Intracellular Na^+ inhibits voltage-dependent N-type Ca^{2+} channels by a G protein $\beta\gamma$ subunit-dependent mechanism

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N-type voltage-dependent Ca^{2+} channels (N-VDCCs) play important roles in neurotransmitter release and certain postsynaptic phenomena. These channels are modulated by a number of intracellular factors, notably by G$\beta\gamma$ subunits of G proteins, which inhibit N-VDCCs in a voltage-dependent (VD) manner. Here we show that an increase in intracellular Na^+ concentration inhibits N-VDCCs in hippocampal pyramidal neurones and in *Xenopus* oocytes. In acutely dissociated hippocampal neurones, Ba^{2+} current via N-VDCCs was inhibited by Na^+ influx caused by the activation of NMDA receptor channels. In *Xenopus* oocytes expressing N-VDCCs, Ba^{2+} currents were inhibited by Na^+ influx and enhanced by depletion of Na^+, after incubation in a Na^+-free extracellular solution. The Na^+-induced inhibition was accompanied by the development of VD facilitation, a hallmark of a G$\beta\gamma$-dependent process. Na^+-induced regulation of N-VDCCs is G$\beta\gamma$ dependent, as suggested by the blocking of Na^+ effects by G$\beta\gamma$ scavengers and by excess G$\beta\gamma$, and may be mediated by the Na^+-induced dissociation of G$\alpha\beta\gamma$ heterotrimers. N-VDCCs may be novel effectors of Na^+ion, regulated by the Na^+ concentration via G$\beta\gamma$.

(Received 30 September 2003; accepted after revision 15 January 2004; first published online 23 January 2004)
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Na^+ ions are crucial for neuronal activity as carriers of depolarization, and play important roles in maintaining the water balance of the body, cardiac contraction, and transport processes. In neurones, short periods of synaptic activity produce large increases in intracellular Na^+ concentration ($[Na^+]_i$), mainly due to Na^+ influx via *N*-methyl-D-aspartate (NMDA) receptor channels. During such periods of activity $[Na^+]_i$ rises from a resting level of 9−13 mM, to ∼30 mM in apical dendrites, and to 30–100 mM in dendritic spines (Rose & Ransom, 1997; Rose & Konnerth, 2001).

Changes in $[Na^+]_i$ regulate a variety of intracellular targets. Many membrane proteins that are engaged in the transport of Na^+ itself (the Na^+−K^+-ATPase and Na^+-coupled transporters and exchangers) are regulated by changes both in $[Na^+]_i$ and extracellular $[Na^+]$ ($[Na^+]_o$) (e.g. Kanner, 1994). In mammalian neurones, elevated $[Na^+]_i$ and/or Na^+ influx via NMDA or AMPA/kainate channels enhances the opening of NMDA receptor channels by a Src protein-dependent mechanism (Yu & Salter, 1998), inhibits voltage-dependent K^+ channels by an unknown mechanism (Van Damme *et al.* 2002) and activates K^+ channels of large conductance (Egan *et al.* 1992). Na^+ also binds to and activates the G protein-gated inward rectifier K^+ channels GIRK (Ho & Murrell-Lagnado, 1999; Petit-Jacques *et al.* 1999), which are normally gated by direct binding of G$\beta\gamma$ subunits (Clapham & Neer, 1997).

Recently, we have described a novel regulatory effect of intracellular sodium (Rishal *et al.* 2003): it promotes the dissociation of heterotrimeric G proteins, G$\alpha\beta\gamma$, into free $G\alpha_{GDP}$ and G$\beta\gamma$, increasing free cellular G$\beta\gamma$ concentration ($[G\beta\gamma]$). This causes a slow, G$\beta\gamma$-dependent activation of GIRK channels (Rishal *et al.* 2003). The physiological impact of the newly described modulation of G proteins by Na^+ is yet to be established.

