

A G Protein-gated K Channel Is Activated via β 2-adrenergic Receptors and G $\beta\gamma$ Subunits in *Xenopus* Oocytes

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ABSTRACT In many tissues, inwardly rectifying K channels are coupled to seven-helix receptors via the Gi/Go family of heterotrimeric G proteins. This activation proceeds at least partially via G $\beta\gamma$ subunits. These experiments test the hypothesis that G $\beta\gamma$ subunits activate the channel even if released from other classes of heterotrimeric G proteins. The G protein-gated K channel from rat atrium, KGA/GIRK1, was expressed in *Xenopus* oocytes with various receptors and G proteins. The β 2-adrenergic receptor (β 2AR), a Gs-linked receptor, activated large KGA currents when the α subunit, G α s, was also overexpressed. Although G α s augmented the coupling between β 2AR and KGA, G α s also inhibited the basal, agonist-independent activity of KGA. KGA currents stimulated via β 2AR activated, deactivated, and desensitized more slowly than currents stimulated via Gi/Go-linked receptors. There was partial occlusion between currents stimulated via β 2AR and the m2 muscarinic receptor (a Gi/Go-linked receptor), indicating some convergence in the mechanism of activation by these two receptors. Although stimulation of β 2AR also activates adenylyl cyclase and protein kinase A, activation of KGA via β 2AR is not mediated by this second messenger pathway, because direct elevation of intracellular cAMP levels had no effect on KGA currents. Experiments with other coexpressed G protein α and $\beta\gamma$ subunits showed that (a) a constitutively active G α s mutant did not suppress basal KGA currents and was only partially as effective as wild type G α s in coupling β 2AR to KGA, and (b) $\beta\gamma$ subunits increased basal KGA currents. These results reinforce present concepts that $\beta\gamma$ subunits activate KGA, and also suggest that $\beta\gamma$ subunits may provide a link between KGA and receptors not previously known to couple to inward rectifiers.

INTRODUCTION

Neural regulation of cardiac function occurs primarily by two pathways: (a) acetylcholine (ACh) released by parasympathetic neurons decreases membrane excitability,

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and thus, heart rate, conduction velocity and contractility, primarily by activating an inwardly rectifying K channel in sinoatrial and atrioventricular nodes and atria. (*b*) Norepinephrine released by sympathetic neurons produces opposite effects primarily by increasing a calcium current. Coupling of cardiac β -adrenergic receptors (β ARs) to calcium channels utilizes a well-established second messenger pathway: activation of the heterotrimeric G protein Gs, stimulation of adenylyl cyclase and phosphorylation of Ca channels by activated protein kinase A (PKA) (Trautwein and Hescheler, 1990). Activation of the inwardly rectifying channel by the muscarinic m2 receptor (m2R) also involves G proteins (Pfaffinger, Martin, Hunter, Nathanson, and Hille, 1985; Breitwieser and Szabo, 1985), but cytoplasmic second messengers are not involved (Soejima and Noma, 1984; Pfaffinger et al., 1985). Instead, activated G proteins are thought to interact directly with the inwardly rectifying channel (Kurachi, Nakajima, and Susimoto, 1986; Yatani, Codina, Brown, and Birnbaumer, 1987a; Logothetis, Kurachi, Golper, Neer, and Clapham, 1987). Because muscarinic stimulation of the inwardly rectifying channel is sensitive to inhibition by pertussis toxin (PTX) (Breitwieser and Szabo, 1985; Pfaffinger et al., 1985), the G proteins involved belong to the Gi/Go family.

Modulation of membrane excitability via G protein-regulated inward rectifying K channels also plays a role in many neuronal tissues (North, 1989; Brown, 1990). Receptor-channel coupling is sensitive to block by PTX, again implying that the relevant G proteins belong to the Gi/Go family. As in the atrium, there is evidence that receptor-channel coupling is membrane delimited in hippocampal neurons (VanDongen, Codina, Mattera, Joho, Birbaumer, and Brown, 1988) anterior pituitary cells (Yamashita, Kajima, Shibuya, and Ogata, 1987; Yatani, Codina, Sekura, Birnbaumer, and Brown, 1987b), and locus ceruleus neurons (Miyake, Christier, and North, 1989). Experiments to date do not eliminate the possible involvement of other membrane-bound intermediates (Clapham, 1994).

Receptor stimulation leads to the formation of two classes of active G protein subunits, GTP-bound α subunits ($G\alpha$ -GTP) and $\beta\gamma$ dimers ($G\beta\gamma$); both classes regulate various effectors (Clapham and Neer, 1993; Birnbaumer, 1992). Which subunit is responsible for channel activation? In early experiments on cell-free excised patches from atrial cells, the inwardly rectifying channel was activated by addition of either α subunits (Codina, Yatani, Grenet, Brown, and Birnbaumer, 1987; Yatani, Mattera, Codina, Graf, Okaber, Padrell, Iyengar, Brown, and Birnbaumer, 1988), $\beta\gamma$ subunits (Logothetis et al., 1987), or both (Logothetis, Kim, Northup, Neer, and Clapham, 1988; Kurachi, Ito, Sugimoto, Katada, and Ui, 1989; Ito, Tung, Sugimoto, Kobayashi, Takahashi, Katada, Ni, and Kurachi, 1992). In recent experiments, Ito et al. (1992) reported that $\beta\gamma$ activation produced a more consistent and higher level of activation than did α subunits. Although direct biochemical evidence is still sparse, strong further evidence favoring $G\beta\gamma$ as the dominant activator is that channel activation by $G\beta\gamma$ subunits could be (*a*) eliminated by preincubation of $G\beta\gamma$ with excess, inactive α (GDP) (Ito et al., 1992); and (*b*) reversed by addition of inactive α (GDP) subunits to the patch (Logothetis et al., 1988; Wickman, Iniguey-Lluhi, Davenport, Taussig, Krapivinsky, Linder, Gilman, and Clapham, 1994).

There is an interesting consequence of the emerging concept that many different $G\beta\gamma$ subunits can activate the G protein-gated inward rectifier (Logothetis, 1987;

Wickman et al., 1994). All seven-helix receptors are thought to liberate Gβγ subunits as a consequence of catalyzing the replacement of bound GDP by GTP on α subunits. As a result, many different receptor types, acting on many different heterotrimeric G proteins, would be expected to liberate Gβγ and therefore to activate the channel under discussion.

Heterologous expression in *Xenopus* oocytes offers an opportunity to test this expectation. cDNA clones have been obtained for many components of the pathway linking seven-helix receptors to G protein-gated K channels, including most recently the channel itself, termed KGA (Dascal et al., 1993a) or GIRK1 (Kubo, Reuveny, Slesinger, Jan, and Jan, 1993b). In the absence of an agreement on systematic terminology, we use the name KGA. We previously showed that the muscarinic m2, serotonin 1A, and δ opioid receptors, when coexpressed with KGA in oocytes, activate robust inward currents in response to agonist application (Dascal et al., 1993a). Coupling of these receptors to KGA utilized G proteins endogenous to the oocyte, since activation did not require injection of any G protein cRNA.

