Na\(^+\) Promotes the Dissociation between \(\text{G}_{\alpha\text{GDP}}\) and \(\text{G}\beta\gamma\), 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 \(\text{G}\beta\gamma\) or of cytosolic Na\(^+\). Na\(^+\) activation is fast, \(\text{G}\beta\gamma\)-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\(^+\)]\(_i\), 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 \(\text{G}\beta\gamma\) scavenger, hence it is \(\text{G}\beta\gamma\)-dependent; but it does not require GTP. We hypothesized that Na\(^+\) elevates the cellular concentration of free \(\text{G}\beta\gamma\) by promoting the dissociation of the \(\text{G}\beta\gamma\) heterotrimers into free \(\text{G}_{\alpha\text{GDP}}\) and \(\text{G}\beta\gamma\). Direct biochemical measurements showed that Na\(^+\) causes a moderate decrease (2-fold) in the affinity of interaction between \(\text{G}_{\alpha\text{GDP}}\) and \(\text{G}\beta\gamma\). Furthermore, in accord with the predictions of our model, slow Na\(^+\) activation was enhanced by mild coexpression of \(\text{G}\alpha_{\text{GTP}}\). Our findings reveal a previously unknown mechanism of regulation of G proteins and demonstrate a novel \(\text{G}\beta\gamma\)-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 \(\text{G}\beta\gamma\).

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 \(\text{G}\beta\gamma\) 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 \(\text{G}\beta\gamma\) scavenger, suggesting mediation by \(\text{G}\beta\gamma\). We hypothesized that Na\(^+\) promotes dissociation of the heterotrimeric \(\text{G}_{\alpha\text{GDP}}\) complex into free \(\text{G}_{\alpha\text{GDP}}\) and \(\text{G}\beta\gamma\); 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 \(\text{G}\beta\gamma\).

**EXPERIMENTAL PROCEDURES**

cDNA Constructs and RNA—RNA was synthesized in vitro from the following DNAs: GIRK1/F137S (9), GIRK1, GIRK2, myristoylated cARK1, and G\(_{\alpha_{3}}\) (10). Amounts of injected RNA were as follows: GIRK1, GIRK2, and GIRK2/F137S, 0.1–0.2 ng/oocyte; GIRK1/E137S, 1–5 ng/oocyte; G\(_{\alpha_{3}}\), 0.5–2 ng/oocyte; cARK1, 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 gentamicin, 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. Patch recordings 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 Axopatch software (Axon Instruments). The results were analyzed as described previously (10). In each patch, 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 G\(_{\alpha_{3}}\) was constructed by inserting the coding sequence of human G\(_{\alpha_{3}}\) into the EcoRI/VolI 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\(_{\alpha_{3}}\) 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 KC1 or 150 mM NaCl) with either 100 μM GDP or GTP\(\gamma\)S. [35S]Methionine-labeled G\(_{\beta}\gamma\) was synthesized in reticulocyte lysate, diluted 1:4, and incubated with GST-

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‡ The abbreviations used are: GIRK, G protein-gated K\(^+\) channel; cARK1, myristoylated C-terminal part of \(\beta\)-adrenergic receptor kinase; \(\text{G}\beta\gamma\), free cellular concentration of \(\text{G}\beta\gamma\); GPCR, G protein-coupled receptor; WT, wild-type; GST, glutathione S-transferase; GTP\(\gamma\)S, guanosine 5'-O-(thio)triphosphate; CHAPS, 3-[3-cholamidopropyl]-dimethylammonio)-1-propanesulfonic acid; NDPK, nucleotide diphosphate kinase.

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**Results**

WT GIRK1/GIRK2 channels were expressed in *Xenopus* oocytes, and Na\(^+\)-induced activation was studied in excised patches (Fig. 1A). 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 G\(\beta\gamma\) scavenger c\(\beta\)ARK (myristoylated C terminus of \(\beta\)-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. 1A).