One candidate effector for G$\beta\gamma$-mediated regulation by Na^+ is the neuronal N-type voltage-dependent Ca^{2+} channel, N-VDCC ($Ca_v2.2$). N-VDCC, and a closely related P/Q-VDCC, play a crucial role in neurotransmitter release (Catterall, 1998). Both N- and P/Q Ca^{2+}

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DOI: 10.1113/jphysiol.2003.056168

channels are widely regulated, usually inhibited, by neurotransmitters acting on G protein-coupled receptors (GPCRs); this is considered to be an important mechanism of neuronal presynaptic inhibition (Miller, 1998). N-VDCCs are also abundant in soma and dendrites (Westenbroek *et al.* 1992); postsynaptic N-VDCCs appear to be critical for associative long-term depression in the hippocampus (Normann *et al.* 2000). A ubiquitous modulation of N- and P/Q-VDCCs is their inhibition by direct binding of $G\beta\gamma$ released from the heterotrimeric $G_{i/o}$ proteins, usually G_o (reviewed by Dolphin, 1998; Zamponi & Snutch, 1998*a*; Ikeda & Dunlap, 1999; Catterall, 2000). This $G\beta\gamma$-mediated inhibition is voltage dependent (VD), because the binding of $G\beta\gamma$ is reduced by depolarization.

Here, we demonstrate that intracellular Na^+ inhibits N-VDCC in a $G\beta\gamma$-dependent manner. While the influx of Na^+ inhibits N-VDCC, the depletion of intracellular Na^+ 'disinhibits' them, suggesting a possible bidirectional regulation by $[Na^+]_i$. These findings reveal a previously unrecognized regulation of neuronal Ca^{2+} channels, and support the idea that Na^+, acting via $G\beta\gamma$, may regulate many membrane proteins.

Methods

cDNA constructs and RNA

RNA was synthesized *in vitro*, using standard methods, from the following DNAs: Na^+ channel subunit NaIIA-α (Dascal & Lotan, 1991); myristoylated cβARK, muscarinic m2 receptor, $G\alpha_o$, $G\beta_1$, $G\gamma_2$ subunits (Peleg *et al.* 2002); N-VDCC α_{1B} subunit (Wakamori *et al.* 1998). The coding parts of various G protein subunits were subcloned into the pGEM-HE or its derivative, pGEM-HJ vectors (Peleg *et al.* 2002). Standard amounts of injected RNA, when not indicated otherwise in the text, were (ng per oocyte^{-1}): N-VDCC α_{1B}, 1.5–2.5; $G\alpha_o$, 0.5–1; cβARK, 5; NaIIA α subunit, 5; $G\beta_1$, 5; $G\gamma_2$, 1; $G\alpha_o$, 0.5; m2 receptor, 0.5.