We chose the β2-adrenergic receptor (β2AR) to test the Gβγ pathway. We show here that when β2AR and KGA are coexpressed in *Xenopus* oocytes, only weak activation of KGA is observed in response to isoproterenol (iso). But when Gas is additionally coexpressed, we observe efficient coupling between the β2AR and KGA. The currents activated by iso are increased 10- to 20-fold by Gas coexpression, and are as large as currents observed in response to muscarinic stimulation. It is important to eliminate interference from the PKA pathway. We have examined the coupling mechanism of β2AR to KGA in oocytes, and we conclude that isoproterenol activation of KGA does not occur via the PKA pathway, but does occur via the expected release of G protein βγ subunits.

EXPERIMENTAL PROCEDURES

Heterologous Expression in Xenopus Oocytes

Stage V–VI *Xenopus laevis* oocytes were prepared as described (Quick and Lester, 1994; Dascal and Lotan, 1992) and injected with one or more cRNAs synthesized in vitro from cDNA clones by the appropriate bacteriophage RNA polymerase after linearization with an appropriate restriction enzyme. Amounts per oocyte are given in parentheses, except where indicated in the text: KGA in pBluescript-KS (Dascal et al., 1993a) (1 ng); cystic fibrosis transmembrane conductance regulator, CFTR, in pAlter1 (McDonough, Davidson, Lester, and McCarty, 1994) (2 ng); muscarinic receptor, m2R, in pGEM3 (gift of E. Peralta, Harvard University, Boston, MA) (Lechleiter, Hellmiss, Duerson, Ennulat, David, Clapham, and Peralta, 1990) (200 pg); beta-2 adrenergic receptor, β2AR, in pSP65 (Kobilka, MacGregor, Daniel, Kobilka, Caron, and Lefkowitz, 1987) (50 pg); Gai2 and Gai3 in pGEM2 (gifts of R. Reed, Johns Hopkins University, Baltimore, MD) (0.05–5 ng); Gβ1 (Fong, Hurley, Hopkins, Miake-Lye, Johnson, Doolittle, and Simon, 1986) and Gγ2 (Gautam, Baetscher, Aebersold, and Simon, 1989) in pFroggy (0.4–6 and 0.2–3 ng, respectively). Gas and Gαolf were amplified from cDNAs with PCR primers that conferred SP6 promoters and alfalfa mosaic virus 5'-untranslated regions and a poly (A) tail, followed by in vitro cRNA synthesis with bacteriophage promoters (Mager, Naeve, Quick, Guastella, Davidson, and Lester, 1993; Uezono, Bradley, Min, McCarty, Quick, Riordan, Chavkin, Zinn, Lester, and Davidson, 1993). After injection, oocytes were incubated for 3–6 d at 19°C before recording. Incubation medium was ND96 (see below) supplemented

with 2.5 mM sodium pyruvate and 50 $\mu\text{g/ml}$ gentamycin and, occasionally, 600 μM theophylline or 5% horse serum.

Electrophysiology and Solutions

KGA activity was assayed by two-electrode voltage clamp using an amplifier (model 8500, Dagan Corp., Minneapolis, MN). Oocytes were placed in a chamber and initially perfused with normal saline, ND96, (96 mM NaCl, 2 mM KCl, 1 mM CaCl_2 , 1 mM MgCl_2 , 5 mM HEPES, pH 7.4–7.5). To increase the current signal through inwardly rectifying K channels, the solution was then changed to a high potassium-containing solution, highK, containing 96 mM KCl, 2 mM NaCl, 1 mM CaCl_2 , 1 mM MgCl_2 , 5 mM HEPES, pH 7.4–7.5. Stimulation and data acquisition were performed using pCLAMP software (Axon Instruments, Foster City, CA).

RESULTS

Effect of Gas Coexpression on $\beta 2\text{AR}$ -KGA Coupling

Xenopus oocytes were injected with in vitro synthesized cRNA encoding KGA and $\beta 2\text{AR}$, and membrane currents were recorded using the two-electrode voltage clamp technique. In all experiments, the membrane potential was clamped at a holding level of -80 mV, and initially perfused with ND96 containing 2 mM KCl. To maximize inward K currents, the bath solution was changed to a high potassium solution (highK), containing 96 mM KCl. This solution change led to the development of an inward current (Fig. 1, *A* and *B*) composed of two components: (*a*) an endogenous inwardly rectifying current observed even in noninjected oocytes; and (*b*) an inwardly rectifying current observed only in KGA cRNA-injected oocytes, and which we attribute to agonist-independent or basal KGA activity (Soejima and Noma, 1984; Okabe, Yatani, and Brown, 1991; Ito, Sugimoto, Kobayashi, Takahashi, Katada, Ui, and Kurachi, 1991; Dascal et al., 1993*b*). The basal KGA currents were not detectably changed ($<10\%$) by coinjection of m2R or $\beta 2\text{AR}$ cRNAs at the levels used in these experiments. The current trace of Fig. 1 *A* was recorded from a cell with injected $\beta 2\text{AR}$ and KGA cRNAs but without coinjected G protein cRNAs and shows that only a small inward current response is evoked by addition of iso to the highK bath solution. The iso-activated current in oocytes coinjected with $\beta 2\text{AR}$ and KGA cRNAs was $40 \text{ nA} \pm 9 \text{ nA}$ (mean \pm SEM, $n = 33$, range 0–240 nA), only a small increment to the basal KGA current of $170 \pm 25 \text{ nA}$ for this group of oocytes. Iso responses were not observed in control cells injected with only KGA cRNA ($n = 9$), only $\beta 2\text{AR}$ cRNA ($n = 4$), $\beta 2\text{AR}$ cRNA and Gas cRNA ($n = 2$), or in noninjected oocytes ($n = 7$).

Since βARs couple to effectors via the heterotrimeric G protein G_s , we tested the effects of coinjecting cRNA for the α subunit, Gas, along with cRNA for $\beta 2\text{AR}$ and KGA. We found that isoproterenol then evoked large inward currents (Fig. 1 *B*). The iso-activated currents were comparable in amplitude to currents evoked by ACh via the m2R: inward current amplitude at saturating agonist concentration was $1015 \pm 126 \text{ nA}$ for ACh versus $1285 \pm 127 \text{ nA}$ for iso (mean \pm SEM, $n = 21$) in cells expressing KGA, Gas, and both m2R and $\beta 2\text{AR}$. Fig. 1 *C* displays a dose-response relation for the iso-activated current as a function of iso concentration. The apparent half-maximal iso concentration, $\sim 80 \text{ nM}$, is in good agreement with typical $\beta 2\text{AR}$

responses. The dependence of the iso-activated current on the amount of Gas cRNA coinjected is shown in Fig. 1 *D* (top). Coinjection of only 200 pg of Gas cRNA is sufficient to augment the iso response by 15-fold compared to oocytes injected with KGA cRNA and β 2AR cRNA alone.

