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Na^+ Promotes the Dissociation between $G\alpha_{GDP}$ and $G\beta\gamma$, Activating G Protein-gated K^+ Channels*

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G protein-gated K^+ channels (GIRK, or Kir3) are activated by the direct binding of $G\beta\gamma$ or of cytosolic Na^+. Na^+ activation is fast, $G\beta\gamma$-independent, and probably via a direct, low affinity (EC_{50}, 30–40 mM) binding of Na^+ to the channel. Here we demonstrate that an increase in intracellular Na^+ concentration, $[Na^+]_{in}$, within the physiological range (5–20 mM), activates GIRK within minutes via an additional, slow mechanism. The slow activation is observed in GIRK mutants lacking the direct Na^+ effect. It is inhibited by a $G\beta\gamma$ scavenger, hence it is $G\beta\gamma$-dependent; but it does not require GTP. We hypothesized that Na^+ elevates the cellular concentration of free $G\beta\gamma$ by promoting the dissociation of the $G\alpha\beta\gamma$ heterotrimer into free $G\alpha_{GDP}$ and $G\beta\gamma$. Direct biochemical measurements showed that Na^+ causes a moderate decrease (~2-fold) in the affinity of interaction between $G\alpha_{GDP}$ and $G\beta\gamma$. Furthermore, in accord with the predictions of our model, slow Na^+ activation was enhanced by mild coexpression of $G\alpha_{i3}$. Our findings reveal a previously unknown mechanism of regulation of G proteins and demonstrate a novel $G\beta\gamma$-dependent regulation of GIRK by Na^+. We propose that Na^+ may act as a regulatory factor, or even a second messenger, that regulates effectors via $G\beta\gamma$.

GIRK[1] (Kir3) channels are crucial for the regulation of heartbeat and for inhibitory actions of many neurotransmitters in the brain. They are activated by direct binding of $G\beta\gamma$ released from heterotrimeric G proteins following activation of G protein-coupled receptors (GPCR) (1–3). GIRK activity also crucially depends on the presence of membrane phosphatidylinositol 4,5-bisphosphate (4). Cytosolic Na^+ has been shown to activate GIRK by a direct, G protein-independent mechanism. The direct activation by Na^+ is fast and exhibits low affinity for Na^+ with an EC_{50} of 30–40 mM Na^+ (4–7).

GIRKs are usually heterotetramers composed of two pairs of homologous subunits. GIRK1/GIRK4 is predominant in the heart; GIRK1/GIRK2 is abundant in the brain. An aspartate, which is absent in GIRK1 but present in the proximal C terminus of GIRK2 (Asp-226) and GIRK4 (Asp-223), is crucial for fast direct gating by Na^+ (6, 8).

We have noticed an additional, slow activating effect of Na^+ on GIRK channels in excised patches of *Xenopus* oocytes. The slow activation occurred both in wild-type (WT) GIRK channels and, surprisingly, in GIRK mutants that lack the fast direct Na^+ regulation. It did not require GTP but was blocked by a $G\beta\gamma$ scavenger, suggesting mediation by $G\beta\gamma$. We hypothesized that Na^+ promotes dissociation of the heterotrimeric $G\alpha_{GDP}\beta\gamma$ complex into free $G\alpha_{GDP}$ and $G\beta\gamma$; the latter activates GIRK. This hypothesis was supported by direct biochemical measurements. Our findings shed new light on mechanisms of regulation of G proteins and GIRK channels by Na^+ and suggest that Na^+ may act as a second messenger that regulates effectors via $G\beta\gamma$.

EXPERIMENTAL PROCEDURES

cDNA Constructs and RNA—RNA was synthesized *in vitro* from the following DNAs: $GIRK1_{F137S}$ (9), GIRK1, GIRK2, myristoylated cβARK, and $G\alpha_{i3}$ (10). Amounts of injected RNA were as follows: GIRK1, GIRK2, and $GIRK2_{D226N}$, 0.1–0.2 ng/oocyte; $GIRK1_{F137S}$, 1–5 ng/oocyte; $G\alpha_{i3}$, 0.5–2 ng/oocyte; cβARK, 5 ng/oocyte. In $GIRK1_{F137S}$ experiments, an antisense oligonucleotide (50 ng) against the endogenous GIRK5 subunit was injected to prevent the formation of GIRK1/5 channels (11).

Xenopus Oocyte Preparation and Electrophysiology—*Xenopus* oocytes were prepared, injected with RNA, and incubated in NDE-96 solution (96 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2.5 mM sodium pyruvate, 50 μg/ml gentamycin, 5 mM Hepes/NaOH, pH = 7.6). Patch-clamp measurements of GIRK activity were done at −80 mV as described previously (10). The electrode solution contained 146 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM NaCl, 10 mM Hepes/KOH. The bath (500 μl) contained a Na^+-free solution (130 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes/KOH, 1 mM EGTA, 2 mM Mg-ATP). The pH of all solutions was 7.4–7.6. NaCl was added in 50 μl of bath solution and mixed manually. Patches that showed >50% rundown of activity within 6 min after addition of Na^+ (about 10% of all patches) were excluded from the study. Currents were filtered at 2 kHz and sampled at 5 kHz using the Axotape software (Axon Instruments). The results were analyzed as described previously (10). In each patch, the Na^+-induced changes in activity (-fold activation) were calculated as -fold change in total open probability, NP_o, relative to basal NP_o measured during the last minute before the addition of Na^+.