Xenopus oocyte preparation and electrophysiology

The care of the *Xenopus laevis* frogs, and the collection of oocytes, which were defolliculated and injected with RNA, was as described previously (Dascal & Lotan, 1991). Briefly, female frogs, maintained at 20 ± 2°C on an 11 h light/13 h dark cycle, were anaesthetized in a 0.15% solution of procaine methanesulphonate (MS222), and portions of ovary were removed through a small incision on the abdomen. The incision was sutured, and the animal was returned to a separate tank until it had fully recovered from the anaesthesia, and afterwards was returned to a large tank where, together with the other postoperational animals, it was allowed to recover for at least 4 weeks until the next surgery. The animals did not show any signs of postoperational distress. All the experiments were carried out in accordance with the Tel Aviv University Institutional Animal Care and Use Committee (permit no. 11-99-47). Oocytes were injected with RNA and incubated in ND96 solution (mM: NaCl, 96; KCl, 2; $CaCl_2$, 1; $MgCl_2$, 1; Hepes/NaOH, 5). Ca^{2+} channel currents were studied using two-electrode voltage clamp as described previously (Ivanina *et al.* 2000) in a high-Na^+ solution (mM: $Ba(OH)_2$, 20; NaOH, 85; KOH, 2; Hepes, 5) or a Na^+-free solution (mM: $Ba(OH)_2$, 20; *N*-methyl-D-glucamine (NMDG), 85; KOH, 2; Hepes, 5). These solutions were titrated with methanesulphonic acid to pH = 7.5. (In one experiment, solutions with 40 mM Ba^{2+} and 50 mM Na^+ or $NMDG^+$ were used.) In most experiments, the oocytes were injected with the Ca^{2+} chelator BAPTA (20 nl of 50 mM solution). The injection of BAPTA had no influence on Na^+-induced changes in I_{Ba}. Agar-cushion electrodes filled with 3 mM KCl with resistances of 0.1–0.4 MΩ were used in all experiments. Data acquisition and analysis were done using the pCLAMP software (Axon Instruments). *I–V* curves were fitted to a standard Boltzmann equation in the form $I_{Ba} = G_{max}(V_m - V_{rev})/(1 + \exp(-(V_m - V_a/K_a)))$, where K_a is the slope factor, V_a is the voltage that causes half-maximal activation, G_{max} is the maximal conductance, V_m is membrane voltage, I_{Ba} is the current measured at the same voltage, and V_{rev} is the reversal potential of I_{Ba}.

Hippocampal neurones in QEHA peptide experiments

Freshly dispersed hippocampal pyramidal CA1–CA3 neurones from 12- to 14-day-old Wistar rats (killed under ether anaesthesia according to Bogomoletz Institute of Physiology, Kiev, guidelines) were prepared as described previously (Panchenko *et al.* 1996) and identified by their characteristic form and partially preserved dendritic arborization. The cells lacked most of their long projections, allowing high-quality voltage control. N-VDCC currents in neurones were recorded with Ba^{2+} as the charge carrier in whole-cell configuration. L- and P/Q-type channels were blocked by 10 μM nitrendipine and 200 nM ω-agatoxin IVA, respectively. The pipette solution contained (mM): Tris-phosphate 70, tetraethylammonium chloride (TEA-Cl) 40, Tris-Cl 20, EGTA 5, Mg-ATP 5, GTP-Tris salt 0.5 (adjusted to pH 7.3 with Tris-OH). When the Na^+ concentration in the pipette solution was elevated, TEA-Cl was reduced accordingly. The external solution contained (mM): TEA-Cl 40, NaCl 100, $BaCl_2$ 10, Tris-Cl 20.

Hippocampal neurones in NMDA experiments

Slices of the hippocampus were incubated in solution containing 5 mg ml^{-1} protease *Aspergillus orizae* (Type XXIII, Sigma) for 30–40 min at 34°C. After enzyme treatment, the slices were rinsed in the same solution without enzyme, but with the addition of 0.5 mM $CaCl_2$ and 0.5 mM $MgCl_2$. The high Na^+ solution and the pipette solution were the same as in QEHA experiments. Na^+-free external solution solutions contained (mM): TEA-Cl 40, NMDG 100, $BaCl_2$ 10, Tris-Cl 20.

Statistics

Data are presented as mean ± S.E.M. Comparison between treatments in the same cells (e.g. effect of NMDA or Na^+ depletion) was done using paired *t* tests. Comparisons between two groups of treatments (e.g. cells with or without coexpressed m-cβARK) were done using two-tailed *t* tests. Multiple group comparisons were done using one-way ANOVA followed by Dunnett's or Student–Newman–Keuls tests.