![Image 215x472 to 563x737](image)

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. 1B). 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\(^+\)\(_{s}\), was 3.9 ± 1-fold (n = 7) above basal (Fig. 1D). It was fully blocked by coexpression of c\(\beta\)ARK (Fig. 1, C and D), implying a G\(\beta\gamma\)-dependent mechanism. We assumed that the additional 2-fold slow increase in GIRK activity (compared with the 1st min after addition of Na\(^+\)\(_{s}\)) reflects an increase in cellular concentration of free G\(\beta\gamma\), [G\(\beta\gamma\)]. 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 G\(\beta\gamma\)-dependent manner? GPCRs activate G proteins by promoting the exchange of GDP for GTP at the G\(\alpha\) subunit followed by dissociation of G\(\alpha\)GTP from G\(\beta\gamma\) (15). Cl\(^-\) also promotes GTP binding to G\(\alpha\) 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 G\(\beta\gamma\), which does not require GTP, is the basal equilibrium between the G\(\beta\)G-protein heterotrimer and free G\(\alpha\)GDP and G\(\beta\gamma\) (17):

\[G_{\alpha}GDPG\beta\gamma = G_{\alpha}GDP + G\beta\gamma\]

**Reaction 1**

We hypothesized that Na\(^+\) promotes the dissociation of the G\(\beta\gamma\) heterotrimer into free G\(\alpha\)GDP and G\(\beta\gamma\) by increasing the K\(_D\) of Reaction 1. This will increase the concentration of free G\(\beta\gamma\), [G\(\beta\gamma\)], activating GIRK in the absence of GTP. The ob-
observed time course of Na⁺ activation (minutes) is consistent with the slow dissociation rate constant of the Gβγ heterotrimer (–0.001 s⁻¹) (17).

To scrutinize this hypothesis, we examined the effect of Na⁺ on binding of Gβγ₁γ₂ to a GST-fused Gα₁₃ protein, GST-Gα₁₃ (Fig. 2A), using a pull-down assay. Gβγ was synthesized in vitro in reticulocyte lysate. Only GST-Gα₁₃-GDP, but not GST or GST-Gα₁₃-GTP, bound Gβγ in this assay (Fig. 2B), confirming specificity and functional activity of GST-Gα₁₃. In three individual experiments like that shown in Fig. 2C (a and b), we examined the dose dependence of binding of Gβγ to GST-Gα₁₃-GDP. The binding was dose-dependent and reached saturation at about 20 μM Gβγ-containing lysate. The affinity of binding was lower in high Na⁺ 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 μM of Gβγ-containing lysate) and at saturating [Gβγ] (40 μM of lysate) (summarized in Fig. 2C, c). With 40 μM 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 μM 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 μM 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 Kᵦ. The effect of Na⁺ was dose-dependent (Fig. 2D, a and b) with a Kᵦ of 13.6 mM Na⁺ (Fig. 2D, c).

The effect of Na⁺ on Gα₁₃-GDP-Gβγ interaction was further studied by SPR. Recombinant Gβγ₁γ₂ was injected in buffers with different concentrations of NaCl (substituted for KCl) across the sensor surface with GST or GST-Gα₁₃ 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-Gα₁₃-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-Gα₁₃-GDP displayed a slower kinetic component, characteristic for a high affinity interaction. The net specific signal of Gβγ-Gα₁₃ 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. 3C). As expected, Gα₁₃-GTP-S binding was reduced in a dose-dependent manner with a Kᵦ of 8 mM Na⁺ (Fig. 3, E and F). Although these data clearly show specific binding of Gβγ to Gα₁₃, the contribution of non-specific 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, Kᵦ, 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-Gβγ.

The findings so far support the hypothesis that the slow activation of GIRK by Na⁺ is due to elevated [Gβγ] that dissociated from Gα₁₃-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 [Gβγ] 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 Gα₁₃-GDP can

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² D. Yakubovich, I. Rishal, and N. Dascal, unpublished results.
scavenge Gβγ and blunt any Gβγ-induced effects. We produced mild overexpression of Gαi3, 3–4-fold over the resting cellular level (by injecting 5 ng of Gαi3 RNA or less), that does not hinder the activation of GIRK by Gβγ (10).