Gas coexpression also affects the basal KGA current. When the bath solution is changed from ND96 to highK, the inward current response (hK current) is smaller in oocytes coexpressing Gas (Fig. 1, *A* and *B*). Fig. 1 *D* (bottom) plots the dose-dependent decrease in hK current as a function of Gas coinjection. In each of three experiments like that of Fig. 1 *D*, augmentation of the iso-activated current had a higher sensitivity to Gas coexpression than did suppression of the hK current. In these experiments we measured the net hK current, which includes both the

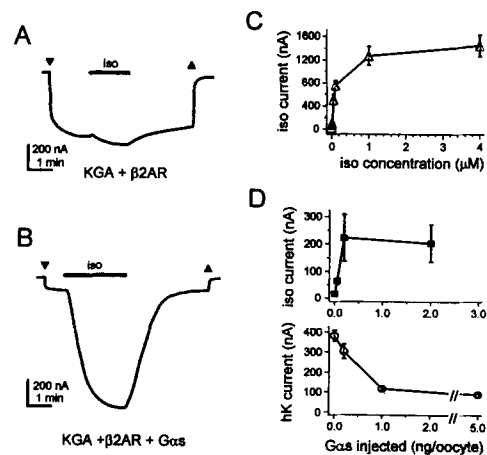


FIGURE 1. The β 2AR couples efficiently to KGA only when Gas is coexpressed. (*A* and *B*) Current traces recorded from oocytes injected with KGA and β 2AR without G proteins (*A*) and with 1 ng Gas (*B*). The voltage is clamped at -80 mV in this and in all subsequent figures. At time indicated by the down arrowhead, the bath solution is switched from ND96 (containing 2 mM KCl) to highK (containing 96 mM KCl), and this leads to an increase in inward current. The β receptor-specific agonist, isoproterenol (iso, 20 nM), is added to the highK solution during the period indicated by the bar. At the time indicated by the up arrowhead, the bath solution is returned to ND96. (*C*) Dependence of iso current on the concentration of iso. The EC50 is ~ 80 nM. (*D*) Dose-dependent effect of Gas cRNA on the iso current and on the basal KGA current. The upper panel plots the amplitude of the current evoked by addition of 20 nM iso to highK solution versus the amount of Gas cRNA injected per oocyte. All groups of oocytes were also injected with cRNA for KGA and β 2AR. The lower panel plots the total inward current evoked when switching from ND96 to highK solution (hK current) as a function Gas cRNA coinjected. Symbols are mean \pm SEM for four to six oocytes.

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endogenous oocyte inward rectifier current and the basal KGA current. In subsequent experiments, we used Ba block to discriminate between these two components (Dascal et al., 1993b), and found that the endogenous inward current is unaffected by Gas coexpression: the endogenous current was 73.6 ± 5.0 nA (mean \pm SEM, $n = 10$) without G protein, and 74.5 ± 4.6 nA (mean \pm SEM, $n = 11$) in oocytes injected with Gas cRNA. Thus, the reduction in hK current at 1–5 ng Gas in Fig. 1 *D* (bottom) reflects the selective and nearly complete block of basal KGA activity.

Comparison of *m*2R and β 2AR Activation of KGA

The current stimulated by isoproterenol in oocytes expressing KGA, β 2AR and Gas appears identical to KGA currents activated via Gi/Go-coupled receptors. Because

the ACh-activated KGA current is blocked almost completely by 200–300 μM Ba^{2+} , whereas the endogenous oocyte current is nearly unaffected by Ba^{2+} at this concentration (Dascal et al., 1993b), we tested the effect of 200 μM BaCl_2 on the iso-activated current (Fig. 2A). Addition of Ba^{2+} to the highK solution blocks the basal KGA current component, and addition of Ba^{2+} to the highK solution plus iso blocks both the current and the basal iso-activated; KGA current; the residual unblocked current in both cases is the endogenous oocyte inward current. The current-voltage (I - V) characteristics of the current activated by ACh or iso are also similar, as illustrated in Fig. 2B which shows I - V curves obtained from the same cell

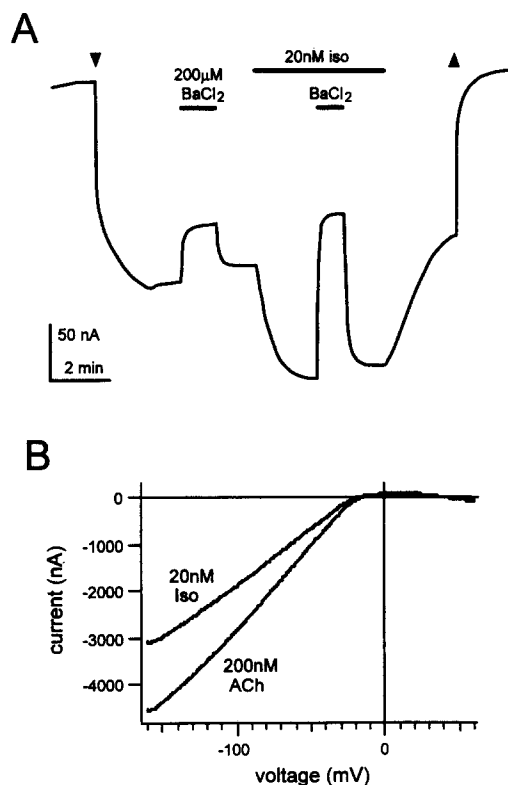


FIGURE 2. The current activated by isoproterenol is blocked by low concentrations of Ba^{2+} and is inwardly rectifying. (A) Current recorded from a cell injected with KGA, $\beta 2\text{AR}$ and 0.5 ng Gas . An inward current develops when the bath solution is switched from ND96 to highK (down arrowhead). Bars above the trace indicate addition of 200 μM BaCl_2 or 20 nM iso to the highK solution. Similar results were obtained in each of three experiments. (B) Current-voltage relations of agonist-activated currents recorded from a cell injected with cRNAs for KGA, 0.5 ng Gas , and both the m2R and $\beta 2\text{AR}$. Agonist concentrations were 200 nM ACh and 20 nM iso, respectively. Current is recorded as the voltage is ramped from -160 mV to $+60$ mV during a 1.6-s interval. Traces shown are obtained by subtracting current in highK solution from the current in highK plus iso or ACh. Similar results were obtained in each of four experiments.

coinjected with cRNA for KGA, Gas , and both m2R and $\beta 2\text{AR}$. Thus, both a Gs-linked receptor and Gi/Go-linked receptors are coupled to KGA in *Xenopus* oocytes.