Results

Regulation of N-VDCCs in *Xenopus* oocytes by coexpressed G$\beta\gamma$, a G$\beta\gamma$ scavenger, and a $G_{i/o}$-coupled neurotransmitter

Initially, we utilized *Xenopus* oocytes to explore the idea that intracellular Na^+ might regulate N-VDCCs. 'Classical' G$\beta\gamma$-dependent regulation of N-VDCCs expressed in *Xenopus* oocytes has been shown in the past (e.g. Roche *et al.* 1995; Bourinet *et al.* 1996). However, we felt it necessary to examine the quantitative aspects of G$\beta\gamma$-dependent regulation, with an emphasis on the effects of moderate changes in free cellular G$\beta\gamma$ concentration ([G$\beta\gamma$]), since only mild changes in [G$\beta\gamma$] are expected to be produced by intracellular Na^+ acting on G proteins (Rishal *et al.* 2003). To achieve this goal, we used two methods: heterologous expression of various amounts of G$\beta\gamma$ by injecting different amounts of RNA, and activation of a coexpressed $G_{i/o}$-coupled GPCR by low and high doses of a neurotransmitter.

G$\beta\gamma$-dependent inhibition of N-type Ca^{2+} channels is relieved by depolarizing prepulses applied just before the standard 'test' pulse used to elicit the current. This phenomenon is called voltage-dependent (VD) facilitation. Overexpression of G$\beta\gamma$ or activation of a neurotransmitter acting via G$\beta\gamma$ causes VD inhibition accompanied by VD facilitation, and usually slows down the kinetics of activation of I_{Ba} ('kinetic slowing') and shifts the current–voltage (*I–V*) curve in the positive direction (Bean, 1989; Ikeda, 1991, 1996; Herlitze *et al.* 1996). The oocytes contain a relatively high resting [G$\beta\gamma$] (Sheng *et al.* 2001), and expressed N-VDCCs formed by their main α_{1B} subunit often exhibit a tonic inhibition by basal levels of G$\beta\gamma$, and, accordingly, a substantial VD facilitation (Roche *et al.* 1995; Roche & Treistman, 1998; see Fig. 1). Tonic VD facilitation is sometimes observed in neuronal cells, for instance in sympathetic neurones (Ikeda, 1991). The presence of tonic G$\beta\gamma$-induced inhibition is best seen in the absence of a coexpressed β subunit of N-VDCC, as in our conditions (Roche & Treistman, 1998), which makes it possible to examine the consequences of bidirectional changes in [G$\beta\gamma$]: a mild increase in resting [G$\beta\gamma$] will reduce I_{Ba} and increase VD facilitation; a decrease in [G$\beta\gamma$] will have an opposite effect.

Figure 1*A* shows a typical experimental protocol. N-VDCCs, formed by α_{1B} subunits, were studied using the whole-cell, two-electrode voltage clamp method. Control (high-Na^+) solution contained 85 mM Na^+ and 20 mM Ba^{2+}. A standard double-pulse protocol (Ikeda, 1991) was used to measure VD facilitation (Fig. 1*A*). A voltage step from a holding potential of −80 mV to 20 mV ('test pulse') was issued to measure the basal I_{Ba}. Fifteen seconds later, the second test pulse was applied again, this time preceded by a 100 ms prepulse to +100 mV. In most oocytes a prominent tonic VD facilitation was observed: I_{Ba} elicited after a depolarizing prepulse (+PP) was 30–100% larger than when the test pulse was given without the prepulse (–PP; Fig. 1*A*).

We next characterized the effects of expression of G$\beta\gamma$ and m-cβARK (C-terminal part of the β-adrenergic receptor kinase). The latter is a high-affinity G$\beta\gamma$ scavenger targeted to the membrane by a myristoyl moiety (Petit-Jacques *et al.* 1999; Peleg *et al.* 2002). The tonic VD facilitation was reduced by m-cβARK from 66.2 ± 7.2% to 28.4 ± 2.1% ($P < 0.01$, Fig. 1*B*; summarized in Fig. 1*C*), confirming the G$\beta\gamma$-dependent character of tonic facilitation. We denote the net change in facilitation caused by a treatment as $\Delta_{facilitation}$ (in this case 37.8%).