Biochemistry and Immunocytochemistry—The DNA of GST-$G\alpha_{i3}$ was constructed by inserting the coding sequence of human $G\alpha_{i3}$ into the *Eco*RI-*Not*I sites of pGEX-4T-1 vector (Amersham Biosciences). The protein was amplified and purified from *Escherichia coli* using glutathione-Sepharose affinity beads. For pull-down assays, GST-$G\alpha_{i3}$ was incubated for 30 min at 4 °C in binding buffer (50 mM Tris, 5 mM $MgCl_2$, 1 mM EDTA, 0.05% Tween 20, pH 7.0 with 150 mM KCl or 150 mM NaCl) with either 100 μM GDP or GTPγS. [^{35}S]Methionine-labeled $G\beta\gamma$ was synthesized in reticulocyte lysate, diluted 1:4, and incubated with GST-

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[1] The abbreviations used are: GIRK, G protein-gated K^+ channel; cβARK, myristoylated C-terminal part of β-adrenergic receptor kinase; [$G\beta\gamma$], free cellular concentration of $G\beta\gamma$; GPCR, G protein-coupled receptor; WT, wild-type; GST, glutathione *S*-transferase; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid; NDPK, nucleotide diphosphate kinase.

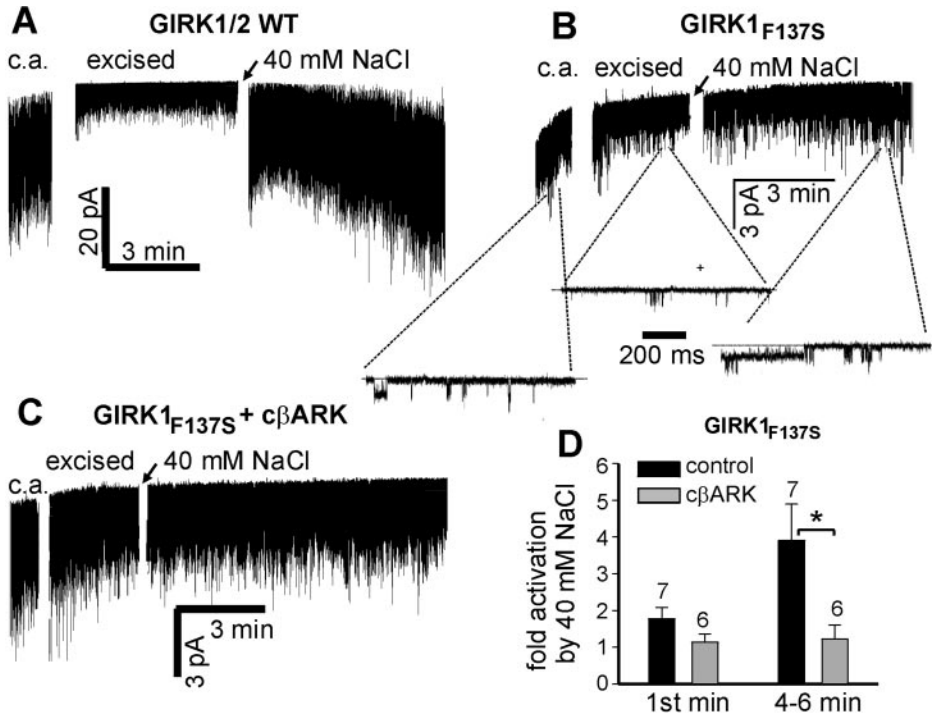

FIG. 1. **Fast and slow activation of GIRK channels by Na^+.** *A*, activation of the WT GIRK1/2 channels by 40 mM NaCl: full time course of a representative experiment. Periods of patch excision and addition of NaCl, accompanied by noise, are blanked. Channel openings are *downward*. *B*, slow activation of $GIRK1_{F137S}$ channels by 40 mM NaCl. *Upper trace*, full time course of the experiment; *lower traces* zoom on shorter periods of activity. *C*, activation of $GIRK1_{F137S}$ channels by 40 mM NaCl is blocked by coexpression of cβARK: a representative record. *D*, summary of effects of 40 mM NaCl on $GIRK1_{F137S}$. Numbers of experiments are shown *above bars*. *, $p < 0.05$. *c.a.*, cell-attached.

$G\alpha_{i3}$ in 300 μl of the above buffer (with 150 mM NaCl or KCl) for 30 min at room temperature in the presence of GDP or GTPγS. The mixture was incubated with glutathione-Sepharose beads and washed, and bound proteins were eluted with 15 mM glutathione and analyzed on a 12% SDS-polyacrylamide gel followed by Coomassie Blue staining and autoradiography as described previously (12).

Surface plasmon resonance (SPR) analysis of GST-$G\alpha_{i3}$-Gβγ interaction was carried out on Biacore 2000 (Biacore AB, Uppsala, Sweden) using anti-GST antibodies (90 ng) covalently attached to the surface of a CM5 sensor chip as described previously (13). Following the capture of GST-$G\alpha_{i3}$, Gβγ was injected across the sensor surface at a flow rate of 15 ml/min in series of concentrations in a buffer containing 50 mM Tris-Cl, 5 mM $MgCl_2$, 1% CHAPS buffer, and either 100 mM KCl or 100 mM KCl. Intermediate concentrations of Na^+ were obtained by mixing the two buffers. The kinetic data were analyzed using BIAEvaluation 3.1 software (Biacore AB) and Sigmaplot (SPSS Inc.).