Evidence that the m2R and $\beta 2\text{AR}$ do not utilize identical pathways to activate KGA is presented in Fig. 3. Agonist-activated currents were recorded from the same oocyte and superimposed in Fig. 3A along with $\beta 2\text{AR}$ stimulation of the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel that is activated by PKA-dependent phosphorylation (Riordan, 1993), in another oocyte. Both the activation and deactivation kinetics are slower for $\beta 2\text{AR}$ -KGA coupling than for

m2R-KGA coupling. The time required for the KGA current to reach 50% of the peak value is 2.9 ± 1.1 s ($n = 4$) for ACh responses and 12.4 ± 3.5 s ($n = 5$) for iso responses. After removal of agonist, the current declines 50% in 11.1 ± 2.9 s ($n = 4$) for the ACh response and 36.6 ± 2.6 s ($n = 5$) for the iso response. The iso-induced CFTR responses, which occur via the cAMP-PKA second messenger pathway, are even slower than the iso-induced KGA responses; the CFTR responses show a pronounced delay after agonist application and very little deactivation on the time scale of these experiments. The contrast with CFTR waveforms suggests that β 2AR

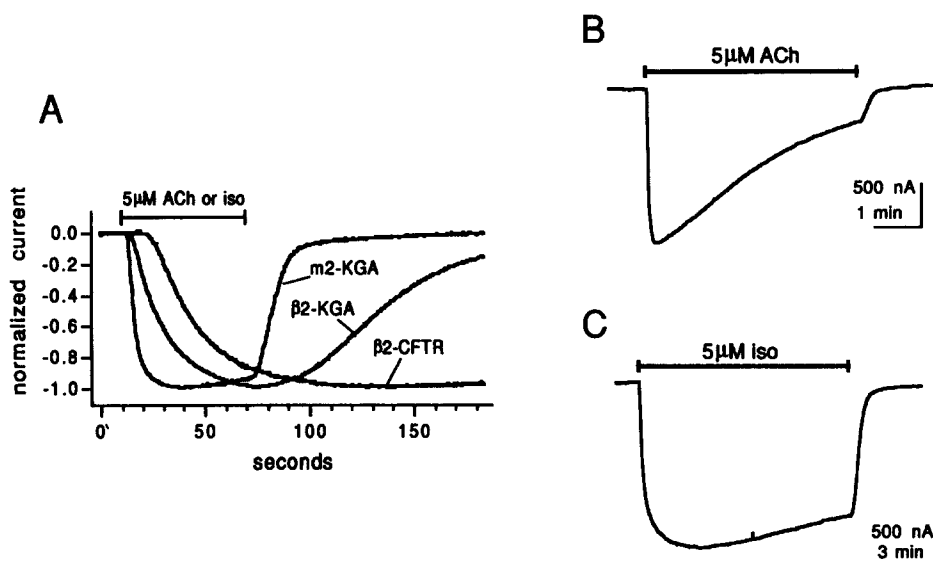


FIGURE 3. Activation, deactivation, and desensitization kinetics are slower for the iso-activated current than for the ACh-activated current. (A) Traces recorded from the same oocyte injected with cRNA for KGA, $G_{\alpha s}$ (0.5 ng), m2R and β 2AR are superimposed. Either 5 μ M ACh or 5 μ M iso was added to highK recording solution for 1 min as indicated by the bar above the traces. A trace is also superimposed for another oocyte injected with cRNA for β 2AR, $G_{\alpha s}$ (0.5 ng), and CFTR and exposed to iso in ND96 for the same time period; note the much slower activation and deactivation. (B and C) Agonists were applied for longer durations to examine the rate of desensitization. Traces are from the same cell and show the response to a 4.5-min perfusion of 5 μ M ACh (B), or a 14-min perfusion of 5 μ M iso (C). Note the different time scales.

does not activate KGA via the cAMP-PKA second messenger pathway. In more direct experiments on this point, we bypassed receptor stimulation and elevated intracellular cAMP levels directly by perfusing the oocyte with a cocktail of drugs that included 10 μ M forskolin, 200 μ M dibutyryl-cAMP, and 100 μ M 3-isobutyl-1-methylxanthine (IBMX). As a positive control, we applied this 'cAMP cocktail' to oocytes expressing the β 2AR and CFTR. In agreement with previous results (McCarty, McDonough, Cohen, Riordan, Davidson, and Lester, 1993; Uezono et al., 1993), Fig. 4A shows that a 2-min application of the cAMP cocktail activated CFTR current about half as

large as that stimulated by a 2-min application of 50 nM iso. In Fig. 4 *B*, a 4-min application of the cAMP cocktail to a cell expressing KGA, β 2AR, *G* α s, and m2R, did not produce an inward current response. Furthermore, the cAMP cocktail did not significantly affect either the amplitude or the kinetics of the ACh response; the trend for the three responses in Fig. 4 *B* is typical of the rundown we and others (Chen and Yu, 1994) have observed for successive agonist applications. Thus, we conclude that (a) isoproterenol activation of KGA does not occur by the cAMP-PKA pathway; and (b) PKA activation does not significantly modulate KGA currents.

Desensitization of KGA currents may occur at several points in the transduction pathway. In particular, desensitization occurs more slowly with increasing amounts of receptor mRNA injected (N. Dascal, unpublished results). For instance, in the experiment of Fig. 3 *B*, with a 200-pg m2R cRNA injection, desensitization was faster than in Fig. 2 *C* of Dascal et al. (1993*a*), which involved several times more m2R

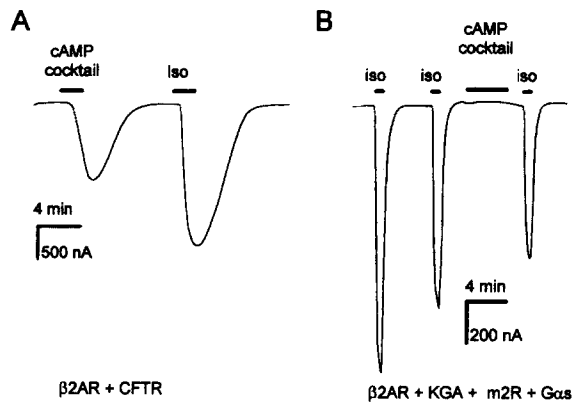


FIGURE 4. KGA activation by isoproterenol is not mediated by protein kinase A. (A) CFTR can be activated by agents that elevate cAMP and activate PKA. This cell was injected with β 2AR and CFTR cRNAs and currents are recorded in ND96. A cAMP cocktail (110 μ M forskolin, 200 μ M dibutyryl-cAMP, and 100 μ M 3-isobutyl-1-methylxanthine [IBMX]) was added for a 2-min interval as indicated, and potently activates CFTR. A 50-nM application of iso also activates CFTR. Similar results were obtained in each of six experiments. (B)

The same cAMP cocktail fails to activate KGA or modulate the response to ACh (200 nM). Current was recorded in highK from a cell injected with KGA, m2R, β 2R, and 0.5 ng *G* α s cRNAs. Similar results were obtained in each of five experiments.

cRNA. Within a given batch of oocytes and for roughly equal KGA currents, we also find that KGA currents evoked by the β 2AR consistently desensitize considerably more slowly than those evoked by the m2R. In the example of Fig. 3 *B* and *C*, ACh responses decay to half the peak value in 2.0 ± 0.2 min ($n = 3$), whereas the iso responses decline by only $\sim 10\%$ in 6.5 min ($n = 3$).