Coexpression of G$\beta\gamma$ at low doses usually enhances VD facilitation (Ikeda & Dunlap, 1999). This was also the case in our experiments (0.2 ng Gβ RNA oocyte^{-1}; Fig. 1*B* and *Ca*). In contrast, an overwhelming excess of [G$\beta\gamma$] is known to overcome the relieving effects of the depolarizing prepulse, to occlude the GPCR-induced modulation of N-VDCCs, and to reduce the extent of VD facilitation (Ikeda, 1996). In our experiments, heavy overexpression of G$\beta\gamma$ (5 ng Gβ RNA oocyte^{-1}) strongly reduced the extent of facilitation and caused kinetic slowing of I_{Ba}

(Fig. 1*B* and *C*). To assess kinetic slowing quantitatively, we calculated the time from the beginning of the depolarizing pulse to 90% of peak amplitude ($t_{90\%}$) at +20 mV. In the experiment shown in Fig. 1, $t_{90\%}$ was 14.3 ± 0.7 ms in cells that expressed the channel alone ($n = 10$). Prominent kinetic slowing was seen at high doses of G$\beta\gamma$ (0.5 ng RNA oocyte^{-1} and more): $t_{90\%}$ increased to 55–60 ms (Fig. 1*Cb*). However, at 0.1 and 0.2 ng Gβ RNA oocyte^{-1}, the kinetic slowing was very mild and did not reach statistical significance. For instance, at 0.1 ng G$\beta\gamma$ RNA oocyte^{-1}, $t_{90\%}$ was 16.5 ± 1.2 ms ($n = 10$), a change of 15.4% only, despite the prominent and statistically significant change in VD facilitation caused by this dose of G$\beta\gamma$ (from 66% to 86%; $\Delta_{\text{facilitation}} = 20\%$. See Fig. 1*C*). The strong reduction in the extent of facilitation caused by the scavenging of G$\beta\gamma$ by cβARK was accompanied by only a very mild, statistically insignificant acceleration of activation ($t_{90\%}$ = 12.7 ± 0.5 ms, an 11% change; $P > 0.05$).

Despite the expectation that coexpression of G$\beta\gamma$ will reduce the average amplitude of I_{Ba} and cβARK will increase I_{Ba}, this did not happen. The amplitude of I_{Ba} was either slightly decreased or increased up to 40% by coexpression of G$\beta\gamma$, but these changes did not reach statistical significance (data not shown). Coexpression of cβARK decreased average I_{Ba} – in the experiment of Fig. 1*C*, from 833 ± 132 nA to 368 ± 76 nA ($P < 0.05$). We assume that coexpressed G$\beta\gamma$ may increase the plasma membrane levels or slow down the degradation of N-type VDCCs, as often happens among interacting proteins; this issue has not been studied any further.

To examine a classical GPCR-induced, G$\beta\gamma$-dependent inhibition of N-VDCCs under our experimental conditions, we looked at the inhibition caused by an agonist (acetylcholine; ACh) activating a typical $G_{i/o}$-coupled GPCR, m2 muscarinic receptor (Fig. 2). The oocytes expressed the m2 receptor, α_{1B}, and Gα_o (coexpression of Gα_o proved important to obtain reproducible inhibition of I_{Ba} by ACh). Addition of ACh reduced I_{Ba} within less than 30 s. The concentrations of ACh were adjusted to produce either mild inhibition (1 nM ACh) or a maximal effect (10 μM ACh, inhibition by 71.5 ± 3.8%, $n = 23$) (Fig. 2*A* and *B*). Inhibition was accompanied by an increase in percentage facilitation, kinetic slowing of activation, and a rightward shift in *I–V* curves (Fig. 2*B*). All these effects were very prominent at the saturating