The level of $GIRK1_{F137S}$ protein in plasma membrane (Fig. 4*C*) was measured as described previously (10). Briefly, large oocyte membrane patches were attached to coverslips, fixated, stained by a specific GIRK1 antibody (Alomone Labs, Jerusalem, Israel), and visualized using a Cy3-conjugated rabbit IgG (Jackson Immunoresearch Laboratories) using a Zeiss LSM 410 confocal microscope.

Statistics—Data are presented as mean ± S.E.; pairwise comparisons were done using a two-tailed *t* test.

RESULTS

WT GIRK1/GIRK2 channels were expressed in *Xenopus* oocytes, and Na^+-induced activation was studied in excised patches (Fig. 1*A*). GIRK activity was first recorded in cell-attached configuration. Then the patch was excised ("inside-out") with its intracellular membrane surface facing the bath solution, which was Na^+- and GTP-free but contained Mg-ATP to preserve membrane phosphatidylinositol 4,5-bisphosphate (4). After excision, the basal activity declined to a new steady level within 0.5–2 min (10). 3–4 min after excision, 40 mM NaCl was added to the bath solution, causing a 6.7 ± 2.5-fold increase ($n = 8$) in channel activity. In agreement with previous studies (7), this *fast* Na^+ activation was not affected by coexpression of the Gβγ scavenger cβARK (myristoylated C terminus of β-adrenergic receptor kinase; data not shown). In addition, in many WT GIRK1/GIRK2 patches, a *slow*, late activation was observed. It started after about 1 min and developed over the next 3–6 min (Fig. 1*A*).

To explore the slow effects of Na^+, we utilized the GIRK1 pore mutant $GIRK1_{F137S}$, which forms functional homotetrameric channels (9) and lacks the C-terminal aspartate crucial for the direct Na^+ effect. Fast activation of homotetrameric $GIRK1_{F137S}$ channels by 40 mM Na^+ was very weak, 1.82 ± 0.36-fold ($n = 7$) (Fig. 1*B*). It might reflect a weak direct effect of Na^+ on the $GIRK1_{F137S}$ channel. In seven of eight patches, the activity continued to increase over the next several minutes, reaching a maximum after 4–7 min. This *slow* activation, measured during a 3-min period between 4 and 6 min after addition of Na^+, was 3.9 ± 1-fold ($n = 7$) above basal (Fig. 1*D*). It was fully blocked by coexpression of cβARK (Fig. 1, *C* and *D*), implying a Gβγ-dependent mechanism. We assumed that the additional 2-fold slow increase in GIRK activity (compared with the 1st min after addition of Na^+) reflects an increase in cellular concentration of free Gβγ, [Gβγ]. Comparable slow activation (2.62 ± 0.45-fold, $n = 6$) was observed already at 10 mM Na^+, which causes little fast activation in WT GIRK channels (5, 14) and no fast activation in $GIRK1_{F137S}$ (1.31 ± 0.16-fold, see Fig. 4).

How could Na^+ activate GIRK in a Gβγ-dependent manner? GPCRs activate G proteins by promoting the exchange of GDP for GTP at the Gα subunit followed by dissociation of $G\alpha_{GTP}$ from Gβγ (15). Cl^- also promotes GTP binding to Gα *in vitro* (16), but Cl^- concentration in our bath solution was already saturating for this effect before the addition of NaCl. Most importantly the activation of $GIRK1_{F137S}$ by NaCl was achieved in a GTP-free solution, ruling out a mechanism involving GDP-GTP exchange. Another source of free Gβγ, which does not require GTP, is the basal equilibrium between the Gαβγ heterotrimer and free $G\alpha_{GDP}$ and Gβγ (17):

$$G\alpha_{GDP}\beta\gamma \leftrightarrows G\alpha_{GDP} + G\beta\gamma$$

REACTION 1

We hypothesized that Na^+ promotes the dissociation of the Gαβγ heterotrimer into free $G\alpha_{GDP}$ and Gβγ by increasing the K_D of Reaction 1. This will increase the concentration of free Gβγ, [Gβγ], activating GIRK in the absence of GTP. The ob-