If the m2R and β 2AR pathways converge at some point, one expects occlusion of the two responses. Fig. 5 presents one of a series of experiments to test this point. For all amounts of KGA cRNA tested (10–1,000 pg), we found partial occlusion between the two pathways. During maximal ACh currents, it was still possible to induce iso currents (Fig. 5), and vice versa (data not shown). Occlusion is shown by the fact that the currents during combined stimulation were substantially less than the sum of the currents for individual stimulation.

G Protein α Subunit Coexpression Studies

Because the iso activation of KGA requires $G_{\alpha s}$ but does not involve adenylyl cyclase, the activated G_s subunits, either $G_{\alpha s}(GTP)$ or $G\beta\gamma$, released by β 2AR stimulation must be affecting KGA by another direct or indirect pathway. To help discriminate between participation by α or $\beta\gamma$ subunits, we tested the effect of coexpression of various G protein subunits on three measures of KGA activity: (a) basal or agonist-independent KGA currents; (b) m2R-KGA currents; and (c) β 2AR-KGA currents. Fig. 6A summarizes the effect of injecting cRNAs for several G protein α subunits on the amplitude of basal KGA current. The results for each G protein tested are expressed as a percentage of the basal current in the control group, which was coinjected with KGA, m2R, and β 2AR cRNAs only (i.e., no exogenous G protein cRNA). The basal KGA current was measured as the component of hK current that was inhibited by 250 μ M BaCl₂ (see for example Fig. 2A), and therefore, the results exclude any contribution of the endogenous inward current. As in Fig. 1, coexpression of $G_{\alpha s}$

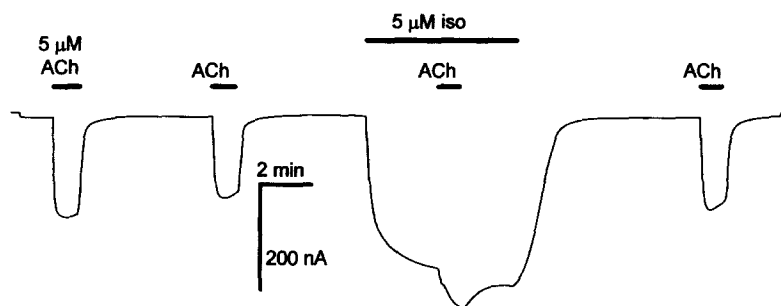


FIGURE 5. The β 2AR and m2R pathways occlude partially. Traces were recorded from an oocyte coinjected with cRNA for β 2AR, m2R, $G_{\alpha s}$ (0.5 ng), and KGA (50 μ g). The ACh and iso concentrations (5 μ M) evoked maximum responses individually (Fig. 1C and data not shown). Similar results were obtained in each of five experiments.

strongly inhibits basal KGA current. $G_{\alpha olf}$, which couples odorant receptors to adenylyl cyclase and shares 88% sequence identity with $G_{\alpha s}$ (Simon, Strathmann, and Gautam, 1991), substituted only partially for $G_{\alpha s}$ in previous experiments on oocyte expression (Uezono et al., 1993). $G_{\alpha olf}$ inhibits the basal current by only 30%; but this difference is highly significant ($P < 0.05$). It is possible that at a higher concentration, $G_{\alpha olf}$ may further reduce the basal current, since basal current inhibition requires a larger amount of injected cRNA than does augmentation of the iso response (see Fig. 1D). Neither $G_{\alpha i1}$, $G_{\alpha i2}$, nor $G_{\alpha s}Q227L$ produced a significant reduction in the basal current ($P > 0.05$ in each case).

The specific reduction of basal KGA current by $G_{\alpha s}$ could result from (a) an inhibitory action produced by a basal activity of $G_{\alpha s}$ subunits or (b) sequestration of a pool of free $\beta\gamma$ subunits which normally stimulate KGA current. To test these possibilities, we employed a mutant $G_{\alpha s}$, $G_{\alpha s}Q227L$; this substitution in a region near the nucleotide binding pocket reduces the GTPase activity of the protein by at least 100-fold (Masters et al., 1989; Graziano and Gilman, 1989). Thus, $G_{\alpha s}Q227L$

has a high fractional occupancy by GTP and is constitutively active, even in the absence of receptor stimulation. When cRNA encoding GasQ227L is coinjected into oocytes along with KGA, β 2AR and m2R, there is no reduction in basal KGA activity compared to controls without G proteins (Fig. 6A). Thus, it is unlikely that the first possibility, an inhibitory action mediated by the α subunit, can account for the reduction in basal current produced by wild-type Gas. This mutant is predominantly in the GTP-bound state and is not complexed with $\beta\gamma$ subunits. Therefore, the inability of GasQ227L to inhibit basal KGA current could be associated with the less efficient sequestration of $\beta\gamma$ subunits in the oocyte.

Coupling of m2Rs to KGA in the atrium is mediated by PTX-sensitive G proteins (Breitwieser and Szabo, 1985; Pfaffinger et al., 1985), and recombinant, preactivated Gai1, Gai2, and Gai3 activated KGA in excised patches from atrial myocytes (Yatani

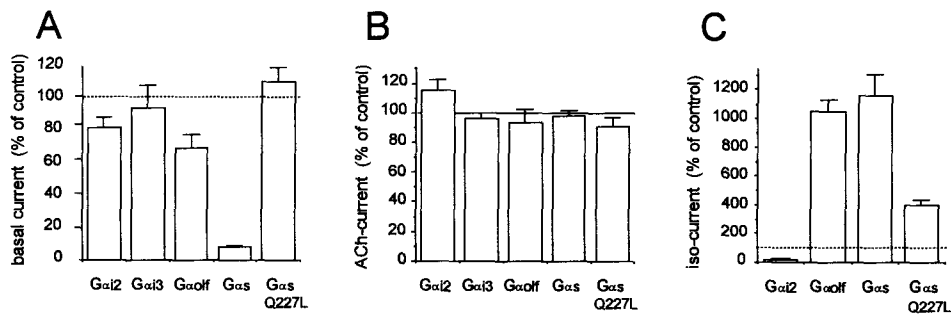


FIGURE 6. Effect of G protein α subunit coexpression on basal and agonist-evoked KGA currents. In A–C, the control group of oocytes were coinjected with KGA, β 2AR, and m2R cRNAs. Other groups of oocytes were coinjected with KGA, β 2AR, and m2R cRNAs and either 2 ng Gai2, 2 ng Gai3, 2 ng Gaolf, 0.5 ng Gas, or 1 ng GasQ227L cRNAs. (A) Basal KGA activity was measured as the Ba-sensitive component of the current in highK solution for each of the G α -coinjected groups and plotted as a percentage of the basal current of the control group (*dashed line*). (B) The current evoked by addition of ACh to the highK solution was measured for each of the G α -coinjected groups and plotted as a percentage of the ACh-evoked current of the control group. (C) The iso-evoked current for each of the G α -coinjected groups is plotted as a percentage of the iso-evoked current of the control group. Values represent mean \pm SEM for four to seven oocytes.