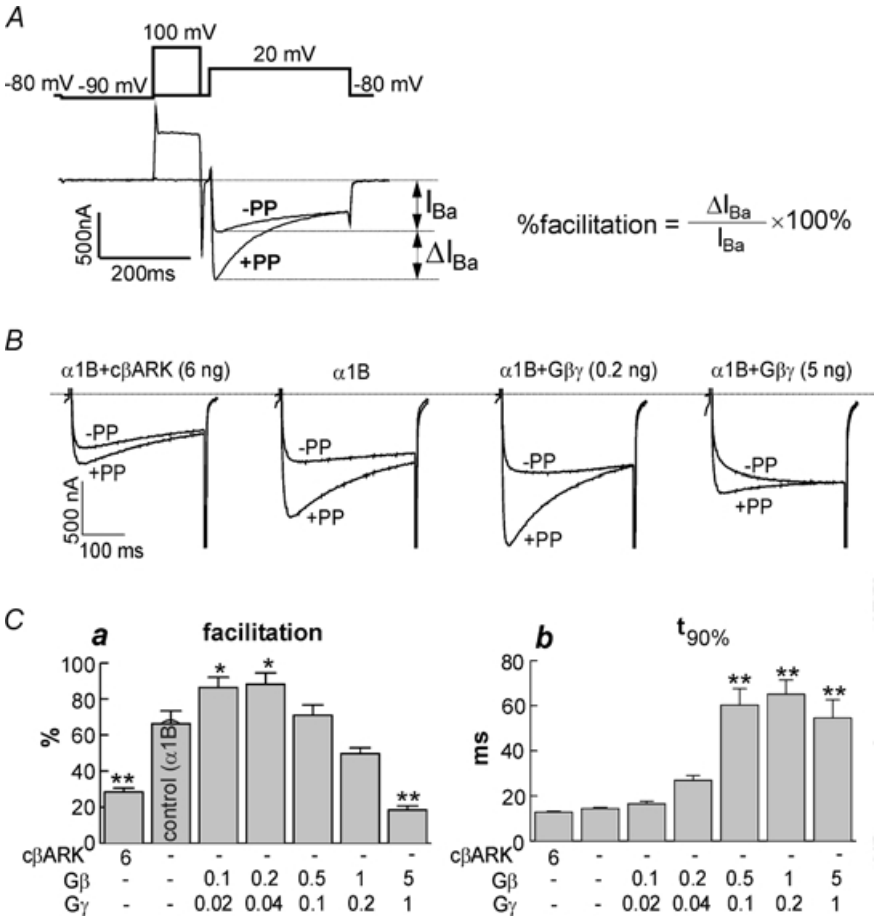

Figure 1. Effects of coexpressed cβARK and G$\beta\gamma$ on N-VDCCs in *Xenopus* oocytes
A, I_{Ba} (lower trace) measured by the double pulse protocol (upper trace). Percentage facilitation was calculated as $\Delta I_{\text{Ba}}/I_{\text{Ba}} \times 100\%$. *B*, effects of coexpressed cβARK and G$\beta\gamma$ on facilitation of α_{1B}. Amounts of coexpressed RNAs are indicated (ng oocyte^{-1}; RNAs of Gβ and Gγ were always injected at 5 : 1 ratio). *C*, summary of effects of cβARK and G$\beta\gamma$ on facilitation (*a*) and $t_{90\%}$ (*b*). Each column represents a group of oocytes injected with the indicated RNAs; $n = 10$ in each group. $^*P < 0.05$, $^{**}P < 0.01$ by one-way ANOVA.

concentration of ACh. However, at 1 nM ACh, despite substantial changes in peak I_{Ba} (inhibition by 42.8 ± 3.8%; $n = 19$) and facilitation (from 8.5 ± 3.4% to 42.3 ± 7.0%), only very mild changes were observed in $t_{90\%}$ (from 9.5 ± 0.3 to 11.6 ± 0.8 ms) and in the *I–V* curve (a shift to the right by less than 2.5 mV; Fig. 2*C*). These results, together with those of Fig. 1, imply that mild changes in $[G\beta\gamma]$, that suffice to cause a substantial inhibition of I_{Ba} and a measurable increase in facilitation, cause only marginal kinetic slowing and *I–V* curve shift.