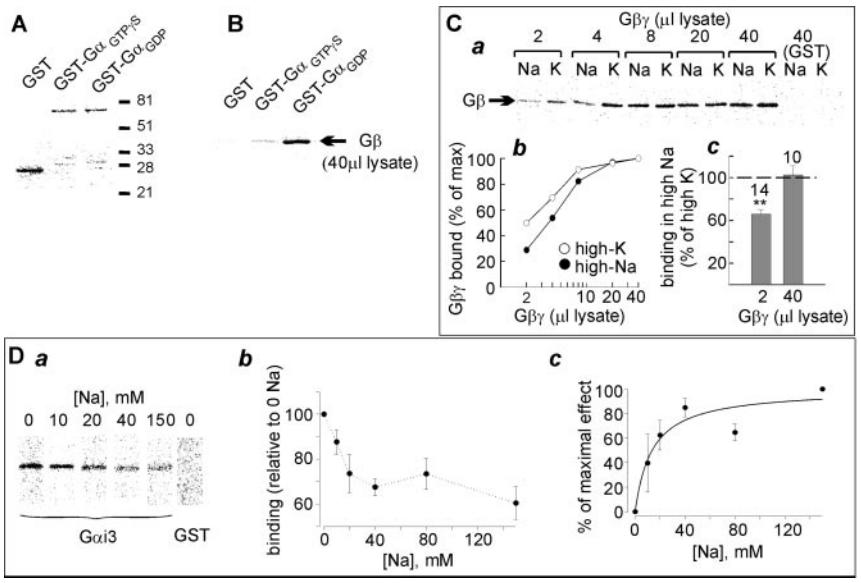

FIG. 2. **High Na^+ reduces the binding of Gβγ to GST-$G\alpha_{i3}$.** *A*, Coomassie Blue staining of GST and GST-$G\alpha_{i3}$ used in the experiment shown in *B*. *B*, pull down of [^{35}S]methionine-labeled Gβγ by GST-$G\alpha_{i3\text{-}GDP}$ but not GST or $G\alpha_{i3\text{-}GTP\gamma S}$ (a representative PhosphorImager autoradiogram). *C*, binding of variable amounts of Gβγ to GST-$G\alpha_{i3\text{-}GDP}$ in solutions with 150 mM K^+, 0 Na^+ (*K*) or 150 mM Na^+, 0 K^+ (*Na*). *a*, a representative experiment. *b*, quantitation of the results shown in *a* relative to maximal signal (150 mM K^+, 40 μl of lysate). *c*, Na^+ reduces binding of Gβγ at low, but not saturating, [Gβγ]. **, $p < 0.01$. *D*, binding of 2 μl of Gβγ lysate to GST-$G\alpha_{i3\text{-}GDP}$ depends on [Na^+]. Intermediate concentrations of Na^+ were obtained by mixing the parental buffers (150 mM KCl and 150 mM NaCl). *a*, a representative autoradiogram. *b*, summary of three separate experiments. Band intensity at each [Na^+] was normalized to the maximal signal, observed at 0 mM Na^+ (150 mM K^+). *c*, the Na^+ dose-response curve, fitted to the Michaelis-Menten equation with a K_i of 13.6 mM (*solid line*). The effect of Na^+ (decrease in binding) at each [Na^+] was presented as percentage of maximal effect (decrease in binding produced by 150 mM Na^+).

served time course of Na^+ activation (minutes) is consistent with the slow dissociation rate constant of the Gαβγ heterotrimer (~0.001 s^{-1}) (17).

To scrutinize this hypothesis, we examined the effect of Na^+ on binding of $G\beta_1\gamma_2$ to a GST-fused $G\alpha_{i3}$ protein, GST-$G\alpha_{i3}$ (Fig. 2*A*), using a pull-down assay. Gβγ was synthesized *in vitro* in reticulocyte lysate. Only GST-$G\alpha_{i3\text{-}GDP}$, but not GST or GST-$G\alpha_{i3\text{-}GTP\gamma S}$, bound Gβγ in this assay (Fig. 2*B*), confirming specificity and functional activity of GST-$G\alpha_{i3}$. In three individual experiments like that shown in Fig. 2*C* (*a* and *b*), we examined the dose dependence of binding of Gβγ to GST-$G\alpha_{GDP}$. The binding was dose-dependent and reached saturation at about 20 μl of Gβγ-containing lysate. The affinity of binding was lower in high Na^+ than in high K^+ solution, but the differences were too small for a statistically reliable estimation of the affinity shift. Therefore, we compared the binding at a low [Gβγ] (2 μl of Gβγ-containing lysate) and at saturating [Gβγ] (40 μl of lysate) (summarized in Fig. 2*C*, *c*). With 40 μl of lysate, the extent of binding in 150 mM NaCl was the same as in 150 mM KCl (102.6 ± 8.2%, $n = 10$). However, at 2 μl of lysate, the binding in the high Na^+ solution was reproducibly weaker than in high K^+ (65.9 ± 4.2%, $n = 14$, $p < 0.001$ compared with 40 μl of lysate). At doses of Gβγ lower than 2 μl of lysate (where a greater effect of Na^+ was expected), the signal in the autoradiograms was too weak for a reliable measurement. The ~34% decrease in Gβγ binding caused by Na^+ at 2 μl of lysate (which gave ~37% of maximum binding in high K^+) corresponds to a 1.9-fold change in K_D. The effect of Na^+ was dose-dependent (Fig. 2*D*, *a* and *b*) with a K_i of 13.6 mM Na^+ (Fig. 2*D*, *c*).

The effect of Na^+ on $G\alpha_{GDP}$-Gβγ interaction was further studied by SPR. Recombinant $G\beta_1\gamma_2$ was injected in buffers with different concentrations of NaCl (substituted for KCl) across the sensor surface with GST or GST-$G\alpha_{i3}$ immobilized via anti-GST antibody (13). Gβγ reveals a relatively high degree of nonspecific binding to GST in this assay (13, 18). However, the extent of Gβγ binding to GST-$G\alpha_{i3\text{-}GDP}$ was always greater than to GST (Fig. 3, *A* and *B*). Also the wash-out of Gβγ from GST was fast as expected for a low affinity process, whereas unbinding of Gβγ from GST-$G\alpha_{i3\text{-}GDP}$ displayed a slower kinetic component, characteristic for a high affinity interaction. The net specific signal of Gβγ-$G\alpha_{i3}$ binding, obtained by subtraction of the GST signal (see Ref. 13), displayed rise and decay phases that were reasonably well fitted by single exponential functions (Fig. 3*C*). As expected, GST-$G\alpha_{i3\text{-}GTP\gamma S}$ bound Gβγ much weaker than GST-$G\alpha_{i3\text{-}GDP}$ (Fig. 3*D*). When the Na^+ concentration was increased, the net binding of Gβγ to GST-$G\alpha_{i3\text{-}GDP}$ was reduced in a dose-dependent manner with a K_i of 8 mM Na^+ (Fig. 3, *E* and *F*). Although these data clearly show specific binding of Gβγ to $G\alpha_{i3}$, the contribution of nonspecific binding, particularly in the first few seconds postinjection and sample wash-out, impaired the precise determination of the kinetic constants in Na^+ and K^+. A limited kinetic analysis on the subtracted traces showed that the dissociation constant of Reaction 1, K_D, was increased by 10 mM Na^+ by ~2-fold (two separate sets of experiments). The calculated 2-fold change is in very good agreement with the pull-down results. The SPR experiments support the conclusion that Na^+ reduces the affinity of interaction between Gα-GDP and Gβγ.