et al., 1988). However, none of the G protein α subunits tested in our coexpression studies, including Gai2, and Gai3, had any reproducible effect on KGA currents activated by the m2R (Fig. 6B) or by the 5-HT1A receptor, although such injection does alter PTX sensitivity (Dascal et al., 1993b). We also tested the effect of G protein α subunits on β 2AR-KGA coupling, and found that Gaolf, in addition to Gas, could augment isoactivated currents (Fig. 6C). Although the iso-current is small in control cells without exogenously expressed G proteins (144 nA in this experiment), the iso current was reduced even further by coexpression of Gai2. Coexpression of the constitutively active mutant, GasQ227L, potentiated isoactivated currents by fourfold compared to control, which is less than the 10-fold increase observed for Gas or Gaolf.

GasQ227L also had a striking effect on the time course of β 2AR-KGA coupling. Fig. 7 compares the KGA current stimulated by a 1-min application of isoproterenol in oocytes expressing KGA, β 2AR, m2R and either wild-type *Gas* (left) or *GasQ227L* (right). The markedly slower deactivation rate of the iso response observed with coexpressed *GasQ227L* indicates that the rate of deactivation of KGA is in part determined by the deactivation (or GTPase) rate of the G protein. This supports the view that kinetic differences between the iso and ACh responses (Fig. 3) may simply reflect the different turnover rates of the heterotrimeric G proteins involved in coupling the two receptors to KGA. We also found a slower activation rate for *GasQ227L* but did not study activation kinetics systematically in view of the small responses.

G Protein $\beta\gamma$ Subunit Coexpression Studies

To study further which G protein subunit may couple to KGA, we tested the effect of $\beta\gamma$ subunit cRNAs in the oocyte expression system. Unlike free α subunits, which exist in both inactive (GDP-bound) and active (GTP-bound) form, free $\beta\gamma$ subunits

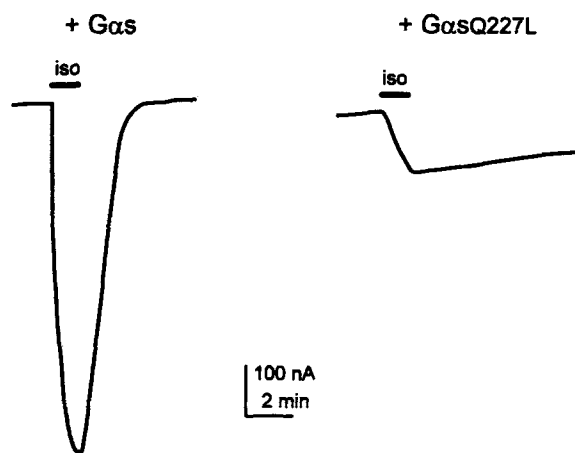


FIGURE 7. The deactivation rate of KGA is decreased in oocytes coexpressing the constitutively active *Gas* mutant. (Left) A typical response in highK to 100 nM iso applied for 1 min for a cell coinjected with KGA, m2R, β 2AR, and 0.5 ng *Gas* cRNAs. (Right) The current was recorded from a cell expressing KGA, m2R, β 2AR, and 1 ng *GasQ227L*. A 1-min application of 100 nM iso evokes a smaller response (see Fig. 6 C) that slowly partially deactivates after iso is removed.

are thought to be constitutively active. Kubo et al. (1993b) reported an increase in agonist-independent KGA current when the three cRNAs for $G\alpha 2$, $\beta 1$, and $\gamma 2$ were coexpressed in oocytes and later showed that equivalent activation occurred if only the $\beta 1$ and $\gamma 2$ subunit cRNAs were injected (Reuveny, Slesinger, Inglese, Morales, Iñiguez-Lluhi, Lefkowitz, Bourne, Jan, and Jan, 1994). We also found activation by $\beta 1\gamma 2$ alone: in oocytes injected with cRNA for these two subunits, there was a 7- to 10-fold increase in agonist-independent KGA current (Fig. 8, A and B). This result, combined with the inhibition of basal current by *Gas*, argues that $\beta\gamma$ subunits underlie agonist-independent KGA activity. There was also a 45% decrease in the ACh-evoked current (Fig. 8 A). The inhibition of the ACh response is presumably due to the fact that there is a limiting amount of channel activity in the oocyte; thus the increase in basal current is achieved at the expense of the agonist-evoked current.

Fig. 8 B presents results for a set of oocytes coinjected with combinations of cRNA

for $\beta 2AR$, $G\alpha s$, $\beta 1\gamma 2$, and KGA. The elevated basal KGA current levels due to $\beta 1\gamma 2$ were decreased by coinjection of $G\alpha s$; thus, $G\alpha s$ suppresses KGA activation due to exogenous $\beta\gamma$ subunits as well as that due to endogenous $\beta\gamma$ subunits. Interestingly, the $\beta 1\gamma 2$ expression had the effect of abolishing the incremental iso responses in the absence of injected $G\alpha s$ cRNA and of reducing the additional iso responses by 30% in the presence of $G\alpha s$. The latter result resembles the reduction found for the m2R pathway (Fig. 8A) and shows that there is occlusion between the $\beta 2AR$ activation pathway and the $\beta\gamma$ activation pathway, again presumably due to a limiting amount of channel activity in the oocyte.

