer *et al.*, 1997). Coexpression of GIRK3 with GIRK2 was shown to suppress GIRK2 channel currents (Kofuji *et al.*, 1995). It is not clear whether more than two types of GIRK subunits can be assembled into a single K_G channel (Wischmeyer *et al.*, 1997).

GIRK1/GIRK4 heteromultimeric K_G channels are likely to correspond to cardiac K_{ACh} channels as mentioned in this Section. GIRK1/GIRK2 channels, however, may represent some neuronal type of K_G channels for the following reasons: (*a*) GIRK2 mRNA is preferentially expressed in the brain (table 3); (*b*) GIRK1 and GIRK2 exhibit overlapping distribution in many areas of the brain at both the mRNA and protein levels (Karschin *et al.*, 1996; Liao *et al.*, 1996); (*c*) both specific anti-GIRK1 and anti-GIRK2 antibodies coimmunoprecipitate GIRK1 and GIRK2 proteins from membrane preparations of the brain (Liao *et al.*, 1996); (*d*) in the mice whose GIRK2 genes are genetically deleted (GIRK2 -/-), the substantial amount of GIRK1 proteins is concomitantly lost in the brain (Signorini *et al.*, 1997); and (*e*) the hippocampal CA1 and CA3 pyramidal neurons of these mice fail to exhibit postsynaptic inhibitory K_G channel currents in response to different inhibitory neurotransmitters (Lüscher *et al.*, 1997). Some neuronal K_G channels may also be composed of GIRK1 and GIRK3 because their transcripts are also expressed together in various regions of the brain (Karschin *et al.*, 1996). It is also possible that GIRK4 is included in neuronal K_G channels (Spauschus *et al.*, 1996; Iizuka *et al.*, 1997) and that some K_G channels are homomultimers of GIRKs2 or 4 or heteromultimers of GIRKs2–4.

Chan *et al.* (1996) found that the synergistic interaction between GIRK1 and GIRK4 for K_G channel current expression can be at least in part ascribed to interaction between phenylalanine at position 137 (F137) in the H5 region of GIRK1 and serine at the corresponding site (S143) in GIRK4 (fig. 8). GIRKs2 and 3 also have conserved serine at this site. They found that coexpression of the wild-type GIRK4 with the mutant GIRK4 whose S143 was replaced with phenylalanine [GIRK4(S143F)] yielded the significantly larger macroscopic current amplitude than the sum of those obtained with either of the subunits alone, as is the case for GIRK1/GIRK4 coexpression. At the single-channel level, GIRK4(S143F)/GIRK4 channels, like GIRK1/GIRK4 channels, opened in clearer bursts and exhibited a significantly longer open time than GIRK4 homomeric channels (Krapivinsky *et al.*, 1995a; Chan *et al.*, 1996). Thus, F137 of GIRK1 may be responsible for the larger macroscopic current amplitude of GIRK1/GIRK4 than GIRK4 channels by stabilizing the channel's open-state conformation. Wischmeyer *et al.* (1997) also obtained similar results by using a mutant GIRK3 subunit bearing a mutation corresponding to GIRK4(S143F) [i.e., GIRK3(S114F)].

However, neither GIRK4(S143F) nor GIRK3(S114F) synergistically interacted with GIRK1 (Chan *et al.*, 1996; Wischmeyer *et al.*, 1997). GIRK1 whose F137 was substituted with serine [GIRK1(F137S)] could form a functional homomeric channel (Chan *et al.*, 1996; Wischmeyer *et al.*, 1997), although coexpression of GIRK1(F137S) with the wild-type GIRK1 yielded current amplitudes intermediate between those obtained with either of the subunits alone (Wischmeyer *et al.*, 1997). These results indicate that F137 of GIRK1 on its own is inhibitory for the K^+ ion flux through the channel pore possibly because of its bulky aromatic side chain (Wischmeyer *et al.*, 1997). Thus, serine derived from other types of GIRK subunits might somehow attenuate this inhibitory effect of F137 and yield the larger current amplitudes of the heteromeic GIRK1-containing channels than those obtained with expression of GIRK1 alone.

It is, however, difficult to explain whole the aspect of the synergistic interaction between GIRK1 and other GIRK subunits only in terms of the interaction between complementary phenylalanine/serine residues in the heteromeric channel pore. For example, GIRK1(F137S)/GIRK4 channels exhibit much larger currents than the GIRK4 homomeric channels (Chan *et al.*, 1996). GIRK1/GIRK4 channels have ~2 times larger macroscopic currents than GIRK4(S143F)/GIRK4 channels. Thus, some region(s) other than F137 of GIRK1 must also be significantly involved in the synergistic interaction between GIRK1 and GIRK4. Similarly, GIRK1(F137S)/GIRK4 channels have substantially larger current amplitudes than homomeric GIRK1(F137S) channels (Chan *et al.*, 1996), indicating that GIRK4 also has some effect(s) on GIRK1 which cannot be ascribed to the serine/phenylalanine interaction.

Kennedy *et al.* (1996) found that GIRK1 cannot translocate to the cell membrane in the absence of GIRK4. They expressed epitope-tagged GIRK1 and GIRK4 in COS cells alone or in combination and examined the localization of the subunits with immunofluorescence

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labeling. When expressed alone, GIRK1 was localized to the cytosol associated with intracellular intermediate filament proteins, whereas GIRK4 was primarily on the plasma membrane. GIRK1 was detected on the plasma membrane when coexpressed with GIRK4. Therefore, GIRK4 may facilitate the membrane-translocation of GIRK1 subunits. It has, however, not been shown whether GIRK2 and GIRK3 also have similar effect of translocation of GIRK1 to the membrane.

E. Tetrameric Structure

Yang *et al.* (1995a) found that the inward rectification of the IRK1 channel was substantially attenuated by replacement of aspartate (D) at position 172 in the M2 region with asparagine (N) and substitution of glutamate (E) at position 225 in the proximal carboxyl terminal region with lysine (K). They analyzed the subunit stoichiometry of the IRK1 channel by using the double mutation. They formed tandem tetramers or trimers consisting of different numbers of wild-type subunits and/or subunits bearing the mutation. When either the tetramer or trimer of the wild-type subunits was expressed in *Xenopus* oocytes, the resultant channel currents exhibited the same extent of inward rectification as those obtained from expression of the wild-type monomers. This was also the case when the tetramer was coexpressed with an excess amount of the mutant monomers, indicating that the number of subunits required for a functional IRK1 channel does not exceed four. In contrast, the inward rectification of the channel currents resulting from expression of wild-type trimers was significantly attenuated by coexpression of the mutant monomers. In this case, the channel currents exhibited approximately the same extent of inward rectification as those obtained from expression of a tetramer consisting of three wild-type subunits and one mutant subunit. These data suggest that a functional IRK1 channel is formed by a tetramer of IRK1 subunits.

Biochemical measurement of the molecular weight of brain K_G channel proteins suggested that these channels also have tetrameric structure (Inanobe *et al.*, 1995a). As stated earlier, GIRK2 and GIRK4 can form functional homomeric channels (fig. 10A) (Lesage *et al.*, 1995; Krapivinsky *et al.*, 1995a; Duprat *et al.*, 1995; Bond *et al.*, 1995; Kofuji *et al.*, 1995; Velimirovic *et al.*, 1996). However, GIRK1 requires other GIRK subunits to form functional K_G channels (Krapivinsky *et al.*, 1995a; Duprat *et al.*, 1995; Velimirovic *et al.*, 1996; Hedin *et al.*, 1996; Chan *et al.*, 1996; Wischmeyer *et al.*, 1997). Tucker *et al.* (1996) assessed the stoichiometry and relative subunit positions in the GIRK1/GIRK4 heteromeric K_G channel by using tandemly linked tetramers consisting of GIRK1 and GIRK4. They found that the most efficient channel comprises two subunits of each type in an alternative array within the tetramer (fig. 10Ba). Through a similar approach, Silverman *et al.* (1996) found that more than one kind of subunit arrangement including

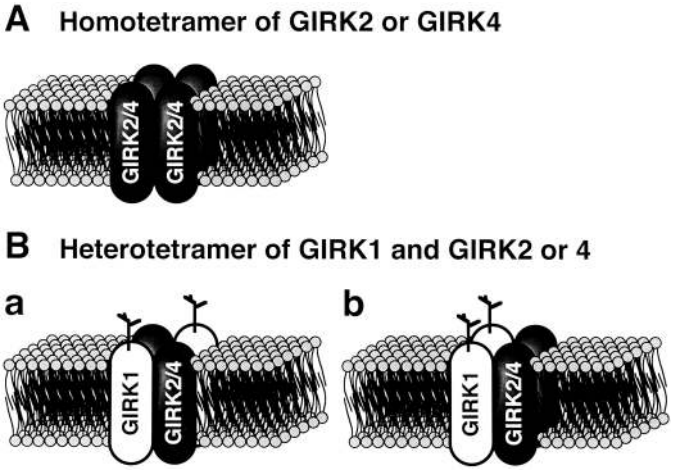

FIG. 10. Tetrameric structure of IRK(Kir2.x) and GIRK(Kir3.x) channels. A putative tetrameric structure of the channels composed of IRK or GIRK subunits in the cell membrane is schematically represented according to the functional and structural studies reported by Lesage *et al.* (1994 and 1995), Krapivinsky *et al.* (1995a), Velimirovic *et al.* (1996), Yang *et al.* (1995b), Silverman *et al.* (1996), and Tucker *et al.* (1996).

(GIRK1) (GIRK1) (GIRK4) (GIRK4) may also be viable (fig. 10Bb). They also obtained similar data with GIRK1/GIRK2 heteromers.

Tinker *et al.* (1996) studied the molecular mechanism of homomeric assembly of IRK1. They concluded that among IRK1, IRK2, and IRK3, the proximal C-terminus and the M2 region equally contribute to polymerization. The proximal C-terminus plays a more significant role in prevention of heteromultimerization between more distantly related channel subunits, such as IRK1 and ROMK1. Tucker *et al.* (1996), however, found that the core region of the GIRK subunit (M1-H5-M2) but neither the C- nor N-terminal domain was important for subunit assembly between GIRK1 and GIRK4. Thus, the mechanism of heteromultimerization of GIRK subunits may not be the same as that of IRK subunits.

F. Molecular Mechanism Underlying G Protein Activation of G Protein-Gated K^+ Channels

1. Interaction between G protein $\beta\gamma$ subunits and subunits of G protein-gated K^+ channels.

a. THE G PROTEIN $\beta\gamma$ SUBUNIT-BINDING DOMAINS IN GIRK1 SUBUNITS. GIRK1 has a significantly longer C-terminus than the constitutively active Kir channel subunits such as IRK1 (Kubo *et al.*, 1993a,b). Reuveny *et al.* (1994) first suggested that the C-terminus of GIRK1 includes an amino acid sequence (between positions 318 and 455) exhibiting a certain level of similarity (~26%) with that of the $G_{\beta\gamma}$-binding site of βARK1 (fig. 8). They also found that truncation of the C-terminus of GIRK1 at leucine (L) at position 403 but not at proline (P) at position 462 resulted in loss of functional expression of a K_G channel in *Xenopus* oocytes coexpressing $G_{\beta1\gamma2}$. Inanobe *et al.* (1995b) directly demonstrated that $G_{\beta\gamma}$ bound to a glutathione S-transferase (GST) fusion protein including the whole C-terminal domain of GIRK1

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(between positions 180 and 501). The fusion protein also bound $G_{\beta\gamma}$ when incubated with purified trimeric G_i in the presence of GTPγS but not in the presence of GDP (see also Inanobe *et al.*, 1995a). Furthermore, the binding of $G_{\beta\gamma}$ to the fusion protein was prevented by $G_{\alpha\text{-GDP}}$ but not $G_{\alpha\text{-GTP}\gamma S}$, indicating that the C-terminal domain of GIRK1 cannot bind with $G_{\beta\gamma}$ included in the trimeric form of the G protein.

By using fusion proteins containing different deleted mutants of the C-terminal domain of GIRK1, Huang *et al.* (1995) narrowed down the $G_{\beta\gamma}$-binding region in the C-terminus of GIRK1 to a 190 amino acid stretch (between positions 273 and 462). $G_{\beta\gamma}$ interacted with the fusion protein in ~1: 1 stoichiometry with calculated K_d of ~0.5 μM. They further found that the $G_{\beta\gamma}$-binding domain was composed of two separate segments between positions 318 and 374 and between positions 390 and 462 (Huang *et al.*, 1997) (figs. 8 and 11). This latter segment did not exhibit a significant $G_{\beta\gamma}$-binding activity by itself but enhanced the $G_{\beta\gamma}$-binding activity of the other segment. The segment between residues 390 and 462 contains a short amino acid sequence similar to the asparagine-X-X-glutamate-arginine (N-X-X-E-R) motif in adenylyl cyclase 2 which is believed to be critical for regulation of the enzyme by $G_{\beta\gamma}$ (fig. 8) (Chen *et al.*, 1995; Huang *et al.*, 1995).