The findings so far support the hypothesis that the slow activation of GIRK by Na^+ is due to elevated [Gβγ] that dissociated from $G\alpha_{GDP}$. This hypothesis not only explains the block of the slow effect of Na^+ by βARK but also allows testable predictions. Thus, mass law dictates that coexpression of Gα should shift the equilibrium in Reaction 1 to the left, increasing [Gαβγ] and reducing free [Gβγ]. This should reduce the basal activity of GIRK, but now addition of Na^+ should cause a larger *relative* increase in [Gβγ] than in control conditions. This prediction is independent of initial conditions chosen to describe channel activation by Gβγ.[2] However, it is important not to overexpress Gα to very high levels at which free $G\alpha_{GDP}$ can

[2] D. Yakubovich, I. Rishal, and N. Dascal, unpublished results.

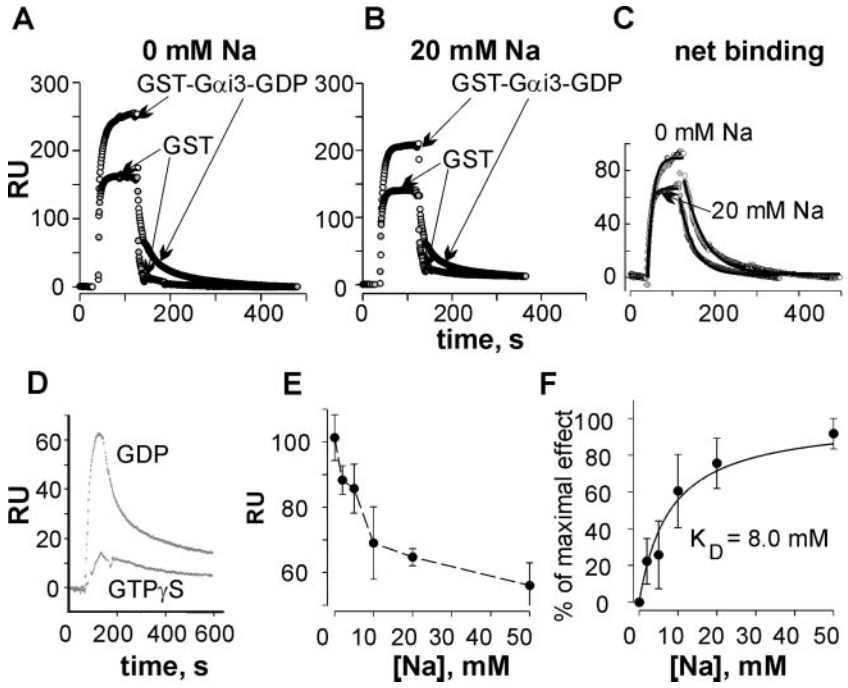

FIG. 3. **Analysis of $G\beta_1\gamma_2$ interaction with GST-$G\alpha_{i3\text{-}GDP}$ or GST-$G\alpha_{i3\text{-}GTP\gamma S}$ by SPR.** GST-$G\alpha_{i3\text{-}GDP}$ (150 ng) was captured on one channel of Biacore, GST-$G\alpha_{i3\text{-}GTP\gamma S}$ was captured on another channel, and recombinant GST was immobilized on a reference channel. *A* and *B*, representative traces showing the SPR signal (in resonance units, *RU*) upon binding of 350 nM $G\beta_1\gamma_2$ to the GST and GST-$G\alpha_{i3\text{-}GDP}$ channels in 0 mM Na^+ (*A*) and in 10 mM Na^+ (*B*). The different Na^+ concentrations were obtained by mixing the parental buffers containing 100 mM KCl (0 Na^+) or 100 mM NaCl (0 K^+). *C*, net GST-$G\alpha_{i3\text{-}GDP}$ SPR signal after subtraction of the nonspecific binding on the GST channel. Rise and decay phases were fitted to monoexponential functions (*solid lines*). *D*, comparison of SPR signal at the GST-$G\alpha_{i3\text{-}GDP}$ and GST-$G\alpha_{i3\text{-}GTP\gamma S}$ channels (nonspecific binding to GST on the reference channel was subtracted). *E*, net binding of $G\beta\gamma$ to GST-$G\alpha_{i3\text{-}GDP}$ is reduced by Na^+: a summary of three separate measurements. *F*, the Na^+ dose-response curve, fitted to the Michaelis-Menten equation with a K_i of 8 mM (*solid line*). The effect of Na^+ (decrease in specific $G\beta\gamma$ binding), at each [Na^+], was presented as percentage of maximal decrease in binding. In two measurements, maximal effect occurred at 50 mM Na^+ and in one measurement at 20 mM.

scavenge $G\beta\gamma$ and blunt any $G\beta\gamma$-induced effects. We produced mild overexpression of $G\alpha_{i3}$, 3–4-fold over the resting cellular level (by injecting 5 ng of $G\alpha_{i3}$ RNA or less), that does not hinder the activation of GIRK by $G\beta\gamma$ (10).