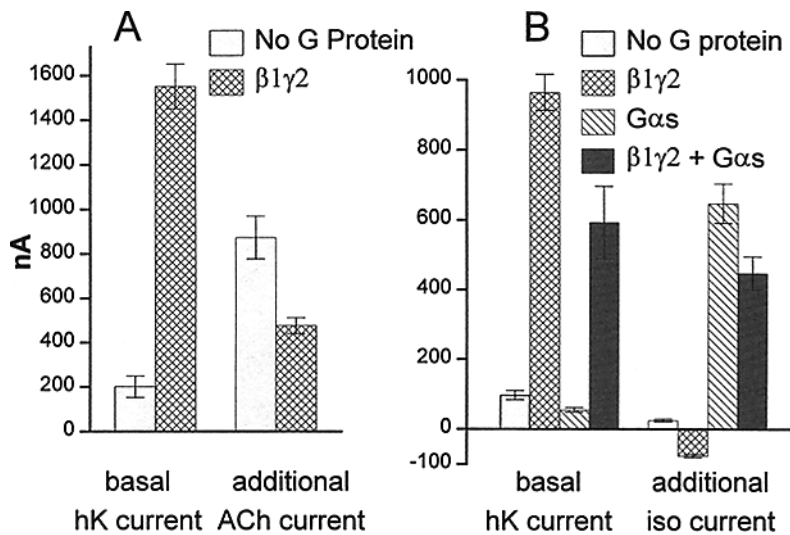


FIGURE 8. Effect of G protein $\beta\gamma$ subunits on basal and agonist-evoked, KGA currents. (A) One group was coinjected with KGA and m2R cRNAs. A second group was coinjected with KGA, m2R and 1.5 ng $\beta 1$ and 0.75 ng $\gamma 2$ cRNAs. Basal KGA current was measured as the current in highK solution blocked by 250 μM BaCl₂. ACh current was activated by adding 200 nM ACh to the highK solution. Bars indicate mean \pm SEM for five to seven oocytes. (B) Groups of oocytes were injected with cRNAs for $\beta 2AR$ and KGA; for $\beta 2AR$, $\beta 1\gamma 2$, and KGA; for $\beta 2AR$, $G\alpha s$, and KGA; and for $\beta 2AR$, $G\alpha s$, $\beta 1\gamma 2$, and KGA. Other procedures as in A. Bars indicate mean \pm SEM for five to six oocytes.

DISCUSSION

Our principal results may be summarized as follows.

- Coexpression in oocytes of the $\beta 2AR$ and KGA cRNAs resulted in a small (~ 40 nA) iso-evoked inwardly rectifying K⁺ current through KGA channels.
- However, coexpression of the $\beta 2AR$, KGA, and $G\alpha s$ resulted in: (i) a large iso-evoked current comparable to that evoked by ACh in oocytes expressing the m2R and KGA; and (ii) a marked reduction in the magnitude of the basal, inwardly rectifying KGA current in high potassium bath solution.

(c) We also find that the iso current is not due to elevation of cAMP and the consequent activation of PKA.

(d) When the constitutively active G α s mutant subunit, Q227L, was substituted for wild-type G α s in the experiments described in (b) above, there was (i) a smaller (~40%) iso-evoked current and (ii) no suppression of the basal hK current. Remarkably, the duration of the evoked current from a 1-min exposure to iso is increased from ~2 to >10 min.

(e) Several lines of evidence indicate that the iso- and ACh-evoked K⁺ currents use partially independent but partially converging pathways. (i) The activation, deactivation, and desensitization kinetics are slower for the iso-evoked current than for the ACh-evoked current. (ii) In oocytes coexpressing β 2AR, m2R, G α s and KGA, there is partial occlusion between the m2R and β 2AR responses. That there is some occlusion indicates that the two pathways must converge at some step in their respective mechanisms.

(f) One striking result is that whereas injection of 0.5 ng of G α s cRNA with the other necessary ingredients greatly enhances the iso-evoked current and suppresses the basal hK current, injection of 2 ng of a G α i cRNA has a negligible effect on the m2R/KGA system.

(g) Coexpression of G β 1 γ 2 with KGA and either m2R or β 2AR results in an increase in the basal hK current and a corresponding reduction in the ACh-evoked or iso-evoked currents, respectively.

Mechanistic Interpretations

We propose the following overall mechanistic interpretation of our results. The mechanism invokes the generally accepted cycle in which an activated receptor interacts with the heterotrimeric G protein to catalyze replacement of bound GDP by GTP; this in turn leads to formation of free G α s(GTP) and G β γ subunits; either or both these moieties activate G protein effectors in many systems. Hydrolysis of bound GTP then leads to recombination of G α s(GDP) with G β γ to reform the heterotrimeric G protein and to deactivation of the effector. We do not have rigorous patch clamp evidence that the β 2AR pathway is membrane delimited, although we show that it does not involve cAMP. The specific mechanistic interpretations are then as follows.

(a) The pathways for activation by both the m2R and β 2AR involve G β γ subunit. Evidence for the principal role of G β γ subunits in the ACh-evoked response in atrial myocytes has been provided by many authors (Logothetis et al., 1987; Kurachi et al., 1989; Ito et al., 1992; Wickman et al., 1994). The first and most obvious support for this result in the present work is the observation that injection of G β γ subunit cRNA augments the basal hK current at the expense of the evoked ACh response (Results summary *g* above and Fig. 8).

(b) Injection of G α s RNA results in an increase by severalfold in the amount of G α s subunits available to participate in the activation pathway. These G α s polypeptides compete effectively with the pool of other G α polypeptides in the oocyte for the available pool of G β γ subunits, thus increasing the total amount of Gs heterotrimers. The response of β 2AR to iso in the oocyte is limited by the endogenous level of available Gs heterotrimer and is enhanced when the level is raised, as in the present experiments.

To explain our data, we must also assume that in oocytes there is only a low level of endogenous $G_{\alpha s}$ available for the β_2 receptor-KGA signal transduction pathway. Full data are not available on this point; but the assumption may be reasonable in view of the facts that the oocyte contains less endogenous mRNA for $G_{\alpha s}$ than for other G_{α} subunits (Onate, Herrera, Antonelli, Birnbaumer, and Olate, 1992) and that much of the endogenous $G_{\alpha s}$ is associated with yolk granules rather than with plasma membrane (L. Jaffe, personal communication). As stated above, we must further assume that the amount of Gs heterotrimer is rate limiting under our experimental conditions for $G\beta\gamma$ liberation by iso-stimulation of β_2 AR. It then follows that the iso/ β_2 AR/ $G\beta\gamma$ /KGA pathway is indeed enhanced by coinjection of $G_{\alpha s}$.

Incidentally, other studies have shown that $G_{\alpha olf}$ acts similarly to $G_{\alpha s}$ in responding to β_2 AR stimulation, albeit with slightly weaker effects (Jones and Reed, 1989; Uezono et al., 1993). Thus, the results reported in Fig. 6 for $G_{\alpha olf}$ are as expected.

The additional observation that basal hK current is suppressed by $G_{\alpha s}$ coexpression requires the plausible assumptions that: (i) the basal hK current principally results from gating by a steady state pool of free $G\beta\gamma$ dimers in the oocyte; and (ii) after injection of $G_{\alpha s}$ cRNA, the pool of free $G\beta\gamma$ dimers is depleted by combination with the expressed $G_{\alpha s}$ subunits in the $G_{\alpha s}(GDP)$ form.