Interestingly, Huang *et al.* (1995, 1997) found that $G_{\beta\gamma}$ also bound to a segment of the N-terminal domain of GIRK1 (between positions 34 and 86) (figs. 8 and 11). The $G_{\beta\gamma}$-binding to the fusion protein of the N-terminus also occurred in 1:1 stoichiometry but exhibited ~10 times lower affinity than $G_{\beta\gamma}$-binding to C-terminal fusion proteins. They also found that the fusion proteins of the N- and C-terminal domains bound together and synergistically enhanced the $G_{\beta\gamma}$-binding activity of each other (Huang *et al.*, 1997).

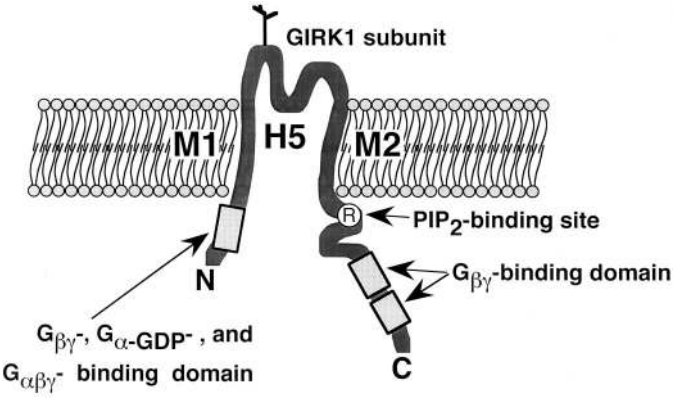

FIG. 11. Domains of GIRK1 involved in G protein-mediated regulation of GIRK1-containing K_G channels. Schematic representation of approximate positions of identified domains of GIRK1 which are responsible for interaction of GIRK1 and G protein subunits or phosphatidilinositol 4,5-bisphosphate (PIP_2). The approximate positions of three identified G protein βγ-binding sites (one in the N- and two in the C-terminal domain) and one trimeric G protein and/or GDP-from of G protein α subunit-binding site in the N-terminal domain are depicted (Huang *et al.*, 1995, 1997; Slesinger *et al.*, 1995). R is the residue corresponding to the arginine of ROMK1 that was reported to play a critical role in interaction of ROMK1 channels with PIP_2 (Huang *et al.*, 1998).

The interaction between $G_{\beta\gamma}$ and the cytoplasmic domains of GIRK1 may indeed underlie the $G_{\beta\gamma}$-induced activation of K_G channels. Huang *et al.* (1995) constructed synthetic peptides possessing the partial amino acid sequence of the predicted $G_{\beta\gamma}$-binding domains of the N- and C-termini. These synthetic peptides inhibited not only the binding of $G_{\beta\gamma}$ to the corresponding fusion proteins, but suppressed GIRK1-containing K_G channel currents activated by $G_{\beta1\gamma2}$. Slesinger *et al.* (1995) expressed chimeras of IRK1 and GIRK1 in *Xenopus* oocytes and examined the response of the resultant channel currents to $G_{\beta1\gamma2}$. The $G_{\beta1\gamma2}$-induced increase in channel activity was observed only when the chimeras contained the N- (between positions 31 and 85) and/or the C-terminal (between positions 325 and 501) domain of GIRK1. A similar result was reported by using chimeras of GIRK1 and IRK2 (Kunkel and Peralta, 1995).

Yan and Gautam (1996) showed with the yeast two-hybrid system that G_β bound with the N-terminus of GIRK1. Different types of G_β interacted with the N-terminal domain of GIRK1 with distinct efficacies. An N-terminal fragment of 100 amino acids of G_β interacted with the N-terminal domain of GIRK1 as effectively as the whole G_β. This N-terminal domain of G_β includes the region responsible for the interaction between G_β and G_α according to the analysis of the crystal structure (Wall *et al.*, 1995; Lambright *et al.*, 1996). Thus, $G_{\alpha\text{-GDP}}$ might prevent $G_{\beta\gamma}$ from interacting with K_G channels by competing with the N-terminus of GIRK1 on the N-terminus of G_β. Binding of G_β to the C-terminus of GIRK1 was not clearly detected in this study. Other domains of G_β or Gγ might, therefore, participate in the interaction between $G_{\beta\gamma}$ and the C-terminus of GIRK1. At present, the whole aspect of the molecular interaction between $G_{\beta\gamma}$ and K_G channel subunits has not been clarified.

b. THE G PROTEIN βγ SUBUNIT-BINDING DOMAINS IN OTHER SUBUNITS OF G PROTEIN-GATED K^+ CHANNELS. Homomeric GIRK2 or GIRK4 channels are also activated by $G_{\beta\gamma}$ (Krapivinsky *et al.*, 1995a; Velimirovic *et al.*, 1996). It was also reported that GIRK4 could mediate the activation of the GIRK1/GIRK4 heteromeric channel by $G_{\beta\gamma}$ (Slesinger *et al.*, 1995; Tucker *et al.*, 1996).

GIRKs2-4 have domains whose amino acid sequences are similar to those of the $G_{\beta\gamma}$-binding domains in the N-terminus and the proximal C-terminus of GIRK1 (between positions 34 and 86 and between residues 318 and 374, respectively) (fig. 8). However, they lack a region corresponding to that at the distal C-terminus of GIRK1 (between positions 390 and 462). Huang *et al.* (1997) showed that the $G_{\beta\gamma}$-binding activity was similar among the N-terminal domains of GIRKs1-4, although the C-terminal domains of GIRKs2-4 exhibited slightly lower $G_{\beta\gamma}$-binding activity than that of GIRK1. They also found that the C-terminal domain of GIRK1 interacted with the N-terminus of GIRK4 and thereby synergisti-

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cally enhanced the $G_{\beta\gamma}$-binding activity (Huang *et al.*, 1997). Therefore, the high-affinity $G_{\beta\gamma}$-binding site in the GIRK1/GIRK4 heteromeric channel might be formed through interaction of the C-terminus of GIRK1 subunit with the N-terminus of the GIRK1 and/or GIRK4 subunits. This interaction may at least in part underlie the higher channel activity yielded by coexpression of GIRK1 and GIRK4 than expression of either of the GIRK subunits alone.

2. Mechanism underlying G protein βγ subunit-induced activation of G protein-gated K^+ channels. As described in Sections II.B.3. and 4., functional analyses of the K_{ACh} channel indicate that the G protein-mediated activation of the channel results from an increase in the functional number of the channels due to the cooperative interaction of $G_{K\beta\gamma}$ and GIRK subunits (Hosoya *et al.*, 1996). However, the molecular mechanism responsible for this phenomenon has not been clearly identified.

Slesinger *et al.* (1995) suggested that the N-terminal domain of GIRK1 may function to suppress the $G_{\beta\gamma}$-independent basal current because chimeras of GIRK1 and IRK1 showed a lower basal activity when they included the N-terminal domain of GIRK1. Dascal *et al.* (1995) proposed that the C-terminal domain of GIRK1 may block the K_G channel pore in a way similar to the "Shaker ball" of the K_v channels because a myristoylated cytosolic C-terminal tail of GIRK1 suppressed the GIRK1 or ROMK1 channel currents. These results suggest that the K_G channel might be intrinsically inhibited by the C- and/or N-terminal domains of GIRK1, and that $G_{K\beta\gamma}$ might activate the channel by removing the inhibition.

Slesinger *et al.* (1995) found that the whole-cell channel currents of IRK1 whose distal C-terminal region was replaced with a part of the C-terminal domain of GIRK1 (between positions 325 and 501) were doubled when coexpressed with $G_{\beta1\gamma2}$ resulted in *Xenopus* oocytes. The doubling of the whole-cell channel current could not be explained only in terms of an increase in P_o because the chimeric channel possessed a P_o as high as ~0.8 in the absence of $G_{\beta1\gamma2}$ at the single channel level. These results support the aforementioned notion that G_K activates the K_{ACh} channel by increasing the functional number of the channels without modulating the fast open-close transitions of the channels (Hosoya *et al.*, 1996).

Huang *et al.* (1998) recently proposed that PIP_2 is critically involved in $G_{\beta\gamma}$-induced activation of K_G channels. They found that PIP_2 by itself induced the maximum activity of heteromeric GIRK1/GIRK4 and homomeric GIRK2 channels in inside-out patch membranes of *Xenopus* oocytes. This PIP_2-induced response seemed to result from direct interaction of PIP_2 with the C-terminus of GIRK subunits because PIP_2 directly bound GST-fusion protein of the C-terminus of GIRK1 in vitro and because the PIP_2-induced channel activity was not inhibited by exogenously applied $G_{i\alpha}$ subunits (probably in the GDP-bound form). However, the PIP_2-induced enhancement of channel activity was not unique to the GIRK channels but also found in ROMK1 and IRK1 channels that had run down in the inside-out configuration. Furthermore, ROMK1 and IRK1 channels were more potently activated by PIP_2 than the K_G channels. However, $G_{\beta\gamma}$ could not activate GIRK1/GIRK4 channels 10 min after patch excision where PIP_2 was expected to be depleted from the membrane. The channels preincubated with $G_{\beta\gamma}$ under these conditions, however, exhibited much higher PIP_2 sensitivity than the control. Specific anti-PIP_2 antibodies suppressed GIRK1/GIRK4 channel currents more slowly in the presence than the absence of $G_{\beta\gamma}$. From these results, they concluded (*a*) that the direct interaction of PIP_2 with the C-terminus of GIRK subunits is prerequisite for K_G channel activity, whereas in the absence of $G_{\beta\gamma}$, the channel has significantly lower PIP_2 sensitivity than other Kir channels and (*b*) that $G_{\beta\gamma}$ activates K_G channels by increasing the PIP_2 sensitivity of GIRK subunits. Therefore, from their point of view, the deactivation of K_G channels is the rundown commonly observed with different types of Kir channels, although the activation of K_G channels is the reactivation of the rundown K_G channels. In this context, $G_{\beta\gamma}$ is a regulator of rundown/reactivation of K_G channels. They suggested that the interaction between the pore-forming C-terminus of GIRK as well as other Kir subunits with PIP_2 in the membrane might lead to opening of the channel pore and that the synergistic interaction between $G_{\beta\gamma}$ and PIP_2 might occur through the pleckstrin homology domain in the C-terminus of GIRK subunits (fig. 11). Although this hypothesis is very attractive, some precautions may be necessary to extrapolate this hypothesis into the mechanism responsible for the physiological activation of native K_G channels by $G_{\beta\gamma}$. For example, in inside-out patch membranes of cardiac myocytes, I_{K1} channels, which may be homomeric IRK2 channels and thus are expected to be more sensitive to PIP_2 than K_G channels, usually run down very promptly. However, the K_{ACh} channel, which may be less sensitive to PIP_2 than the I_{K1} channel, can be consistently and strongly activated by $G_{\beta\gamma}$ applied even tens minutes after patch excision (Kurachi, 1995). Therefore, it may be important to analyze the effect of PIP_2 on the interaction between $G_{\beta\gamma}$ and native K_G channels.