In agreement with the prediction, in $G\alpha_{i3}$-expressing oocytes, slow activation of $GIRK1_{F137S}$ by 10 mM Na^+ was 10.3 ± 2.3-fold (n = 10), significantly greater than without $G\alpha_{i3}$ (Fig. 4*A* and summarized in Fig. 4*B*). Slow activation by Na^+ was still fully blocked by coexpression of cβARK (Fig. 4, *A* and *B*). Another GIRK channel mutant known to lack the direct Na^+ activation, $GIRK1/GIRK2_{D226N}$ (14), was also slowly activated by 10 mM Na^+ in $G\alpha_{i3}$-expressing cells (11.6 ± 2.8-fold, n = 9), and this effect was inhibited by cβARK ("activation" by 1.04 ± 0.13-fold, n = 4). The dramatic effect of $G\alpha_{i3}$ on slow Na^+-induced activation strongly supports the G protein-dependent character of this phenomenon. The extent of slow activation by 10 mM Na^+, in the presence of $G\alpha$, is comparable to the direct effect of 40 mM Na^+, although it is still severalfold smaller than the 20–800-fold activation by saturating doses of $G\beta\gamma$ (10). A simple kinetic model based on our hypothesis and on known or estimated parameters of $G\alpha_{GDP}$-$G\beta\gamma$ and $G\beta\gamma$-GIRK interactions correctly described the slow GIRK activation by Na^+. The calculations showed that a ~1.8-fold increase in K_D of Reaction 1 by 10 mM Na^+ fully accounted for the 2-fold slow activation of $GIRK1_{F137S}$ under control conditions and for an ~8-fold activation after coexpression of $G\alpha_{i3}$.[2]

It appeared (although a systematic study has not been conducted) that coexpression of $G\alpha_{i3}$ did not cause the expected reduction in basal activity of $GIRK1_{F137S}$ (*e.g.* compare the cell-attached levels of channel activity in Fig. 4*A*, *a* and *b*). The unexpectedly high basal activity could reflect an increase in membrane levels of $GIRK1_{F137S}$ caused by $G\alpha_{i3}$ as described for WT GIRK1/GIRK2 (10). Indeed immunostaining of the expressed channels in large plasma membrane patches (Fig. 4*C*) showed a net 6.4-fold increase of $GIRK1_{F137S}$ protein by $G\alpha_{i3}$ (after subtraction of background observed in uninjected oocytes) compared with channels expressed alone.

The dose dependence of the slow Na^+ effect was studied in oocytes coexpressing $GIRK1_{F137S}$ and $G\alpha_{i3}$. Maximal activation was observed at 10 mM Na^+ (Fig. 4*D*). At higher [Na^+] the effect was smaller, consistent with the previously reported inhibition, at high [Na^+], of GIRK channel constructs lacking the fast Na^+ activation (6). The mechanism of this inhibition is unknown. The half-maximal activation dose (EC_{50}) appeared to be slightly above 5 mM Na^+, but considering the interference of the inhibitory effect at high [Na^+], the actual EC_{50} of slow Na^+ activation is probably higher.

DISCUSSION

Na^+ Regulates Basal Equilibrium between Free and $G\alpha_{GDP}$-bound $G\beta\gamma$—Our results strongly suggest that Na^+ reduces the affinity of interaction between $G\alpha_{GDP}$ and $G\beta\gamma$. This hypothesis initially arose following the observation of slow, $G\beta\gamma$-dependent activation by Na^+ of mutant GIRK channels lacking the direct Na^+ modulation. We then demonstrated weakening of $G\alpha_{GDP}$-$G\beta\gamma$ binding using direct *in vitro* binding assays. Although the detected change in affinity was small, the results obtained by two independent methods (a pull-down assay and surface plasmon resonance) were in good agreement. Both methods, as well as estimates obtained from patch-clamp data, indicate that Na^+ causes a ~2-fold decrease in the affinity of interaction between $G\alpha_{i3\text{-}GDP}$ and $G\beta\gamma$. The EC_{50} or K_i of the Na^+ effect estimated by electrophysiological and the two biochemical methods are also in good agreement (~6–14 mM), falling within the physiological range of $[Na^+]_{in}$. The proposed hypothesis made it possible to explain the observed inhibition