Note that despite the strong facilitation of ACh-inhibited I_{Ba} produced by the prepulse, the facilitated (+PP) I_{Ba} in the presence of ACh still remained smaller than the facilitated I_{Ba} measured before ACh (Fig. 2*A*). The depolarizing prepulse relieved only 24.5% of inhibition produced by 10 μM ACh, and about 39% of inhibition produced by 1 nM ACh.

Regulation of N-VDCCs in *Xenopus* oocytes by depletion or influx of Na^+

According to our model (Rishal *et al.* 2003), Na^+ enhances the dissociation of $G\alpha\beta\gamma$ in the reaction:

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

An increase in $[Na^+]_i$ should increase $[G\beta\gamma]$, inhibit N-VDCCs and produce VD facilitation; a decrease in $[Na^+]_i$ should have opposite effects. The following phenomena may be expected. (1) Influx of Na^+ into the cell should inhibit N-VDCCs and increase VD facilitation. (2) Resting $[Na^+]_i$ in amphibian oocytes is about 10 mM (Dascal, 1987), close to the EC_{50} of the Na^+ effect on $G\alpha\beta\gamma$ (Rishal *et al.* 2003). Partial depletion of submembrane $[Na^+]_i$ by incubation in Na^+-free extracellular solution should reduce $[G\beta\gamma]$ and remove part of the tonic inhibition of N-VDCCs. (3) m-cβARK should strongly attenuate all effects of Na^+. (4) An excess of $G\beta\gamma$ should attenuate (occlude) changes caused by Na^+ depletion.

These predictions have been tested experimentally. To study the effect of Na^+ influx, we coexpressed the neuronal voltage-dependent Na^+ channel (type IIA). The level of expression of Na^+ channels was adjusted to give Na^+ currents of 10–50 μA (Fig. 3*A*, upper trace). Satisfactory quality of voltage control during I_{Na} was confirmed by measuring the actual membrane voltage with an independent voltage electrode inserted into the oocyte in addition to the two standard electrodes (Fig. 3*A*, lower trace). Na^+ influx was forced by tetanic stimulation (TS: a train of 2 ms voltage steps from −80 to 0 mV at 100 Hz for 100 s), applied under voltage clamp conditions, in Ca^{2+}- and Ba^{2+}-free ND96 solution containing 96 mM Na^+. I_{Ba} was measured before and after TS in a Na^+-free, high-Ba^{2+} solution (Fig. 3*B*). Although the TS protocol included repetitive depolarizations, the parameters were chosen such as not to inactivate I_{Ba}. This was confirmed by applying TS in the presence of 0.1 μM tetrodotoxin (TTX), which blocked the Na^+ currents by > 90%. This TS did not change I_{Ba} (98.2 ± 3.0% of control; $n = 9$).

In the absence of TTX, the TS protocol reduced I_{Ba} and enhanced the facilitation. Figure 3*Ba* shows a representative experiment. The first record (left trace) was done about 1 min after the transfer of the oocyte from normal ND96 to the Na^+-free, high-Ba^{2+} solution; the TS was performed in 96 mM Na^+, and the solution was switched again to Na^+-free. I_{Ba} was measured 30 s after