(c) The constitutively active $G_{\alpha s}$ mutant, Q227L, has a greatly reduced, but evidently nonzero k_{CAT} for GTP hydrolysis (Graziano and Gilman, 1989). Thus, in the oocyte, a much smaller fraction of the mutant subunit is in the heterotrimer form, and a larger fraction in the $G_{\alpha s}$ (GTP) form. This explains how the enhancement of the iso current and the suppression of basal hK current are less for the mutant than for wild-type $G_{\alpha s}$ subunits. Furthermore, because the ratio of free $G_{\alpha s}(GDP)/G_{\alpha s}(GTP)$ is much lower for the mutant, the rate of removal of $G\beta\gamma$ subunits by heterotrimer formation is reduced and the evoked current has a longer duration after an iso pulse (Results summary *d* and Fig. 7). The half-time for deactivation, ~ 10 min, is in rough agreement with the GTPase rate of Q227L (Graziano and Gilman, 1989). That the Q227L mutant produces no basal KGA activation contrasts with the basal activation produced by $G\beta_1\gamma_2$ and provides further evidence for the primacy of the free $G\beta_1\gamma_2$, rather than the $G_{\alpha s}(GTP)$ subunit, in activating the channel.

That the Q227L mutant potentiates the iso current by \sim fourfold (Fig. 6 C) without affecting basal KGA current is reminiscent of the result, in native heart, that intracellular GTP- γ -S produces marked increases in the ACh-activated currents before it increases the basal current (Karschin, Ho, Labarca, Elroy-Stein, Moss, Davidson, and Lester, 1991). We previously suggested the implication that activation of each channel requires the cooperative action of several identical G proteins (Karschin et al., 1991); according to present concepts, these G proteins are $\beta\gamma$ subunits.

(d) Injection of $G_{\alpha i}$ subunit cRNAs does not have a statistically significant effect on increasing the evoked response to ACh and suppressing the basal current, whereas $G_{\alpha s}$ RNA has a pronounced effect on the iso current and in suppressing basal hK currents. To interpret this result, we assume that the endogenous level of available G_i is high enough to be saturating and not rate limiting. However, to explain the absence of suppression of hK current, and the fact that $G_{\alpha s}$ RNA does not detectably affect the m2R response, we must further assume (i) that the fractional increase in

G α i levels due to the injected G α i cDNA is also small and/or (ii) that G α i and G α s interact with at least partially distinct populations of endogenous G $\beta\gamma$ subunits.

(e) The time course of activation is similar to that for deactivation, but these processes are much slower for the β 2AR pathway than for the m2R pathway, and slower still for the β 2AR with coexpression of GasQ227L. These facts suggest that a common step dominates both activation and deactivation but that this step differs in rate among these three cases. It may seem paradoxical that a common step dominates both these events; we explain as follows. In classical concepts of channel gating, rate constants for activation are the sum of the rate constants for opening and closing; rate constants for deactivation are simply the rate of closing. We assume that the rate constant for opening is much less than that for closing in all these cases in the oocyte expression system; this closing rate constant therefore dominates both activation and deactivation kinetics. This assumption explains the relatively modest activation of KGA: current levels are much smaller than for other inward rectifiers expressed in oocytes (Ho, Nichols, Lederer, Lytton, Vassilev, Kanazirska, and Herbert, 1993; Kubo et al., 1993a), and single-channel open probabilities are <5% (W. Schreibmayer, N. Dascal, N. Davidson, and H. A. Lester, unpublished observations). This assumption also explains that KGA activation is slower, even for the m2R pathway, in oocytes than in native atrial cells (~3 s vs 300 ms) where open probabilities appear to be higher than in the oocyte expression system (Kurachi et al., 1992). That the Q227L mutation gives the slowest activation/deactivation rate of all suggests that the step under consideration, which closes the channel, is closely linked to GTP hydrolysis. We do not yet know how GTP hydrolysis removes G $\beta\gamma$ from the activated channel to deactivate the channel; but the most likely mechanism would be combination of G α (GDP) with G $\beta\gamma$.

We believe that the statements in paragraphs *a–e* above are the most straightforward premises and interpretations relevant to our results. We also note that, in principle, a more complete analysis of the effect of heterologous expression of one particular receptor or G protein subunit on the activation of G protein effectors would require knowledge of (1) the concentrations of at least all the abundant G proteins of the α and $\beta\gamma$ types, (2) the GTPase rates for the several subunits and the association rate constants of the several G α (GDP) species with the many G $\beta\gamma$ species, (3) the preferences for association and activation among receptors, G α , and G $\beta\gamma$ subtypes, (4) complementary preferences for effector activation, and finally (5) any relevant compartmentalization of the various components. This information is not available for any system. For this reason, we regard statement *d* above as more tenuous than the others.

Absence of PKA Effects on KGA

Our observations that PKA does not detectably modulate KGA activation in oocytes agree with reports in the literature. In native tissues such as the heart, KGA is not known to be one of the downstream effectors of β AR stimulation. Elevation of intracellular cAMP failed to modulate the muscarinic response in rabbit atrioventricular nodal cells (Trautwein, Taniguchi, and Noma, 1982) or in bullfrog atrial cells (Nargeot, Nerbonne, Engels, and Lester, 1983). However, there are two previous reports that β AR stimulates KGA modestly in cardiac tissue (Logothetis, 1987; Kim,

1990); the two reports differ on whether the pathway is membrane delimited or involves cAMP (Logethetis, 1987). A recent report tested the effect of perfusion of membrane-permeable cAMP analogue or injection of the catalytic subunit of PKA on KGA expressed in oocytes, and noted that both treatments prevented the desensitization of KGA responses stimulated via the κ -opioid receptor (Chen and Yu, 1994). However, it was not determined whether this was due to phosphorylation of the κ -receptor or KGA.

Functional Implications

Our results provide additional support for the concept that KGA is activated most strongly via free G $\beta\gamma$ subunits and are, in fact, a necessary consequence of this idea. Furthermore, given present concepts about the interchangeability among G $\beta\gamma$ subunits, we would expect KGA to be activated by receptors that couple to other heterotrimeric G proteins, such as Gq, G11, and Gz as well. We have not undertaken systematic experiments on this point.

On the other hand, the pathway that we describe is novel because KGA is being activated via Gs-coupled receptors. We may ask whether our finding has any implications for understanding normal physiology. There are no known instances in normal (or patho-) physiology where a KGA-type current can be activated by β 2AR stimulation. If the mechanism proposed here is correct, this is presumably due to the relative concentrations and/or localizations of different receptors and G-protein subunits in cells in which KGA-type expression has been studied. The possibility that in other cells and tissues where G-protein-gated inward rectifiers operate, some pathways involving liberation of G $\beta\gamma$ from heterotrimers other than members of the Gi family awaits further exploration.

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