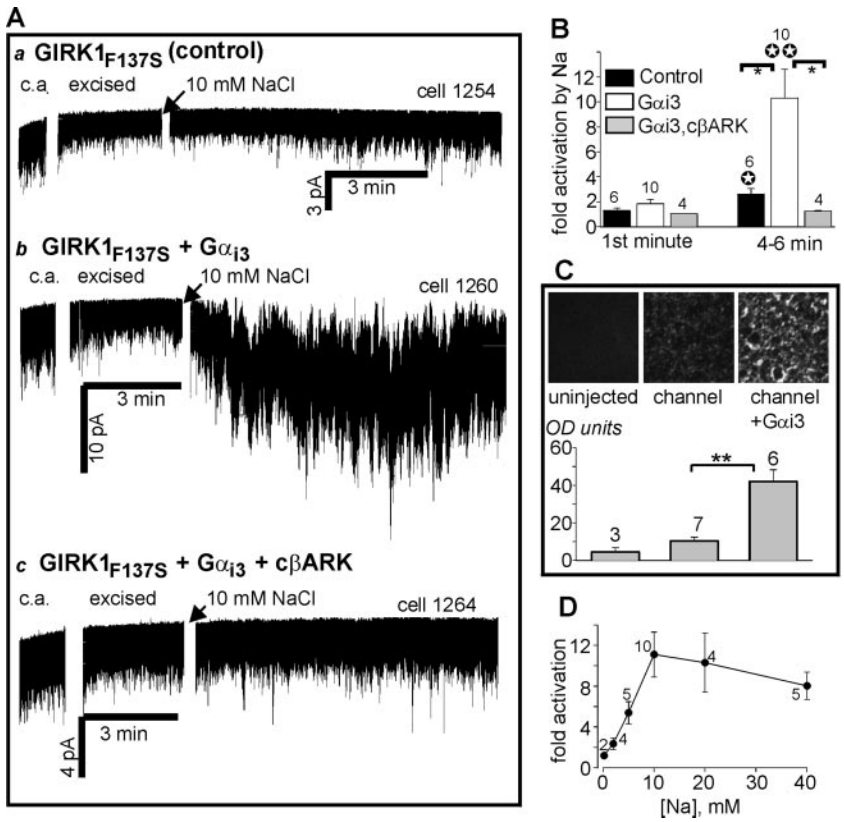

FIG. 4. **Coexpression of $G\alpha_{i3}$ enhances the NaCl-induced activation of $GIRK1_{F137S}$.** *A*, examples of the effect of 10 mM NaCl on $GIRK1_{F137S}$ activity in a cell expressing only the channel (*a*), channel and $G\alpha_{i3}$ (*b*), and channel, $G\alpha_{i3}$, and cβARK (*c*). *B*, summary of the effects of 10 mM Na^+ and coexpression of $G\alpha_{i3}$ and cβARK. *, $p < 0.05$ by one-way analysis of variance; ✪, $p < 0.05$; ✪✪, $p < 0.01$ as compared with 1st min by *t* test. *C*, *upper panel*, confocal images of $GIRK1_{F137S}$ protein in large patches of plasma membrane (field, 25 × 25 μm): representative oocytes of one batch. $GIRK1_{F137S}$ and $G\alpha_{i3}$ RNAs (5 ng/oocyte) were injected. *Lower panel*, summary of experiments presented in the *upper panel*. Intensity of labeling (*OD units*) was measured with TINA software (Raytest, Straubenhardt, Germany). **, $p < 0.001$. *D*, dose dependence of slow Na^+ activation (measured during 1 min of maximal activity between 4–8 min after addition of NaCl). $GIRK1_{F137S}$ (5 ng) was coexpressed with $G\alpha_{i3}$ (0.5–5 ng of RNA/oocyte). *c.a.*, cell-attached.

of the slow Na^+-induced activation by the Gβγ scavenger, cβARK, and to predict a novel physiological effect: a dramatic enhancement of slow Na^+ activation of GIRK by coexpressed Gα. Interestingly *Xenopus* oocytes possess an unusually high basal level of free Gβγ (19, 20), and coexpression of Gα may actually mimic the "usual" cellular condition of low [Gβγ].

Na^+ has long been known to modulate the binding of agonists to many GPCRs, uncoupling these receptors from G proteins. Initially Na^+ had been suspected to regulate G proteins directly, but later studies demonstrated a pivotal role of Na^+ interaction with a conserved aspartate residue located in the transmembrane region of many GPCRs (for review, see Ref. 21). Finally, although NaCl has been shown to promote GDP-GTP exchange at the G protein, the active agent was Cl^- rather than Na^+ (16). Thus, no direct effects of Na^+ on G proteins are known, and this report is the first demonstration of such regulation.

Two Mechanisms of Regulation of GIRK by Na^+—Na^+ regulation of GIRK channels is considered of high physiological importance since it is believed to determine part of their basal activity in intact cells and to underlie the negative chronotropic effect of cardiac glycosides in the heart (2, 5). Our data imply that, in GIRK channels, Na^+ acts both directly and indirectly (via Gβγ) to modulate basal activity. The EC_{50} of the well characterized, direct, Gβγ-independent activation by Na^+ is 30–40 mM. The slow, Gβγ-dependent activation of GIRK by Na^+ has a definitely lower EC_{50}: maximal effect is attained at 10 mM Na^+. Despite the small magnitude of $G\alpha_{GDP}$-Gβγ affinity change by Na^+, 10 mM Na^+ caused an impressive 4–10-fold activation of GIRK. Therefore, the slow mechanism may contribute substantially to the basal activity of the channel in intact cells where the resting $[Na^+]_{in}$ is 5–10 mM.

The observed features of slow Na^+ modulation of GIRK conform to the model in which [Gβγ], elevated because of the direct effect of Na^+ on Gαβγ dissociation, activates the channel. Yet at present we cannot exclude contribution of additional mechanisms, for instance activation of a nucleotide diphosphate kinase (NDPK), which catalyzes the transfer of phosphate from ATP to GTP. This might, in principle, promote Gαβγ dissociation and activate GIRK (see the discussion in Ref. 2). However, Na^+ has been reported to inhibit NDPK (22) and should have inhibited GIRK if NDPK were involved.