3. Interaction between subunits of G protein-gated K^+ channels, G_α proteins, and membrane agonist receptors. Huang *et al.* (1995) found that $G_{\alpha\text{-GDP}}$ and the entire heterotrimeric G protein can bind to fusion proteins containing the N-terminal domain of GIRK1 (fig. 11). This observation raises the possibility that membrane agonist receptors, G_K, and K_G channels, might form a functional complex in native K_G channel systems. Namely, once G_K associated with a GIRK1 subunit is activated, the dissociated $G_{K\beta\gamma}$ would promptly access

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the $G_{K\beta\gamma}$-binding site on the GIRK1 subunit. The $G_{K\alpha\text{-GTP}}$ dissociated from the G_K, however, may be quickly converted to $G_{K\alpha\text{-GDP}}$ by the aid of RGS proteins, leading to reassociation of $G_{K\alpha\text{-GDP}}$ with the GIRK1 subunit. This $G_{K\alpha\text{-GDP}}$ would effectively sequestrate the $G_{K\beta\gamma}$ from the $G_{K\beta\gamma}$-binding site on the GIRK1 subunit, and the cycle would be complete. Indeed, it has been shown that K_G channels coexpressed with RGS proteins in *Xenopus* oocytes are deactivated much faster than expected from the intrinsic rate of $G_{\beta\gamma}$ dissociation from GIRK subunits (Doupnik *et al.*, 1997; Krapivinsky *et al.*, 1995c; Saitoh *et al.*, 1997). In the classical view of receptor/G protein interaction (Levitzki, 1981), association of an agonist-bound receptor with $G_{\alpha\text{-GDP}}$ is the rate-limiting step for the receptor-mediated activation of effectors. However, the fast conversion of $G_{K\alpha\text{-GTP}}$ to $G_{K\alpha\text{-GDP}}$ due to the enhanced GTP_i hydrolysis rate evoked by RGS proteins might not provide a sufficient time for $G_{K\alpha\text{-GTP}}$ to be dissociated from receptor. In the continuous presence of an agonist bound to the receptor, therefore, the receptor would promptly restart the next round of the G protein cycle. The resultant increase in GDP/GTP exchange might balance the accelerated GTPase activity and thereby maintain the steady-state concentrations of $G_{K\alpha\text{-GTP}}$ and $G_{K\beta\gamma}$ at a certain level, which would explain why RGS proteins do not significantly decrease the steady state response of the K_G channel system (Doupnik *et al.*, 1997; Saitoh *et al.*, 1997). A distinct but similar hypothesis of a functional receptor/G protein/effector complex was recently proposed by Ross' group based on an extensive analysis of the reconstituted M_1-muscarinic/G_q/phospholipase C-β1 system (Biddlecome *et al.*, 1996).

The maximum response of the K_{ACh} channel is reached within several hundred milliseconds after application of ACh (fig. 1) (Breitwieser and Szabo, 1988). Neuronal K_G channels are also known to respond to agonist in a comparable fast time course (Surprenant and North, 1988; Sodickson and Bean, 1996). The model of G protein/RGS protein/channel interaction described above might be applicable to the steady-state response in the continuous presence of agonists, but cannot explain this initial fast response. Slesinger *et al.* (1995) raised the possibility that GIRK1 may directly interact with M_2-muscarinic receptors through its hydrophobic core region (M1-H5-M2). They found that a chimera of IRK1 and GIRK1 containing the C-terminus but not the hydrophobic core region of GIRK1 could be activated by coexpressed $G_{\beta1\gamma2}$, but not through coexpressed M_2-muscarinic receptors. The ability to respond to receptor stimulation was endowed by transplantation of the hydrophobic core region of GIRK1 to the chimera. When the receptor is indeed kept in the vicinity of G_K through association with a GIRK1 subunit, the agonist-induced initial response is expected to be substantially accelerated. However, this hypothesis needs further verification because Kofuji *et al.* (1996a) showed that GIRK1 whose hydrophobic core region was substituted by that of ROMK1 could be activated through coexpressed M_2-muscarinic receptors. In addition, it has not been examined whether there are additional molecules which impose a topological restriction on the receptor/G_K/K_G channel system which enables efficient signal transmission between the molecules. These issues remain unanswered.

4. Possible mechanisms underlying specific signal transduction in the receptor/G protein/G protein-gated K^+ channel system. In atrial myocytes, the K_{ACh} channel is activated by stimulation of M_2-muscarinic and A_1 adenosine receptors. However, β_1-adrenergic stimulation, which would also induce dissociation of $G_{\beta\gamma}$ from G_s, never activates the K_{ACh} channel in cardiac atrial myocytes.

Such specific signal transduction cannot be explained in terms of the different affinities of distinct types of $G_{\beta\gamma}$ for K_G channels. Wickman *et al.* (1994) compared the effects of recombinant $G_{\beta\gamma}$s ($G_{\beta1\gamma1}$, $G_{\beta1\gamma2}$, $G_{\beta1\gamma5}$, $G_{\beta1\gamma7}$, $G_{\beta2\gamma5}$, and $G_{\beta2\gamma7}$) on the K_{ACh} channel in inside-out patch membranes of atrial myocytes, and found less than 10-fold difference in potency among these $G_{\beta\gamma}$s except for $G_{\beta1\gamma1}$. $G_{\beta1\gamma1}$ is a major component of $G_{\beta\gamma}$ of the retinal G protein, transducin. Native transducin $\beta\gamma$ subunits are more than 100-fold less potent than other $G_{\beta\gamma}$s (Yamada *et al.* 1994a). A lack of specificity of $G_{\beta\gamma}$ in vitro was confirmed when K_G channel currents in *Xenopus* oocytes expressing GIRK1 could be activated through coexpressed β_2-adrenergic receptors (Lim *et al.*, 1995).

An alternative explanation for receptor-specific transduction may be that a particular set of receptor, G protein, and GIRK subunits is compartmentalized into a microdomain in the native K_G channel system. However, when ACh and adenosine are applied together to cardiac atrial myocytes they activate the current in a less-than-additive manner (Kurachi *et al.*, 1986a,b; Bünemann *et al.*, 1995). Similar phenomena have been reported for different K_G channel systems (North, 1989) including stimulation of 5-HT_{1A} and $GABA_B$ receptors in rat hippocampal CA1 pyramidal cells (Andrade *et al.*, 1986), μ-opioid, M_2-muscarinic, and $GABA_B$ receptors in rat lateral parabrachial neurons (Christie and North, 1988), and $GABA_B$ and D_2 dopamine receptors in rat substantia nigra zona compacta neurons (Lacey *et al.*, 1988). Thus, if compartmentalization existed, it would have to be formed in such a way that certain classes of receptors are excluded from a K_G channel system, although the others can share the system. The molecular mechanism by which this may be achieved remains to be elucidated.

IV. Voltage-Dependent Properties of G Protein-Gated K^+ Channels

In previous sections, we considered the G protein-mediated activation of K_G channels. In this section, we will deal with voltage-dependent properties of K_G chan-

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nels. As described in the section II.B.1., all K_G channels exhibit the inward rectification property where ionic currents flow through K_G channels more readily in the inward than the outward direction. The mechanism of inward rectification has been mainly studied with the constitutively-active, classical Kir channels such as IRK1 channels. Most of our understanding of the mechanism of inward rectification of K_G channels is, therefore, based on these results. However, the inward rectification of K_G channels is not identical with that of the classical Kir channels especially in terms of its voltage-dependency and kinetics. In this section, we first review the present understanding of the mechanism of inward rectification of the classical Kir channels, and then deal with specific issues regarding K_G channels.

A. Inwardly-Rectifying K^+ Channels

1. Voltage-dependent change in inwardly rectifying K^+ channel activity. K^+ channels mediate a flow of K^+ ions depending on an electrochemical gradient of K^+ ions across the plasma membrane (i.e., V_m - E_K) (Hille, 1992b). Thus, the macroscopic K^+ current flowing through K^+-selective channels can be expressed as:

$$I = gK^*(V_m - E_K) \qquad [3]$$

where I is a macroscopic K^+ current, and gK is a macroscopic chord conductance of the K^+ channels. Comparison of equations 2 and 3 indicates that gK is $N^*P_o^*\gamma$. If gK is constant, the I-V relationship should be linear. In the presence of the physiological transmembrane gradient of K^+ concentration; however, it slightly bends outward as predicted by the constant field theory (Hille, 1992b).

The inward rectification of Kir channels occurs because gK decreases as V_m increases in the positive direction. The relationship between gK and V_m (G-V relationship) is usually expressed by the following Boltzmann's equation:

$$\frac{gK}{gK_{max}} = \frac{1}{1 + exp\left(\frac{\Delta V - \Delta V_h}{v}\right)} \qquad [4]$$

where gK_{max} is the maximum gK available at sufficiently negative V_m, ΔV is the V_m relative to E_K (i.e., V_m - E_K), ΔV_h is ΔV at which gK is the half-maximum, and v is a slope factor that has a dimension of voltage and indicates how steeply gK decreases as ΔV increases (Hagiwara and Takahashi, 1974). This equation indicates that gK of Kir channels is a function of the V_m relative to E_K but not the absolute value of V_m. When $[K^+]_o$ is changed, neither ΔV_h nor v significantly changes (Hagiwara and Takahashi, 1974; Hagiwara and Yoshii, 1979; Leech and Stanfield, 1981; Gunning, 1983; Kurachi, 1985; Harvey and Ten Eick, 1988; Cohen *et al.*, 1989; Silver and DeCoursey, 1990). However, when intracellular K^+ concentration is changed, this rule does not hold (Hagiwara and Yoshii, 1979; Leech and Stanfield, 1981; Cohen *et al.*, 1989; Silver and DeCoursey, 1990). Therefore, the gK of Kir channels may be determined by V_m and $[K^+]_o$. The gK_{max} increases approximately in proportion to the square root of $[K^+]_o$ (Hagiwara and Takahashi, 1974) due to the $[K^+]_o$-dependent increase in γ (Sakmann and Trube, 1984a).

Kir channels are significantly heterogenous in terms of their G-V relationship (Hille, 1992b). The constitutively-active, classical Kir channels composed of the IRK subunits have the steepest G-V relationship (strong inward rectifiers) (Kubo *et al.*, 1993a; Takahashi *et al.*, 1994; Morishige *et al.*, 1994; Makhina *et al.*, 1994; Pärier *et al.*, 1994). The v and ΔV_h values for the strong inward rectifiers are normally 5 to 10 mV and ~−10 mV, respectively (Leech and Standfield, 1981; Hestrin, 1981; Gunning, 1983; Kurachi, 1985; Tourneur *et al.*, 1987; Harvey and Ten Eick, 1988; Cohen *et al.*, 1989; Silver and DeCoursey, 1990). Thus, the gK of the channels is approximately one fourth of the gK_{max} at E_K and becomes virtually negligible around E_K + *60 mV*. Strong inward rectifiers can, therefore, hold V_m close to E_K in the absence of action potentials, but their outward currents are effectively shut off once an action potential is generated. However, ROMKs, BIR, and uK_{ATP}-1 channels are weak inward rectifiers because they only marginally rectify at highly depolarized potentials (Ho *et al.*, 1993; Inagaki *et al.*, 1995a; Yamada *et al.*, 1997). In the presence of a physiological K^+ gradient, they can even exhibit outwardly rectifying I-V curves as predicted by the constant-field theory. Therefore, these channels strongly impair action potential generation and fix V_m to E_K. The extent of inward rectification of K_G channels and K_{AB}-2 channels is intermediate between those of the strong and weak inward rectifiers (Kubo *et al.*, 1993b; Bond *et al.*, 1994 and 1995; Lesage *et al.*, 1995; Krapivinsky *et al.*, 1995a; Duprat *et al.* 1995; Kofuji *et al.*, 1995; Doupnik *et al.*, 1995b; Takumi *et al.*, 1995; Isomoto *et al.*, 1996; Velimirovic *et al.*, 1996).

When V_m is suddenly shifted from one value to another, strong inward rectifiers alter gK through at least two kinetically distinct processes (Leech and Standfield, 1981; Hestrin, 1981; Gunning, 1983; Kurachi, 1985; Tourneur *et al.*, 1987; Harvey and Ten Eick, 1988; Cohen *et al.*, 1989; Silver and DeCoursey, 1990; Newman, 1993). Upon depolarization, gK decreases first instantaneously to a certain extent and then continues to decline slowly to the steady state level. The reverse is true for hyperpolarization of the membrane where gK increases first instantaneously and then more slowly. The slow component of these changes is called the relaxation of the currents. This component can be fitted with a single exponential function with the time constant (τ) determined by channel-types and V_m at which the relaxation takes place. The τ of mammalian strong inward rectifiers largely falls within a range of <10 msec, having a peak around E_K and smaller values at both more posi-

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tive and negative potentials (Leech and Standfield, 1981; Hestrin, 1981; Gunning, 1983; Kurachi, 1985; Tourneur *et al.*, 1987; Harvey and Ten Eick, 1988; Cohen *et al.*, 1989; Silver and DeCoursey, 1990).