In agreement with the prediction, in Gαi3-expressing oocytes, slow activation of GIRK1F137S by 10 mM Na+ was 10.3 ± 2.3-fold (n = 10), significantly greater than without Gαi3 (Fig. 4A and summarized in Fig. 4B). 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, GIRK1GIRK2D226N (14), was also slowly activated by 10 mM Na+ in Gαi3-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 Gαi3 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 Gα, is comparable to the direct effect of 40 mM Na+, although it is still several-fold smaller than the 20–800-fold activation by saturating doses of Gβγ (10). A simple kinetic model based on our hypothesis and on known or estimated parameters of Gαi3-Gβγ and Gβγ-GIRK interactions correctly described the slow GIRK activation by Na+. The calculations showed that a 1.8-fold increase in Kd of Reaction 1 by 10 mM Na+ fully accounted for the 2-fold slow activation of GIRK1F137S under control conditions and for an ~8-fold activation after coexpression of Gαi3.2

It appeared (although a systematic study has not been conducted) that coexpression of Gαi3 did not cause the expected reduction in basal activity of GIRK1F137S (e.g. compare the cell-attached levels of channel activity in Fig. 4A, a and b). The unexpectedly high basal activity could reflect an increase in membrane levels of GIRK1F137S caused by Gαi3 as described for WT GIRK1/GIRK2 (10). Indeed immunostaining of the expressed channels in large plasma membrane patches (Fig. 4C) showed a net 6.4-fold increase of GIRK1F137S protein by Gαi3 (after subtraction of background observed in un.injected oocytes) compared with channels expressed alone.

The dose dependence of the slow Na+ effect was studied in oocytes coexpressing GIRK1F137S and Gαi3. Maximal activation was observed at 10 mM Na+ (Fig. 4D). 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 (EC50) appeared to be slightly above 5 mM Na+, but considering the interference of the inhibitory effect at high [Na+], the actual EC50 of slow Na+ activation is probably higher.

**DISCUSSION**

**Na+ Regulates Basal Equilibrium between Free and Gαi3-Gβγ-bound Gβγ**—Our results strongly suggest that Na+ reduces the affinity of interaction between Gαi3-Gβγ and Gβγ. This hypothesis initially arose following the observation of slow, Gβγ-dependent activation by Na+ of mutant GIRK channels lacking the direct Na+ modulation. We then demonstrated weakening of Gαi3-Gβγ 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 Gαi3-Gβγ and Gβγ. The EC50 or Kd 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+[i]. The proposed hypothesis made it possible to explain the observed inhibition...
of the slow Na\(^+\)-induced activation by the G\(\beta\gamma\) scavenger, cARK, and to predict a novel physiological effect: a dramatic enhancement of slow Na\(^+\) activation by G\(\beta\gamma\) by coexpressed Ga. Interestingly Xenopus oocytes possess an unusually high basol level of free G\(\beta\gamma\) (19, 20), and coexpression of Ga may actually mimic the "usual" cellular condition of low [G\(\beta\gamma\)].

Na\(^+\) has long been known to mediate 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 G\(\beta\gamma\)) to modulate basal activity. The EC\(_{50}\) of the well characterized, direct, G\(\beta\gamma\)-independent activation by Na\(^+\) is 30–40 mM. The slow, G\(\beta\gamma\)-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\(_{\text{GDP}}\)-G\(\beta\gamma\) 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\(^+\)]\(_{\text{in}}\) is 5–10 mM.

The observed features of slow Na\(^+\) modulation of GIRK conform to the model in which [G\(\beta\gamma\)], elevated because of the direct effect of Na\(^+\) on Ga\(\beta\gamma\) 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 Ga\(\beta\gamma\) 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\(^+\)]\(_{\text{in}}\) is tightly regulated by pumps and exchangers, being maintained in resting cells below 10 mM. Yet [Na\(^+\)]\(_{\text{in}}\) often widely fluctuates. In neurons, even relatively short periods of synaptic activity produce very large increases in [Na\(^+\)]\(_{\text{in}}\) 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 G\(\beta\gamma\). The effective range of Na\(^+\) concentrations that regulate the Ga\(\beta\gamma\) \(\rightleftarrows\) G\(_{\text{GDP}}\) + G\(\beta\gamma\) equilibrium is close to the resting physiological range of [Na\(^+\)]\(_{\text{in}}\). Therefore, regulation of G proteins by Na\(^+\) may be a servo-type mechanism that sensitively responds to bidirectional changes in [Na\(^+\)]\(_{\text{in}}\) rather than to increases alone. We propose that Na\(^+\) regulation of the dynamic equilibrium between bound and free G\(_{\text{GDP}}\) and G\(\beta\gamma\) can have a substantial biological effect by regulating a host of effectors of G\(\beta\gamma\) (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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