Na^+ as a Second Messenger or a Servo-type Intracellular Regulator—Sodium ions are crucial for neuronal activity as carriers of depolarization and also regulate many physiological processes (fluid balance and secretion, cardiac contraction, glutamate-induced neuronal excitation, etc.) by a variety of molecular mechanisms from a direct binding of Na^+ (GIRK and Na^+-dependent transporters and exchangers (23)) to activation of Src by Na^+ via an unknown intermediate (*N*-methyl-D-aspartate receptors (24)). Basal $[Na^+]_{in}$ is tightly regulated by pumps and exchangers, being maintained in resting cells below 10 mM. Yet $[Na^+]_{in}$ often widely fluctuates. In neurons, even relatively short periods of synaptic activity produce very large increases in $[Na^+]_{in}$, reaching ~30 mM in apical dendrites and >100 mM in dendritic spines (25). These considerations raise the possibility that Na^+ may, in some cases, act as a second messenger that regulates intracellular targets when a substantial change in concentration occurs.

Our findings suggest a new mechanism of Na^+-dependent regulation of cellular processes: via Gβγ. The effective range of Na^+ concentrations that regulate the $G\alpha\beta\gamma \leftrightarrows GG\alpha_{GDP} + G\beta\gamma$ equilibrium is close to the resting physiological range of $[Na^+]_{in}$. Therefore, regulation of G proteins by Na^+ may be a servo-type mechanism that sensitively responds to bidirectional changes in $[Na^+]_{in}$ rather than to increases alone. We propose that Na^+ regulation of the dynamic equilibrium between bound and free $G\alpha_{GDP}$ and Gβγ can have a substantial biological effect by regulating a host of effectors of Gβγ (1), some of which are second messenger-generating enzymes that may further amplify Na^+ effects. This establishes a possible novel way of communication between electrical activity and other cellular processes.

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REFERENCES

1. Clapham, D. E., and Neer, E. J. (1997) *Annu. Rev. Pharmacol. Toxicol.* **37,** 167–203
2. Dascal, N. (1997) *Cell. Signal.* **9,** 551–573
3. Yamada, M., Inanobe, A., and Kurachi, Y. (1998) *Pharmacol. Rev.* **50,** 723–757
4. Sui, J. L., Petit-Jacques, J., and Logothetis, D. E. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 1307–1312
5. Sui, J. L., Chan, K. W., and Logothetis, D. E. (1996) *J. Gen. Physiol.* **108,** 381–391
6. Ho, I. H., and Murrell-Lagnado, R. D. (1999) *J. Biol. Chem.* **274,** 8639–8648
7. Petit-Jacques, J., Sui, J. L., and Logothetis, D. E. (1999) *J. Gen. Physiol.* **114,** 673–684
8. Zhang, H., He, C., Yan, X., Mirshahi, T., and Logothetis, D. E. (1999) *Nat. Cell Biol.* **1,** 183–188
9. Chan, K. W., Sui, J. L., Vivaudou, M., and Logothetis, D. E. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 14193–14198
10. Peleg, S., Varon, D., Ivanina, T., Dessauer, C. W., and Dascal, N. (2002) *Neuron* **33,** 87–99
11. Hedin, K. E., Lim, N. F., and Clapham, D. E. (1996) *Neuron* **16,** 423–429
12. Ivanina, T., Blumenstein, Y., Shistik, E., Barzilai, R., and Dascal, N. (2000) *J. Biol. Chem.* **275,** 39846–39854
13. Doupnik, C. A., Dessauer, C. W., Slepak, V. Z., Gilman, A. G., Davidson, N., and Lester, H. A. (1996) *Neuropharmacology* **35,** 923–931
14. Ho, I. H., and Murrell-Lagnado, R. D. (1999) *J. Physiol.* (*Lond.*) **520,** 645–651
15. Gilman, A. G. (1987) *Annu. Rev. Biochem.* **56,** 615–649
16. Higashijima, T., Ferguson, K. M., and Sternweis, P. C. (1987) *J. Biol. Chem.* **262,** 3597–3602
17. Sarvazyan, N. A., Remmers, A. E., and Neubig, R. R. (1998) *J. Biol. Chem.* **273,** 7934–7940
18. Xu, J., Wu, D., Slepak, V. Z., and Simon, M. I. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 2086–2090
19. Sheng, Y., Tiberi, M., Booth, R. A., Ma, C., and Liu, X. J. (2001) *Curr. Biol.* **11,** 405–416
20. Lutz, L. B., Kim, B. E., Jahani, D., and Hammes, S. R. (2000) *J. Biol. Chem.* **275,** 41512–41520
21. Gierschik, P., McLeish, K., and Jakobs, K. H. (1988) *J. Cardiovasc. Pharmacol.* **5,** Suppl. 12, S20–S24
22. Marshall, L. J., Muimo, R., Riemen, C. E., and Mehta, A. (1999) *Am. J. Physiol.* **276,** C109–C119
23. Seal, R. P., and Amara, S. G. (1999) *Annu. Rev. Pharmacol. Toxicol.* **39,** 431–456
24. Yu, X. M., and Salter, M. W. (1998) *Nature* **396,** 469–474
25. Rose, C. R., and Konnerth, A. (2001) *J. Neurosci.* **21,** 4207–4214