2. Mg^{2+} and polyamine block. The voltage-dependent change in gK of strong inward rectifiers recently turned out to be caused by blockade of the channel pore by intracellular cations such as $Mg^{2+}{}_i$ and polyamines (Matsuda *et al.*, 1987; Vandenberg, 1987; Matsuda, 1988 and 1991; Lopatin *et al.*, 1994; Ficker *et al.*, 1994; Fakler *et al.*, 1994). In other words, the apparent gating of Kir channels results from exogenous elements. Consistent with this, they lack in their primary structure the domain corresponding to the voltage-sensing S4 region of voltage-dependent K^+ channels (Ho *et al.*, 1993; Kubo *et al.*, 1993a). Polyamines are aliphatic amines such as putrescine, spermidine and spermine, which are endogenous metabolic intermediates derived from arginine. They normally exist in submillimolar concentrations in the cytosol of almost all cell-types (Watanabe *et al.*, 1991). Putrescine, spermidine, and spermine bear 2, 3, and 4 positive charges per molecule, respectively. Thus it is likely that polyamines and $Mg^{2+}{}_i$ interact with Kir channels via the electrostatic force created between their own positive charges and negatively charged amino acid residues within the channel pore (Stanfield *et al.*, 1994; Taglialatela *et al.*, 1994; Lu and MacKinnon, 1994; Wible *et al.*, 1994; Lopatin *et al.*, 1994; Ficker *et al.*, 1994; Fakler *et al.*, 1995; Yang *et al.*, 1995a)

The interaction of charged substance with their receptor sites within the channel pore is affected by the transmembrane electric field (Hille, 1992b). As a result, the apparent potency of Mg^{2+}/polyamines is greater at more depolarized V_m. The apparent potency of a blocker with a larger valance is more strongly affected by V_m (Hille, 1992b). Thus, spermidine and spermine block Kir channels in a more steeply voltage-dependent manner than $Mg^{2+}{}_i$ or putrescine (Lopatin *et al.*, 1994). Therefore, it is likely that the steep voltage-dependency of *gK* of strong inward rectifiers mainly arises from the blockade by spermidine and spermine (Lopatin *et al.*, 1994; Ficker *et al.*, 1994; Fakler *et al.*, 1994; Ishihara *et al.*, 1989 and 1996). It has not been clarified how the effect of these intracellular cations on *gK* of Kir channels depends on ΔV and not V_m.

Upon a sudden depolarization (or hyperpolarization), the IRK1 channel is blocked (or unblocked) by $Mg^{2+}{}_i$ and putrescine quasi-instantaneously, but the effects of spermidine and spermine are slower and occur in a time-dependent manner (Ficker *et al.*, 1994; Ishihara *et al.*, 1996). Thus, the instantaneous and time-dependent components of the *gK* change may arise from the effects of $Mg^{2+}{}_i$/putrescine and spermidine/spermine blocks, respectively. When the membrane is depolarized to potentials less negative than $\sim E_K + 20\ mV$, inward rectification is predominantly caused by spermine and spermidine (Ishihara *et al.*, 1986, 1989). Upon much stronger depolarization, however, the channels may be first blocked by $Mg^{2+}{}_i$/putrescine which is followed by the block by spermidine/spermine that further suppresses *gK* in a time-dependent manner. The G-V relationship at these potentials is, therefore, determined by the balance between the two mechanisms with distinct voltage dependencies. As a result, the G-V relationship of strong inward rectifiers usually becomes slightly less steep at ΔV between ~+30 mV and +100 mV than expected from the data at more negative potentials (Ishihara *et al.*, 1989; Yamashita *et al.*, 1996). This phenomenon seems to be physiologically significant because action potentials usually cover a range of potentials between E_K and $E_K + \sim 100\ mV$.

3. Mg^{2+}/polyamine block sites in the inwardly rectifying K^+ channel pore. The molecular mechanism underlying the Mg^{2+}/polyamine block has been elucidated through comparison of the molecular structures of the strong inward rectifier IRK1 and the weak inward rectifier ROMK1 channels. At E_K −20 mV, the K_d value of Mg^{2+} and spermine for IRK1 channels are respectively ~1 mM and ~10 μM (Lopatin *et al.*, 1994), which are ~1000 and ~1000,000 times smaller than the corresponding values for ROMK1 channels (Nichols *et al.*, 1994; Yang *et al.*, 1995a). Mutagenesis studies revealed that at least two negatively-charged amino acid residues found in IRK1 (aspartate (D) at position 172 in the M2 region and glutamate (E) at position 224 in the proximal C-terminus) but not in ROMK1 are responsible for this discrepancy (fig. 12) (Stanfield *et al.*, 1994; Taglialatela *et al.*, 1994; Lu and MacKinnon, 1994; Wible *et al.*, 1994; Lopatin *et al.*, 1994; Ficker *et al.*, 1994; Yang *et al.*, 1995a). For convenience, we shall designate these two positions R1 and R2, respectively. The two acidic residues are likely to interact with the blocking particles independently of each other (Yang *et al.*, 1995a). Other residues may also participate in the blocking to a lesser extent because ROMK1 exhibits weak but significant inward rectification (Ho *et al.*, 1993). IRK2 and IRK3 also have the aspartate (D) and glutamate (E) residues at the analogous positions, although both BIR and uK_{ATP}-1 lack acidic residues at either of the sites (Takahashi *et al.*, 1994; Morishige *et al.*, 1994; Ho et al, 1993; Inagaki *et al.*, 1995a and b).

B. Inward Rectification of G Protein-Gated K^+ Channels

1. Inward rectification of the muscarinic K^+ channel. The K_{ACh} channel exhibits clear inward rectification irrespective of ACh concentrations and thus G_K activity (figs. 13Aa and b). The G-V relationship of the K_{ACh} channel can be fitted with Eq. 4 with ΔV_h and v of ~0 and ~20 mV, respectively (fig. 13Ac). Both the values are significantly larger than those of strong inward rectifiers but much smaller than those of weak inward rectifiers. The Boltzmann's fit deviates from the measured values at ΔV positive to ~+40 mV. Thus, the

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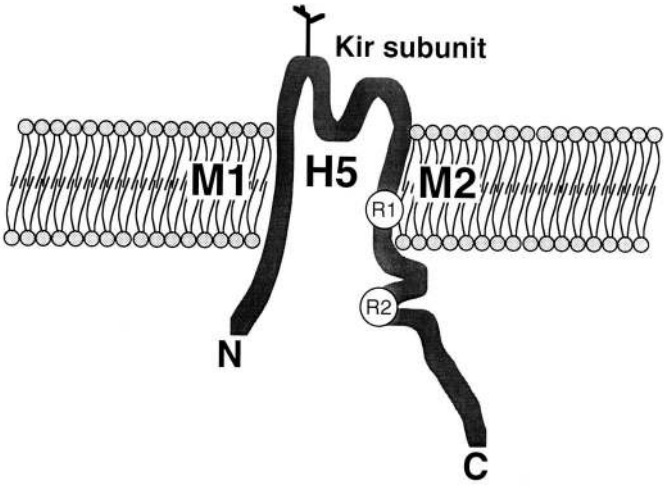

Kir subunits	Amino acid residues at R1	R2
ROMK1	N	G
IRK1-3	D	E
GIRK1	D	S
GIRK2-4	N	E
K_{AB}-2	E	G
uKATP, BIR	N	S

FIG. 12. Proposed Mg^{2+}/polyamine interaction sites in Kir subunits. A schematic representation of proposed Mg^{2+}/polyamine interaction sites in Kir subunits. R1 and R2 indicate the approximate position of the negatively charged amino acid residues in IRK1 that are responsible for the high sensitivity of the channel to intracellular Mg^{2+} and polyamine: aspartate at position 172 and glutamate at position 224, respectively (Stanfield *et al.*, 1994; Taglialatela *et al.*, 1994; Lu and MacKinnon, 1994; Wible *et al.*, 1994; Lopatin *et al.*, 1994; Ficker *et al.*, 1994; Yang *et al.*, 1995a). The table shows the amino acid residues at the sites analogous to R1 or R2 in different types of Kir subunits. N indicates asparagine; G, glycine; D, aspartate; E, glutamate; and S, serine.

actual G-V relationship is less steep at depolarized potentials than predicted from the data at more negative potentials as is the case for strong inward rectifiers (Ishihara *et al.*, 1989)

Upon a sudden change in V_m, gK of K_{ACh} channel also changes first instantaneously and then time-dependently (figs. 13Aa,b). However, this relaxation of the K_{ACh} channel is much slower than that of mammalian strong inward rectifiers. This slow relaxation presumably has an important functional meaning in regulation of action potential configuration of some cell-types such as the pace-making sinoatrial nodal cells of the heart. However, this issue has not been extensively investigated to our knowledge. The relaxation of the K_{ACh} channel occurs with a time course that can be fitted with a biexponential function with τ of <10 and 50 to 150 msec (fig. 13B). The former value is similar to, but the latter is ~5 to 10 times larger than that of mammalian strong inward rectifiers. The slow τ of the K_{ACh} channel is less steeply voltage-dependent than the τ of the strong inward rectifiers and monotonically decreases as the membrane is depolarized (Osterrieder *et al.*, 1981; Iijima *et al.*, 1985; Simmons and Hartzell, 1987; Kurachi, 1990). These results suggest that the slow relaxation of the K_{ACh} channel may arise from a molecular mechanism distinct from that underlying the relaxation of mammalian strong inward rectifiers. Furthermore, the slow τ of the K_{ACh} channel becomes smaller as the ACh concentration, therefore G protein activity is increased (figs. 13B, C).

2. Mg^{2+}/polyamine block of G protein-gated K^+ channels. Fig. 14A shows the effect of $Mg^{2+}{}_i$ and intracellular polyamines on the single K_{ACh} channel current (Yamada and Kurachi, 1995). In these experiments an inside-out patch was obtained from a rabbit atrial myocyte with pipette solution containing 145 mM K^+ and 0.5 μM ACh. The patch was excised in a solution containing 145 mM K^+ and 1.4 mM free Mg^{2+}. Application of a nonhydrolyzable GTP analogue, GTPγS induced inward K_{ACh} channel currents at −60 mV. This channel activity continued even after washout of GTPγS because the nucleotide irreversibly activated G_K in the membrane. Depolarization of the membrane to +40 mV resulted in appearance of small outward currents. The unitary amplitude of these outward currents was increased after removal of $Mg^{2+}{}_i$. Under these conditions, the single-channel I-V relationship was virtually linear, indicating that γ had been reduced by Mg^{2+} block at the depolarized potential (Horie and Irisawa, 1987, 1989). The IC_{50} of $Mg^{2+}{}_i$ has been reported to be ~300 μM at ΔV of +40 mV (Horie and Irisawa, 1987 and 1989), whereas cytosolic Mg^{2+} concentrations are estimated to be in millimolar range (Hess *et al.*, 1982; Gupta *et al.*, 1984; Alvarez-Leefmans *et al.*, 1986, Blatter and McGuigan, 1986). Thus, Mg^{2+} block is likely to underlie the physiological inward rectification of the single K_{ACh} channel conductance (γ)

Spermine (10 μM) applied to the internal side of the patch membrane in the absence of $Mg^{2+}{}_i$ almost completely suppressed the outward currents. Fig. 14B shows the concentration-dependent effect of spermine at this potential. It is clear that spermine reduced P_o in a concentration-dependent manner without altering γ (figs. 14Ba-c). The IC_{50} of spermine was ~10 nM at ΔV of +40 mV (fig. 14Bd). Thus, spermine is much more potent than $Mg^{2+}{}_i$ in suppressing outward K_{ACh} channel currents at this potential. Not only spermine but spermidine and putrescine also inhibit outward currents of the K_{ACh} channel although putrescine was much less potent than the other two (Yamada and Kurachi, 1995). Besides the K_{ACh} channel (a GIRK1/GIRK4 heteromer), the GIRK1/GIRK2 and GIRK2/GIRK4 heteromultimers are also shown to be blocked by $Mg^{2+}{}_i$ and intracellular polyamines at V_m positive to E_K (Velimirovic *et al.*, 1996; Lesage *et al.*, 1995).

Figure 14C shows the relationship between V_m and P_o of the K_{ACh} channel in the presence and absence of 100

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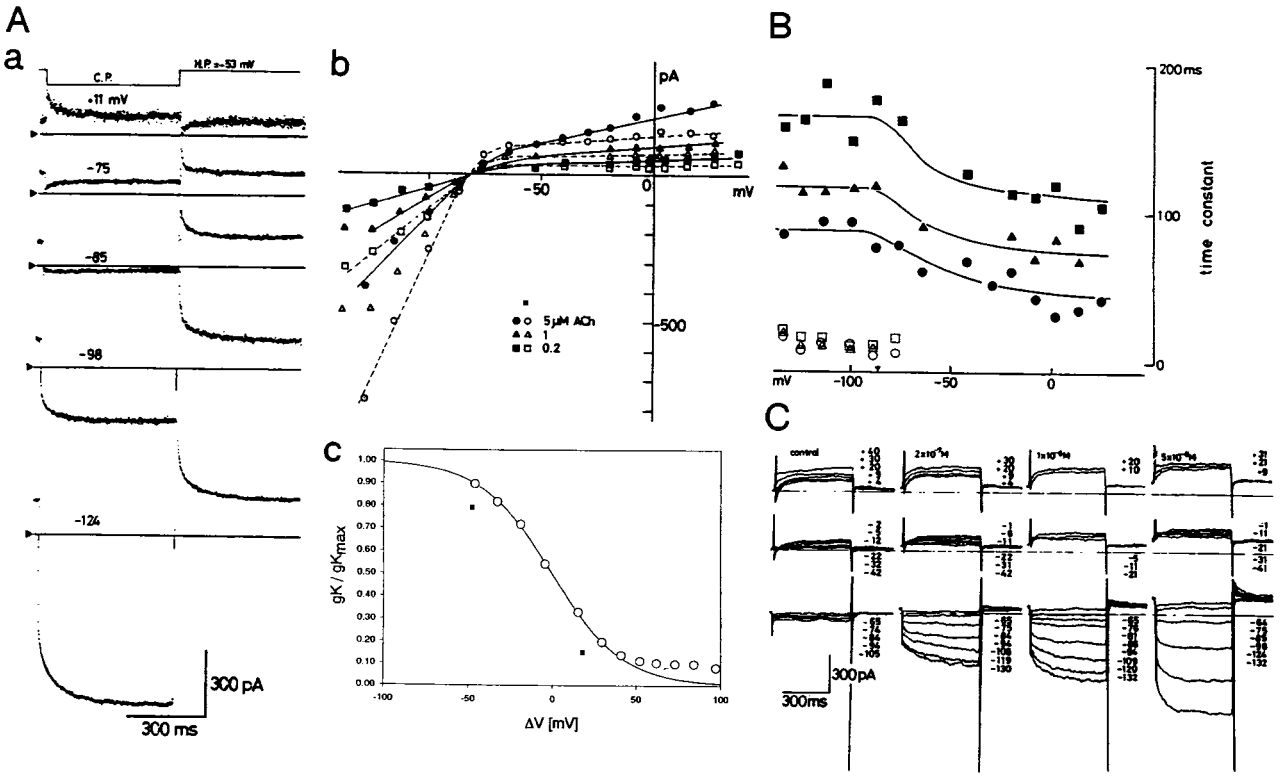

FIG. 13. Inward rectification of the muscarinic K^+ channel. These data were obtained from the whole-cell recording of the muscarinic K^+ channel current in a single sinoatrial node cell of rabbit. A: a, The channel current induced by 5 μM acetylcholine applied to the bath. The extracellular K^+ concentration was 5.4 mM. Five hundred msec command pulses (c.p.) were applied from the holding potential of −53 mV to the potentials indicated above each trace. Note that in each trace, the command pulse induced an instantaneous jump followed by a time-dependent change (relaxation) of the channel current. This was also the case when the membrane potential was returned to the holding potential. b, The relationship between the membrane potential and the channel currents induced by different concentrations of acetylcholine. The magnitude of the currents was measured immediately (closed symbols) or 500 msec (open symbols) after application of the command pulses. The reversal potential was ∼−80 mV under each condition. c, The relationship between the membrane potential relative to the K^+ equilibration potential (∼−80 mV under these conditions) (ΔV) and the macroscopic chord conductance (gK) of the muscarinic K^+ channel current induced by 5 μM acetylcholine (symbols). In this graph, gK is normalized to its maximum value (gK_{max}) predicted by the fit of the data with Boltzmann's equation (Equation 4 in the text) (line in the graph). The best fit was obtained with V_h and v of −0.32 and 21.0 mV, respectively. B: The voltage-dependence of the time constants of the relaxation of the muscarinic K^+ channel currents induced by 0.2 μM (squares), 1 μM (triangles), and 5 μM (circles) of acetylcholine. The voltage-clamp protocol was the same as in A. The relaxation of each acetylcholine-induced current could be fitted with a biexponential function with a smaller (open symbols) and a larger (closed symbols) time constant. The inverted triangle on the voltage-axis indicates the reversal potential of the channel currents. C: The channel currents observed in the sinoatrial node cell in the presence and the absence of different concentrations of acetylcholine. Command steps were applied from −53 mV to various membrane potentials indicated to the right of each current trace, and the obtained currents are superimposed. Note that the higher concentration of acetylcholine induced not only larger currents but accelerated relaxation at every membrane potential. [Reproduced with permission from Kurachi (1990)].

nM spermine. The same concentration of spermine decreased P_o more strongly as V_m became more positive probably due to the voltage-dependent increase in the apparent potency of the blocker. Under these conditions, P_o decreased to ∼20% at E_K *+40 mV*. Because cytosolic concentrations of polyamines are estimated to be in a submillimolar range (Watanabe *et al.*, 1991), these data indicate that polyamine block also underlies the physiological inward rectification of the K_{ACh} channel current by reducing P_o at depolarized potentials.

When V_m was suddenly depolarized from −60 mV to +40 mV, the P_o of the channel decreased in a time-dependent fashion (fig. 14Da, the upper two rows). The bottom row shows the ensemble averaged current under each condition. The decay of the outward current became faster as spermine concentration was increased, probably because the on-rate of the block increased in the presence of higher concentration of spermine (Yamashita *et al.*, 1996). In the presence of physiological concentrations of spermine, the kinetics of polyamine block would become much faster and exceed those of the slow relaxation. Therefore, polyamine block is unlikely to be the mechanism of the slow relaxation of the K_{ACh} channel. In other words, the inward rectification and the slow relaxation of K_{ACh} channels might be attributable to distinct mechanisms. This issue will be dealt with in Section IV.B.4.

3. The Mg^{2+}/polyamine-binding sites in G protein-gated K^+ channels. The K_G channels have an unique arrangement of negative charges at the R1 and R2 sites (figs. 8 and 12). GIRK1 has negatively-charged aspartate at R1 (D173) but uncharged serine (S) at R2 (Kubo *et al.*, 1993b; Dascal *et al.*, 1993). However, all GIRKs 2–5 have uncharged asparagine (N) at R1 and negatively-

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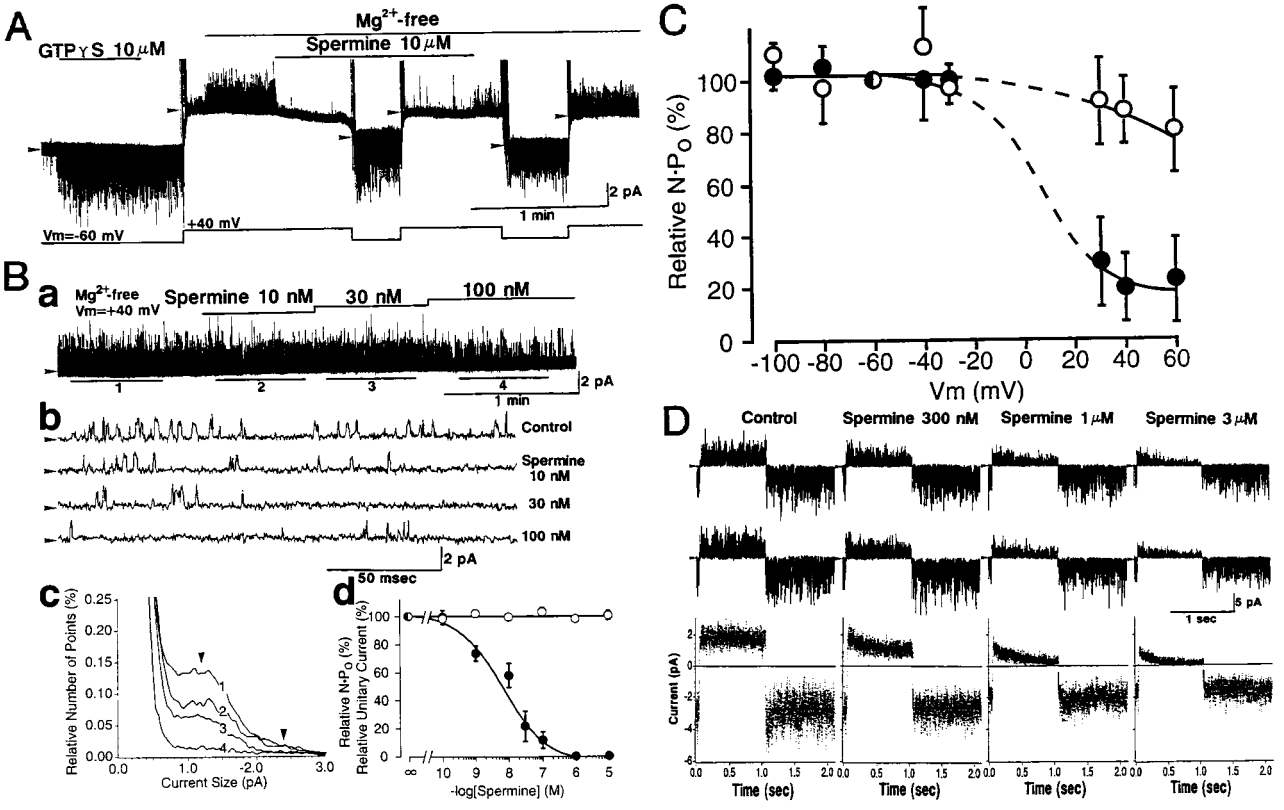

FIG. 14. Effect of spermine on the muscarinic K^+ channel. All data shown were obtained from muscarinic K^+ channels in inside-out patch membranes of rabbit atrial myocytes exposed to symmetrical 145 mM K^+ solutions. The pipette also contained 0.5 μM acetylcholine. A: Effect of 10 μM spermine applied to the internal side of the patch membrane. The protocol of perfusion of the intracellular side of the patch membrane and the change in holding membrane potential are indicated above and below the current trace, respectively. Arrowheads indicate the zero current levels. Large and small spikes observed at + 40 mV in the presence of spermine were occasional opening of ATP-sensitive K^+ and muscarinic K^+ channels, respectively. B: Concentration-dependent inhibitory effect of internal spermine on outward muscarinic K^+ currents at + 40 mV. a, After the muscarinic K^+ channels were irreversibly activated by GTPγS the indicated concentrations of spermine was applied to the intracellular side of the patch membrane in the absence of intracellular Mg^{2+}. The $N{\cdot}P_o$ of the muscarinic K^+ channels was 0.096, 0.084, 0.046, and 0.014 in the presence of 0, 10, 30, and 100 nM spermine, respectively. The bars and numbers below the current trace correspond to the numbers in the graph c. b, High-speed current traces obtained from the same patch as a under the conditions indicated to the right of each current trace. c, The current amplitude histogram obtained from the same current record as a. The numbers in the graph correspond to those in a and indicate the portions of the record from which each histogram was constructed. Bin width is 0.02 pA, and the number of points in each bin is expressed as a percentage of the total number of points recorded. d, Concentration-dependent effect of spermine on the unitary current amplitude (open circles) and $N{\cdot}P_o$ (closed circles). Data were normalized to the values obtained in the absence of spermine. Symbols and bars indicate the mean ± SE. Lines were drawn by eye. Unitary channel current amplitude in 1 and 10 μM spermine was measured by eye because very few openings of muscarinic K^+ channels were available in this concentration range. C: Voltage-dependent change in $N{\cdot}P_o$ of muscarinic K^+ channels in the presence (closed circles) and absence (open circles) of 100 nM spermine. ATP (1 mM) was added to the intracellular side of patch membranes. $N{\cdot}P_o$ is expressed as a percentage of the value recorded at −60 mV. Symbols and bars indicate the mean ± SE. Lines were drawn by eye. D: The time-dependent change in muscarinic K^+ currents in the presence or absence of 300 nM, 1 μM, and 3 μM spermine. Muscarinic K^+ channels in an inside-out patch membrane were preactivated with 10 μM GTPγS, and then Mg^{2+} was removed from the intracellular side of the patch membrane. As shown at the top, 1 sec duration command steps to + 40 mV were applied every 5 sec from a −60 mV holding potential. Reading from left to right, progressively higher concentrations of spermine (300 nM−3 μM) were applied to the intracellular side of the patch membrane. The membrane was depolarized ~30 to 40 times in each condition. The first and second rows show representative current traces. In each of the traces, the baseline current was subtracted with a computer. Arrowheads indicate the zero current level. Time and current scales are shown to the right beneath the second row. The third row shows the ensemble average current obtained from 20 successive pulses under each condition. The zero current level is indicated by a straight line. Each of the ensemble average current traces could be fitted by a single exponential both at + 40 and −60 mV. At + 40 mV, the time constant obtained with the best fit was 9.9 sec in the control; 1.3 sec in the presence of 300 nM spermine; 0.38 sec, 1 μM; 0.18 sec, 3 μM. [Reproduced with permission from Yamada and Kurachi (1995)].

charged glutamate (E) at R2 (Lesage *et al.*, 1994 and 1995; Isomoto *et al.*, 1996; Krapivinsky *et al.*, 1995a). Recently, Kofuji *et al.* (1996a) examined the functional significance of D173 of GIRK1 by replacing this residue with non-charged asparagine [GIRK1(D173N)]. The G-V relationship of K_G channels in *Xenopus* oocytes expressing GIRK1(D173N) was much less steeply voltage-dependent and shifted leftward along the voltage-axis compared with that for the wild-type GIRK1: v and ΔV_h were respectively ~40 and ~−50 mV for GIRK1(D173N), and ~20 and ~−30 mV for the wild-type GIRK1. These data indicate that the $Mg^{2+}{}_i$/polyamine block at D173 of GIRK1 is in fact crucial for the inward rectification of K_G channels that contain GIRK1.

Although homomultimeric GIRK2 and GIRK4 channels show clear inward rectification, their *gK* is hardly saturated even at E_K *−100 mV* (Lesage *et al.*, 1994; Bond *et al.*, 1995; Iizuka *et al.*, 1995). However, when the

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G-V curve of GIRK4 channels constructed from the data shown by Chan *et al.* (1996) were roughly fitted with equation 4, the v and ΔV_h values were estimated to be ~30 mV and ~−40 mV, respectively. The difference in G-V relationship between GIRK1 and GIRK4 channels might be due to the distinct location of the negatively charged residues in the channel pore. However, the v and ΔV_h values of GIRK1/GIRK4 heteromeric channels are ~20 and ~−20 mV, respectively as estimated from the data reported by Krapivinsky *et al.* (1995a). GIRK1/GIRK4 channels therefore have significantly smaller v and more positive ΔV_h values than GIRK4 homomeric channels, and a more positive ΔV_h value than GIRK1 channels. Further studies are needed to clarify how the G-V relationship of GIRK1/GIRK4 channels is determined by the negatively charged residues at R1 of GIRK1 and at R2 of GIRK4. The G-V relationship of GIRK1/GIRK4 channels is not identical with that of the native K_{ACh} channel (Iizuka *et al.*, 1995) and exhibits a more negative ΔV_h value. These results suggest that subunit composition might not be the same between the native K_{ACh} channel and the heterologously-expressed GIRK1/GIRK4 channel, and/or that some additional factors other than GIRK1 or GIRK4 might be included in the native K_{ACh} channel.

4. Slow relaxation of G protein-gated K^+ channels containing GIRK1. No mammalian strong inward rectifiers show such slow relaxation as the K_{ACh} channel does (fig. 13) (Leech and Standfield, 1981; Kurachi, 1985; Tourneur *et al.*, 1987; Harvey and Ten Eick, 1988; Cohen *et al.*, 1989; Silver and DeCoursey, 1990). GIRK2 and GIRK4 homomultimeric channels show fast relaxation kinetics similar to those of IRKs (Lesage *et al.*, 1995; Krapivinsky *et al.*, 1995a; Iizuka *et al.*, 1995; Duprat *et al.*, 1995; Bond *et al.*, 1995; Kofuji *et al.*, 1995; Velimirovic *et al.*, 1996). The heteromeric K_G channels containing GIRK1, however, exhibit the slow relaxation after a voltage step similar to that of the K_{ACh} channel (i.e., a native GIRK1/GIRK4 heteromer) (Kubo *et al.*, 1993b; Krapivinsky *et al.*, 1995a; Iizuka *et al.*, 1995; Duprat *et al.* 1995; Kofuji *et al.*, 1995; Doupnik *et al.*, 1995b; Velimirovic *et al.*, 1996; Isomoto *et al.*, 1996), although the relaxation kinetics of GIRK1/GIRK2 channels may be slightly faster than those of GIRK1/GIRK3 or GIRK1/GIRK4 channels (Wischmeyer *et al.*, 1997). Therefore, it is likely that GIRK1 is responsible for the slow relaxation.

Kofuji *et al.* (1996a) recently found that a phenylalanine residue at position 137 (F137) in the H5 region of GIRK1 is responsible for the slow relaxation (fig. 8). All other GIRK subunits bear conserved serine (S) at the position analogous to F137 of GIRK1. Interestingly, this residue was identified by Chan *et al.* (1996) and Wischmeyer *et al.* (1997) as the residue responsible for the synergistic enhancement of macroscopic current amplitudes induced by coexpression of GIRK1 and other GIRK subunits. When GIRK1 whose F137 was replaced with serine [GIRK1(F137S)] was expressed alone or in combination with GIRK3 in *Xenopus* oocytes, the resultant macroscopic K_G channel currents no longer exhibited the slow relaxation (Kofuji *et al.*, 1996a; Wischmeyer *et al.*, 1997). Kofuji *et al.* (1996a) showed that the G-V relationships of the channel currents observed in oocytes expressing either the wild-type GIRK1 or the mutant GIRK1(F137S) alone were not significantly different. It is also noteworthy that the GIRK1(D173N), which retains F137, did not exhibit steep inward rectification (Kofuji *et al.*, 1996a). Taken together, these results indicate that inward rectification of K_G channels containing GIRK1 might be determined primarily by the Mg^{2+}/polyamine block, although the intrinsic gating arisen from F137 might mainly serve to modulate the relaxation kinetics.

Doupnik *et al.* (1995b) and Kofuji *et al.* (1996a) conducted the envelope test for the K_G channel current in *Xenopus* oocytes expressing GIRK1. In these experiments, the activation kinetics of the current at E_K *−80 mV* were examined after varying durations of depolarizing prepulses. As the duration of the prepulse was elongated, a slow relaxation component of the inward tail became progressively larger until it occupied ~50% of the total inward current with τ of ~200 msec, whereas the faster component(s) reciprocally decreased (Doupnik *et al.*, 1995b; Kofuji *et al.*, 1996a). In contrast, the outward current during the prepulse was promptly suppressed by more than 90% within the initial 50 msec, and only the remaining fraction was slowly decreased (Kofuji *et al.*, 1996a). These results suggest that at depolarized potentials, the channel currents might be first strongly and promptly suppressed by Mg^{2+}/polyamine, and then further slightly depressed by an intrinsic gating gradually substituting for part of the Mg^{2+}/polyamine block. Upon repolarization, the component which had been blocked by Mg^{2+}/polyamine might be relieved first, followed by reactivation of the component affected by intrinsic gating. Such an interactive mechanism might remind one of the interaction between $Mg^{2+}{}_i$ and polyamines on strong inward rectifiers (Ishihara *et al.*, 1989). Although the latter phenomenon can be explained in terms of interaction for the common binding sites shared by $Mg^{2+}{}_i$ and polyamines in IRK subunits (Yamashita *et al.*, 1996), it is not known what molecular mechanism underlies the interaction between intrinsic gating and Mg^{2+}/polyamine block.

There are also some other unsettled issues regarding the molecular mechanism of the slow relaxation. First, neither homomeric GIRK4(S143F) nor heteromeric GIRK1(F137S)/GIRK3(S114F) channels exhibit the slow relaxation (Chan *et al.*, 1996; Wischmeyer *et al.*, 1997), indicating that interaction of F137 with some other part(s) of the same GIRK1 subunit is prerequisite for the slow relaxation. Furthermore, Wischmeyer *et al.* (1997) found that GIRK1/GIRK1(F137S) "homomeric" K_G channels showed the fast relaxation kinetics. Thus, it

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may be that a GIRK1-containing K_G channel can exhibit the slow relaxation only when such intramolecular interaction occurs in all the GIRK1 subunits included in the channel. These results indicate that we are still not aware of all the molecular mechanisms underlying the slow relaxation of GIRK1-containing K_G channels. Second, the kinetics of the slow relaxation may vary depending on the subunit composition of K_G channels containing GIRK1. The slow relaxation of GIRK1/GIRK4 channels is much faster than that of the K_G channel yielded with expression of GIRK1 alone in *Xenopus* oocytes (Iizuka *et al.*, 1995). GIRK1/GIRK2 channels may have even faster relaxation kinetics than GIRK1/GIRK3 or GIRK1/GIRK4 channels (Wischmeyer *et al.*, 1997). It is not yet known how GIRK subunits other than GIRK1 regulate the slow relaxation. Finally, the relaxation kinetics of the K_{ACh} channel become faster in the presence of higher ACh concentrations (figs. 13B and C). Such a receptor- (or G_K-) dependent change in the slow relaxation kinetics cannot be reproduced in oocytes expressing either GIRK1 alone (Doupnik *et al.*, 1995b) or GIRK1 plus GIRK4 (Iizuka *et al.*, 1995). The agonist- (or G_K-) dependent regulation of the kinetics might, therefore, require some unidentified factor(s) existing in cardiac myocytes but not in oocytes. Further studies are necessary to fully elucidate the molecular mechanism underlying the slow relaxation of native K_G channels containing GIRK1.

V. Pharmacological Properties of G Protein-Gated K^+ Channels

The pharmacological properties of K_G channels including the K_{ACh} channel have not been extensively investigated. However, it is well known that like other Kir channels (Hagiwara and Takahashi, 1974; Hagiwara *et al.*, 1976, 1978; Gay and Stanfield, 1977; Standen and Stanfield, 1978; Constanti and Galvan, 1983; Harvey and Ten Eick, 1989), cesium and barium ions effectively block various native K_G channels (Carmeliet and Mubagwa, 1986; Gähwiler and Brown, 1985; Inoue *et al.*, 1988; Mihara *et al.*, 1987; Surprenant and North, 1988; Pennefather *et al.*, 1988; Penington *et al.*, 1993; Lacey *et al.*, 1987 and 1988; Gerber *et al.*, 1991; Dousmanis and Pennefather, 1992; Sodickson and Bean, 1996). These cations, when applied extracellularly, block K_G channels in a steeply voltage-dependent manner with higher potency at more negative membrane potentials (but see Sodickson and Bean, 1996). Thus, the cations seem to go through the transmembrane electric field before binding to their receptor sites in the channel pore. Depending on experimental protocols and cell type, the reported K_d values of these cations for K_G channels vary but largely fall into a concentration range of 10^{-3}-10^{-2} M for cesium ions and 10^{-5} to 10^{-4} M for barium ions at negative membrane potentials. These values are ~10 times more than those required to block the classical Kir channels (Hagiwara and Takahashi, 1974; Hagiwara *et al.*, 1976, 1978; Gay and Stanfield, 1977; Standen and Stanfield, 1978; Constanti and Galvan, 1983; Harvey and Ten Eick, 1989). Similar values were reported for the cesium or barium blockade of the recombinant K_G channels (Velimirovic *et al.*, 1996; Lesage *et al.*, 1995; Bond *et al.*, 1995). Although barium ions are known to block the classical Kir channel in a clear time-dependent manner (Hagiwara *et al.*, 1978; Constanti and Galvan, 1983), some K_G channels were reported to be instantaneously inhibited by the ions (Carmeliet and Mubagwa, 1986; Dousmanis and Pennefather, 1992, but see Velimirovic *et al.*, 1996). K_G channels are also known to be blocked by millimolar concentrations of rubidium ions (Katayama *et al.*, 1997; Mihara *et al.*, 1987; Surprenant and North, 1988).

Recent studies indicated that cesium, rubidium, and strontium ions block a recombinant strong inward rectifier IRK1 channel at least in part through binding to a negatively charged aspartate at the R1 position in the putative channel pore (D172) (Reuveny *et al.*, 1996; Abrams *et al.*, 1996). As we have already seen in Section IV.A.3., this acidic residue is conserved among various constitutively active Kir channels and may serve as a receptor site for the intracellular Mg^{2+} and polyamines as well (fig. 12) (Stanfield *et al.*, 1994; Taglialatela *et al.*, 1994; Lu and MacKinnon, 1994; Wible *et al.*, 1994; Lopatin *et al.*, 1994; Ficker *et al.*, 1994; Yang *et al.*, 1995a). GIRK1 but not GIRKs2–4 bear this residue. This may at least in part explain the lower sensitivity of K_G channels to these cation blockers than classical Kir channels.

K_G channels are also fully blocked by quinidine and quinine with the IC_{50} of 10 μM (Kurachi *et al.*, 1987a; Nakajima *et al.*, 1989; Katayama *et al.*, 1997). Verapamil also inhibits the ACh-activated cardiac K_{ACh} channel with the IC_{50} of 1 μM although its effect is partially mediated by suppression of the M_2-muscarinic receptor/G_K system (Ito *et al.*, 1989). To our knowledge, K_G channels are rather insensitive to other well-known channel blockers such as tetraethylammonium, 4-aminopyridine, apamine, charybdotoxin, disopyramide, and procainamide (Inoue *et al.*, 1988; Lacey *et al.*, 1987 and 1988; Nakajima *et al.*, 1989; Katayama *et al.*, 1997).

VI. Localization of the G Protein-Gated K^+ Channel in Different Organs

$G_{K\beta\gamma}$ seems to be the physiologically functional arm of G_K activating K_G channels not only in the heart but also in the brain and endocrine organs. However, the molecular mechanisms of G protein-regulation of ion channels have been found to be more complicated than we had thought. In AtT20 cells which had been transfected with the α_{2A}-adrenergic receptor, adrenergic agonists can inhibit the Ca^{2+} current and adenylyl cyclase and activate a K^+ current (Surprenant *et al.*, 1992). A point mutation of the receptor removes activation of the K^+ current, but not inhibition of Ca^{2+} current and adenylyl cyclase. This indicates that the G protein coupling to the K^+ channel

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is different from that to the Ca^{2+} channel and adenylyl cyclase although the receptor is the same. G proteins may thus be more specific to each receptor and to each signaling system than we are currently assuming or than we can determine in vitro. In *Xenopus* oocytes, however, when β_2-adrenergic receptors, G_s protein, and GIRK1 are coexpressed, β-adrenergic agonists could induce activation of K_G channel current (Lim *et al.*, 1995). Accordingly, the affinity of particular G protein subunits for the K_G channel may not be sufficient to explain specific activation of K_{ACh} channel by G_K. Actually, various combinations of recombinant $G_{\beta\gamma}$ (except for $G_{\beta1\gamma1}$) have similar efficacy and potency in activating K_{ACh} channels (Wickman *et al.*, 1994). However, the receptor specificity in cardiac atrial myocytes is well documented by extensive studies (Kurachi, 1995). It is often argued that receptor specificity could arise from compartmentalization of the appropriate receptors and channels, although little evidence has been shown for such compartmentalization. We do not know whether different mechanisms underlie receptor specificity in different organs. In other words, we have not yet fully answered the question how information specifically passes from a membrane receptor to the effector, the K_G channel, *via* G proteins.

Because the signal transduction mechanisms are not necessarily the same among heart, neurons, and endocrine cells, it is worthwhile at present to summarize the observations on the localization of K_G channels in these organs. Apparently, GIRK1 and GIRK4 immunoreactivities diffusely distribute in the cell membrane of cardiac myocytes, whereas those of GIRK1 and/or GIRK2 are localized to specialized segments of neuronal membrane, such as presynaptic axonal termini and postsynaptic dendritic regions (fig. 15). Thus, we may tentatively classify the system based on the apparent distribution into two categories: (*a*) homogeneously distributed system and (*b*) localized system. In these systems, different mechanisms may underlie signal transduction.

A. Cardiac Atrial Myocytes

The cardiac K_{ACh} channel is the prototype of K_G channels and is a heteromultimer of GIRK1 and GIRK4 (Krapivinsky *et al.*, 1995a). Immunohistochemistry using a specific antibody showed that GIRK1 is homogeneously localized on the cell membrane of atrial but not ventricular myocytes (fig. 15A). This is consistent with the electrophysiological studies of cardiac myocytes. The electrophysiological experiments also suggested that some topological restriction may exist in cardiac atrial myocytes, because K_{ACh} channels in the cell-attached membrane patch are activated by ACh or adenosine when they are applied to the pipette solution, but not when they are added to the bathing solution (Soejima and Noma, 1984).

It was found that either G_i- or G_s-coupled receptors, when expressed together with GIRK channels in *Xenopus* oocytes, can activate the channels (Lim *et al.*, 1995). This may indicate that under the conditions where compartmentalization does not exist, as in *Xenopus* oocytes, the $\beta\gamma$ subunits released from G_s may be able to activate K_G channels. Because β-adrenergic agonists never activate these channels in cardiac myocytes, there must be some mechanism to guarantee the specificity of the native G_K/K_{ACh} channel system in cardiac atrial myocytes. Further studies are needed to elucidate the mechanism.

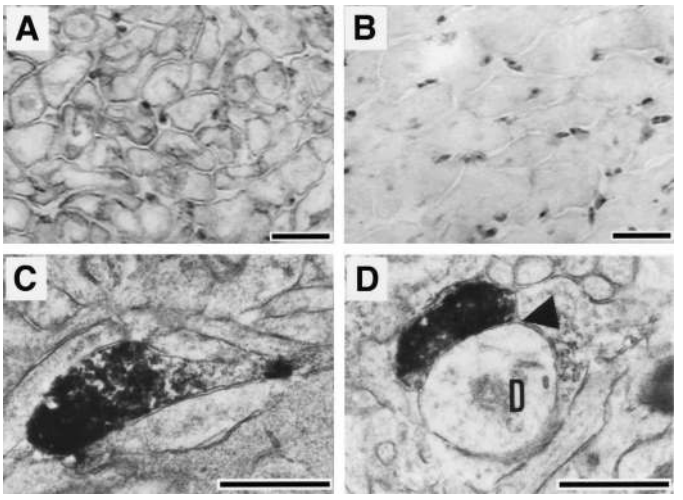

FIG. 15. Different subcellular localization of GIRK1 proteins. A and B: Immunohistochemical analyses for the GIRK1 proteins in the rat atrium (A) and ventricle (B). Homogeneous immunoreactivity was found on the plasma membranes of the atrial, but not of the ventricular myocytes. C and D: Electron microscopic analyses for the GIRK1 immunoreactivity in the rat paraventricular nucleus of the hypothalamus. Obvious GIRK1 immunoreactivity was present at the axonal terminals (probably on the vesicles) neighboring upon a dendrite (D) (reproduced with permission from Morishige *et al.*, 1996). All GIRK1 immunoreactivities were developed with diaminobenzidine-horseradish peroxidase method.

B. Neurons

1. Differential cellular and subcellular distribution of GIRK subunits. Cellular and subcellular localization of GIRK1, 2, and 4 subunits in the rat and mouse brains has been studied with specific antibodies against individual GIRK proteins (Ponce *et al.*, 1996; Liao *et al.*, 1996; Iizuka *et al.*, 1997). No corresponding studies have been done on GIRK3 proteins to our knowledge. The followings are the summary of currently available information.

Liao *et al.* (1996) showed that antibodies against GIRK1 or GIRK2 proteins immunocoprecipitated both GIRK1 and GIRK2 proteins from membranes of the cerebral cortex, indicating the presence of GIRK1/GIRK2 heteromultimers in this region. In the mice whose GIRK2 genes were genetically deleted (GIRK2 -/-), virtually all GIRK1 and GIRK2 proteins disappeared from the cerebral cortex, indicating that most of GIRK1 proteins are assembled with GIRK2 subunits (Signorini *et al.*, 1997). GIRK1 and GIRK2 immunoreactivities are found in the somata and apical dendrites of pyramidal cells in the somatosensory cortex (Ponce *et*

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al., 1996; Liao *et al.*, 1996). The GIRK1 immunostaining is stronger in the dendrites than in the somata (Ponce *et al.*, 1996), whereas strong GIRK2 immunoreactivity is found both in the cell body and apical dendrite of layer V pyramidal neurons (Liao *et al.*, 1996). GIRK4 immunoreactivity is also exist in the cerebral cortex (Iizuka *et al.*, 1997).

In the layer IV of the neocortex, fiber-like immunostainings of GIRK1, but not GIRK2, protein can be seen in addition to the staining of pyramidal neurons (Ponce *et al.*, 1996; Liao *et al.*, 1996). Ponce *et al.* (1996) argued that these stainings correspond to those of the terminals of thalamocortical projections, the axons of thalamic relay neurons, because the stainings markedly reduced on the side ipsilateral but not contralateral to thalamus lesioned with kainic acid. Consistent with this, neurons in most thalamic nuclei express GIRK1 mRNA strongly (Karschin *et al.*, 1994 and 1996), and the somata of relay neurons and neuropiles in thalamus are stained with anti-GIRK1 antibodies (Ponce *et al.*, 1996). The presence of GIRK subunits in presynaptic compartments is also suggested in other regions. Morishige *et al.* (1996) found that in the paraventricular nucleus of the hypothalamus, GIRK1 immunoreactivity seems as "beads on a string," a typical staining pattern of nerve terminals. Electron microscopy immunohistochemistry revealed that the immunoreactivity resides in terminal buttons but not in the dendrites (figs. 15B and C). Liao *et al.* (1996) also found that GIRK1 and GIRK2 immunoreactivities exist in axon-like fibers in the lateral septal region.

In the hippocampus, virtually all GIRK1 subunits seem to form heteromultimers with GIRK2 proteins (Liao *et al.* 1996; Signorini *et al.*, 1997). GIRK1 immunoreactivity is strongest in the stratum lacunosum-moleculare and the adjacent stratum radiatum, followed in intensity by the deep portion of the stratum radiatum and the stratum pyramidalis, whereas the stratum oriens is only weakly stained (Ponce *et al.*, 1996; Liao *et al.*, 1996; Drake *et al.*, 1997). Drake *et al.* (1997) examined the detailed subcellular localization of GIRK1 immunoreactivity in CA1 pyramidal neurons with an electron microscope. They found that the immunoreactivity almost exclusively exists in perisynaptic areas of the fine dendrites and dendritic spines of pyramidal neurons locating in the stratum lacunosum-moleculare and the stratum radiatum (Drake *et al.*, 1997). In the dentate gyrus, immunostaining of GIRK1 is strong in the molecular layer, moderate in the granular layer, and sparse in the polymorphic layer (Ponce *et al.*, 1996; Liao *et al.*, 1996; Drake *et al.*, 1997). An overall immunostaining pattern of GIRK2 in the hippocampus is similar to that of GIRK1 (Liao *et al.* 1996).

Strong immunoreactivities of GIRK1 and GIRK2 but not GIRK4 are found in the cerebellar granule cell layer (Ponce *et al.*, 1996; Liao *et al.* 1996; Iizuka *et al.*, 1997). The immunoreactivities are largely confined to the glomeruli where granule cell dendrites form synapses with mossy fiber terminals (Ponce *et al.*, 1996; Liao *et al.* 1996). Electron microscopy immunohistochemistry revealed strong immunostaining of GIRK1 protein on distal dendrites of granule cells surrounding unstained mossy fiber terminals (Ponce *et al.*, 1996). Purkinje cells have weak or no GIRK1 and GIRK2 immunoreactivities but a certain level of GIRK4 immunoreactivity throughout the soma (Ponce *et al.*, 1996; Liao *et al.* 1996; Iizuka *et al.*, 1997). Deep cerebellar nuclei exhibit distinct immunostaining of GIRK1 and GIRK2 proteins in a clear somatodendritic pattern (Ponce *et al.*, 1996; Liao *et al.* 1996). Axons of basket cells surrounding the proximal segment of Purkinje cell axons are likely to be relatively rich in GIRK4 immunoreactivity (Iizuka *et al.*, 1997). Although GIRK1 and GIRK2 proteins are coimmunoprecipitated from cerebellar membranes (Liao *et al.*, 1996), a significant amount of GIRK1 proteins remains in the cerebellum of the GIRK2-/- mice (Signorini *et al.*, 1997), indicating that GIRK2 is not the sole partner of GIRK1 in the cerebellum. This observation is consistent with the fact that GIRK2 mRNA is expressed only by granule cells in the cerebellum, whereas significant amounts of GIRK1 transcripts are found in the other regions of the cerebellum as well (DePaoli *et al.*, 1994; Karschin *et al.*, 1994 and 1996).

Substantia nigra and the ventral tegmental area are unique in that abundant GIRK2 subunits exist although little or no GIRK1 protein is expressed (DePaoli *et al.*, 1994; Ponce *et al.*, 1996; Liao *et al.*, 1996). GIRK4 is expressed in these regions at the mRNA level but not at the protein level (Iizuka *et al.*, 1997). GIRK2-specific immunoreactivities are found in both the cell body and dendrites of dopaminergic neurons in the pars compacta and in the dendrites in the pars reticulata (Liao *et al.*, 1996; Yoshimoto *et al.*, 1997).

Immunostaining of GIRK1 proteins in the brain stem exhibits a clear somatodendritic pattern in several nuclei including the superior olive, the nucleus of the trapezoid body, the reticular formation and the central nuclei of the inferior colliculus (Ponce *et al.*, 1996). GIRK2 immunoreactivities are also found in these neurons basically in a somatodendritic pattern (Liao *et al.*, 1996). Immunoreactivity of GIRK4 is also found in the neuronal fiber plexus in the inferior olivary, pontine, and facial nuclei (Iizuka *et al.*, 1997).

These immunohistochemical analyses delineate a great deal of complexity of neuronal K_G channel systems in the brain. To summarize, GIRK1, 2, and 4 subunits exist mostly in the somatodendritic subcellular compartment and sometimes in the axon-like fibers, although some neurons may have these proteins in both the compartments.

2. Functional significance of differential subcellular distribution of GIRK subunits. Differential subcellular distribution of GIRK subunits in neurons would be intimately correlated with the functional task of K_G chan-

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nels in each neuron. For example, in the CA3 hippocampal pyramidal neurons, a selective $GABA_B$ agonist baclofen causes both presynaptic and postsynaptic inhibition in the wild-type mice but only presynaptic inhibition in GIRK2 -/-mice (Lüscher *et al.*, 1997). These results indicate that GIRK1/GIRK2 channels in these neurons are selectively activated by the postsynaptic $GABA_B$ receptor because of the postsynaptic localization of the GIRK subunits in these neurons. In hippocampal CA1 pyramidal neurons, Drake *et al.* (1997) found that GIRK1 immunoreactivity is found mainly associated with the dendritic membranes in the vicinity of the asymmetric (stimulatory) but not symmetric (inhibitory) type of synapses. These observations raise the possibility that the GIRK1-containing K_G channels in these neurons might serve to modulate the propagation of neuronal inputs originated at the excitatory synapses to the soma, as well as the back propagation of the action potential from the soma to the synapses on a synapse-to-synapse basis. To more concretely identify the functional roles of K_G channels in these neurons, it is important to determine the type(s) of neurotransmitter receptors, G proteins and nerve terminals associated with these channel subunits.

It is surprising that GIRK subunits also exist in the axon of some neurons (Ponce *et al.*, 1996; Morishige *et al.*, 1996; Liao *et al.*, 1996). In fact, many G protein-coupled receptors inhibit release of neurotransmitter presynaptically (Nicoll, 1988; North, 1989; Thompson *et al.*, 1993; Wu and Saggau, 1997). In most cases, however, the presynaptic inhibition has been ascribed to inhibition of presynaptic Ca^{2+} channels and/or direct inhibition of exocytotic machineries (Scanziani *et al.*, 1992; Thompson *et al.*, 1993; Dittman and Regehr, 1996; Takahashi *et al.*, 1996; Wu and Saggau, 1997). The functional role of the presynaptic K_G channels needs to be clarified in future studies.

C. Endocrine Cells

Electrophysiological studies indicate that K_G channels activated by somatostatin and/or dopamine exist in endocrine cells of anterior pituitary lobe (Pennefather *et al.*, 1988; Einhorn and Oxford, 1993). This system would be essential for the inhibitory regulation of hormone secretion. However, there is no information available on subcellular distribution of GIRK subunits in these and other endocrine cells so far.

VII. *Weaver* Mutant Mice and the GIRK2 Gene

Weaver mice have been studied intensively over the past 25 years for insights into the normal processes of neuronal development and differentiation (Hess, 1996). Homozygous animals (*wv/wv* mice) suffer from severe ataxia due to death of cerebellar granular cells. The animals also represent a model of Parkinsonism because dopaminergic input to the striatum is lost during the first few weeks after birth due to the death of dopaminergic neurons in the substantia nigra. Male *wv/wv* mice are sterile: spermatogenesis fails to proceed normally past the third postnatal week leading to a complete failure of sperm production.

Recently, it was shown that *wv/wv* mice have their neurological abnormalities because of a point mutation of guanine 953 to adenine in the GIRK2 gene (Patil *et al.*, 1995). This mutation causes a change of the corresponding amino acid from glycine (G) at position 156 to serine (S), which is in the ion selectivity filter of the potassium channel in the H5 region. In this way, the "finger print" K^+ channel sequence of glycine-tyrosine-glycine (G-Y-G) is altered to serine-tyrosine-glycine (S-Y-G) in GIRK2 with the *weaver* mutation (GIRK2 *wv*). This results in a striking change in the selectivity of homomultimeric GIRK2 channels. Wild-type GIRK2 channels are highly selective for K^+ ions with the permeability ratio P_{Na}/P_K of <0.05, and are virtually impermeable to Cs^+ (Lesage *et al.*, 1994; Slesinger *et al.*, 1996; Kofuji *et al.*, 1996b). However, homomultimeric GIRK2 *wv* channels allow K^+, Na^+, Rb^+, and Cs^+ to permeate with P_{Na}/P_K of 0.5 to 0.95, P_{Rb}/P_K of ~0.8 and P_{Cs}/P_K of 0.9 to 1.0, but are still impermeable to Ca^{2+}, N-methyl-D-gulcamine, and anions (Slesinger *et al.*, 1996; Kofuji *et al.*, 1996b; Navarro *et al.*, 1996). Thus, the *weaver* mutation renders GIRK2 channels nearly nonselective among monovalent cations.

The *weaver* mutation also has another unexpected effect on GIRK2 channels. Compared with wild-type GIRK2 channels, GIRK2*wv*-channels have a large G protein-independent basal current and a small G protein-induced increase in a current amplitude (Slesinger *et al.*, 1996; Kofuji *et al.*, 1996b; Navarro *et al.*, 1996). This may be because the pore mutation impairs the gating which is crucial for the regulation of channel activity in response to G protein activity. However, Slesinger *et al.* (1996) found that as a larger amount of GIRK2*wv* cRNA was injected into *Xenopus* oocytes, expressed GIRK2*wv*-channels exhibited a larger basal current and a smaller response to G protein stimulation. Thus, it is also possible that the Na^+ influx through GIRK2*wv*-channels increases the intracellular Na^+ concentration, which in turn directly activates GIRK2*wv*-channels and occludes the channels' response to G protein stimulation (Lesage *et al.*, 1995; Sui *et al.*, 1996).

As with the wild-type GIRK2 subunits, GIRK2*wv* subunits form a heteromultimeric channels with GIRK1 (Slesinger *et al.*, 1996; Liao *et al.*, 1996). However, coexpression of GIRK1 and GIRK2*wv* in *Xenopus* oocytes yields current amplitudes not larger than the sum of those obtained with either of the subunits alone. Liao *et al.* (1996) found that in the *wv/wv* brain, the amounts of both GIRK1 and GIRK2 proteins were reduced although the amount of the unglycosylated form of GIRK1 proteins increased compared with the control. Thus, some fraction of the GIRK1/GIRK2*wv* complex might be re-

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tained in the endoplasmic reticulum and not be expressed to the membrane. Furthermore, GIRK1/GIRK2*wv* channels also lack K^+ selectivity and have an impaired response to G protein stimulation (Slesinger *et al.*, 1996; Kofuji *et al.*, 1996b; Navarro *et al.*, 1996; Liao *et al.*, 1996). All these in vitro studies indicate that the effect of the *weaver* mutation can be pleiotropic depending on the expression level of GIRK2*wv* subunits and the presence of other types of GIRK subunits coexisting with GIRK2*wv* subunits.

During the early postnatal development of the mouse cerebellum, the external granule cell layer (EGL) consists of a mitotically active outer layer and a later developing inner postmitoic layer (Goldowitz and Smeyne, 1995). As development proceeds, the postmitotic granule cells migrate out of the EGL to the internal granule cell layer (IGL). In the *wv*/*wv* cerebellum, the granule cells undergo an apoptotic process before the migration. GIRK2 mRNA can be already detected in granule cell precursors in the prenatal mouse cerebellum (Kofuji *et al.*, 1996b). Both GIRK1 and GIRK2 immunoreactivities are found in the mouse cerebellum by the postnatal day (PND) 3 and in the EGL and the newly forming IGL on PND4 (Slesinger *et al.*, 1996). On PND19 and PND27, an anti-GIRK2 and anti-GIRK1 antibodies give a nearly uniform staining in the cerebellum of *wv*/*wv* mice compared with the discrete staining pattern in the wild-type littermates (Liao *et al.*, 1996). This abnormal staining pattern of the *wv*/*wv* cerebellum corresponds to a loss of granule cells in the mutant mice by these ages. Therefore, the temporal expression pattern of GIRK2 and GIRK1 in the mouse cerebellum consists with the time course of the development of the neuronal deficits in the *wv*/*wv* cerebellum.

However, how the malfunction of GIRK2-containing K_G channels leads to the death of the cerebellar granule cells has not been unequivocally identified. The simplest explanation is that the basal or neurotransmitter-induced Na^+ influx caused by the mutation imposes a heavy metabolic burden on granule cells and thereby causes the premature death or prevention of differentiation of the neurons. Kofuji *et al.* (1996b) found that charged channel blockers MK-801 and QX-314 and a Ca^{2+} channel blocker verapamil more potently inhibited GIRK1/GIRK2*wv* channels than wild-type GIRK1/GIRK2 channels. They also found that these blockers potently inhibited the aberrant, constitutively-active Na^+ conductance in cultured *wv*/*wv* granule cells and promoted the survival and differentiation of the neurons in vitro. These results strongly suggest the causal relationship between the Na^+ current caused by the *weaver* mutation and the deterioration of *wv*/*wv* granule cells. Slesinger *et al.* (1996) reported that *Xenopus* oocytes injected with GIRK2 *wv* cRNA also died much faster than those injected with the wild-type GIRK2 cRNA and that the survival period of oocytes was shorter when the larger amount of GIRK2 *wv* cRNA was injected. However, Surmeier *et al.* (1996) reported that they could not detect such aberrant Na^+ currents in their cultured *wv*/*wv* granule cells. Instead, they found that somatostatin and a metabotropic glutamate receptor agonist trans-ACPD induced significantly smaller K_G channel currents in these cells than in the wild-type granule cells. Accordingly, they speculated as follows. In the postnatal cerebellum, the postmitotic, premigratory granule cells are known to be exposed to elevated levels of extracellular glutamate, which may trigger the migration of granule cells from the EGL. On this occasion, the GIRK2-containing K_G channels in the wild-type granule cells may be activated by glutamate and serve to counteract the depolarization caused by stimulation of *N*-methyl-D-aspartate glutamate receptors. However, *wv*/*wv* granule cells may be continuously depolarized by glutamate and die due to excessive Ca^{2+} entry because they lack the K_G channels. It is difficult to reconcile these different two observations. However, Signorini *et al.* (1997) found that the morphology of the cerebellum and midbrain dopaminergic neurons and the fertility of GIRK2 -/- mice are different from those of the *wv*/*wv* mice and indistinguishable from those of the wild-type (GIRK2 +/+) mice. Thus, the loss of GIRK2-containing K_G channels in the *wv*/*wv* mice may not be the primary cause of the *weaver* phenotype. They further argued that the cerebellum of the heterozygous *wv*/- mice is histologically more similar to *wv*/+ mice than that of +/+ or *wv*/*wv* mice. This observation favors the hypothesis that the gain-of-function and gene dosage mechanisms are responsible for the developmental defects in *weaver* mutants.

GIRK2 proteins are widely expressed in different regions in the brain as described in the section VI.B. However, only limited regions such as the cerebellar cortex, substantial nigra, and hippocampus are severely affected by the *weaver* mutation (Hess, 1996). Even within the same regions, damages are not homogeneous. For example, granule cells in the lateral cerebellar hemispheres are more resistant to the *weaver* mutation than midline neurons. Such inhomogeneity could be accounted for by several factors including the pleiotropic effects of the mutation depending on the expression levels of GIRK2 and the other GIRK subunits (Slesinger *et al.*, 1996) as well as the environmental factors that determine the inherent vulnerability of each neuron (Hess, 1996). Further studies are necessary to answer why some neurons are more susceptible to the *weaver* mutation.

VIII. Conclusions

Until 1993, the G protein-activation of inwardly rectifying K^+ channel systems was mainly studied in cardiac myocytes with electrophysiological techniques. The recent rapid progress in the molecular biology of K_G channels has disclosed an unimagined complexity of this

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channel system. Although many aspects of regulation of the K_G channels have been elucidated by the efforts of many laboratories listed in this review, there also have emerged many unclarified but possibly important mechanisms that may underlie the physiological regulation of K_G channels in organs that include heart, brain, and endocrine tissues. We cannot yet explain the molecular mechanisms responsible for receptor-specific control of K_G channels. Because the expression of the different GIRK genes and coupling to different receptor subtypes occurs throughout the central nervous system, the role of the K_G channel and its regulation by G proteins in neural systems requires more attention than it has received to date. Phenomena described for K_{ACh} channels in cardiac atrial myocytes, such as desensitization, deactivation, and cross-talk with other signaling systems have not yet been examined at all in other tissues.

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