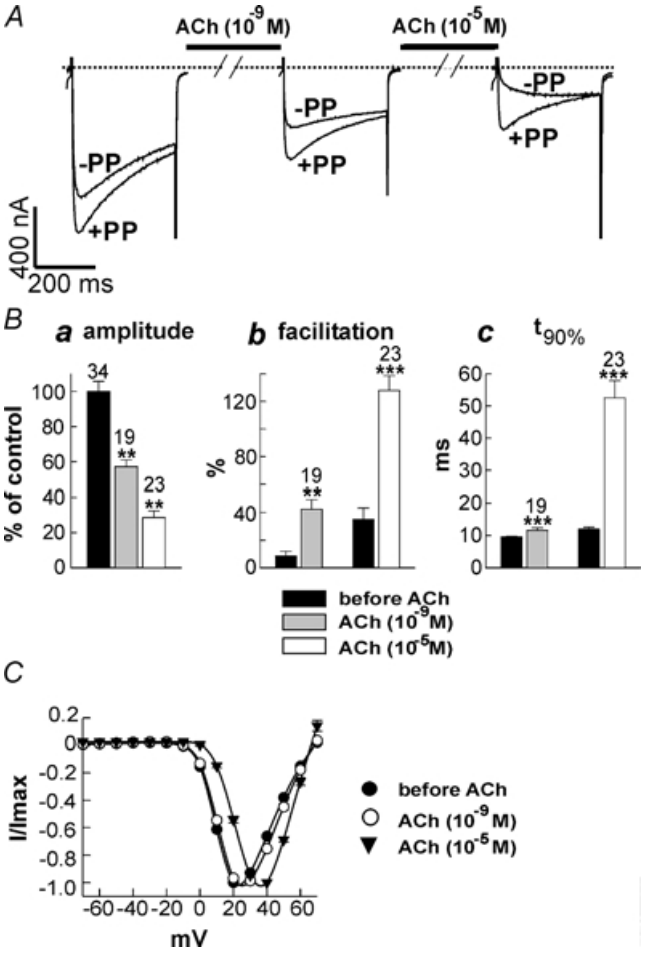

Figure 2. Inhibition of N-VDCCs expressed in *Xenopus* oocytes by a $G_{i/o}$-coupled neurotransmitter

A, typical records of the effects of ACh (10^{-9} or 10^{-5} M) on I_{Ba}. *B*, summary of the effects of ACh on amplitude (*a*), facilitation (*b*), and $t_{90\%}$ (c) of I_{Ba}. Numbers above columns correspond to the number of oocytes tested. $^{**}P < 0.01$, $^{***}P < 0.001$. *C*, normalized average current–voltage (*I–V*) curves of Ba^{2+} currents in 8 oocytes of one donor, and the effect of 10^{-9} and 10^{-5} M ACh.

the switch (middle trace), and in most oocytes also 3–4 min later to monitor the recovery of I_{Ba} (right trace). On average, TS reduced I_{Ba} by 17.9 ± 2.9%, and facilitation became greater than before TS by 7.7 ± 1.7% ($n = 10$). Three minutes later I_{Ba} fully recovered to 101.7 ± 2.6% ($n = 10$) of initial amplitude.

When internal Na^+ was depleted by prior 7–10 min incubation in Na^+-free solution (see below for details), the effects of Na^+ influx were amplified (Fig. 3*Bb*). On average, TS reduced I_{Ba} by 34.5 ± 2.4% ($n = 12$, $P < 0.001$; Fig. 3*B*); facilitation that was 27.4 ± 3.6% before TS, became 45.5 ± 6.9% after TS ($n = 12$, $P < 0.001$; Fig. 3*C*). I_{Ba} partially recovered after 3–4 min (to 85.7 ± 3.8% of control; $n = 9$; see Fig. 3*Bb*; longer recovery periods have not been tested). In this series of experiments, only minor changes in activation kinetics have occurred that did not reach statistical significance (data not shown).

Coexpression of the G$\beta\gamma$ scavenger m-cβARK fully prevented the decrease in I_{Ba} and the change in facilitation caused by TS (Fig. 3*C*). Thus, the effects of Na^+ influx were G$\beta\gamma$ dependent. The changes caused by the TS were not due to alterations in $[Na^+]_o$, because both before and after TS, I_{Ba} was measured in Na^+-free solution.

As in the case of ACh-induced inhibition, the depolarizing prepulse did not fully remove the inhibition caused by Na^+ influx; the PP-facilitated I_{Ba} was still inhibited by TS-induced Na^+ influx by 25.8 ± 2.5% ($n = 12$). This is still 0.75 of the 34.5% inhibition of the non-facilitated I_{Ba}. Thus, the PP relieved only about 25% of inhibition caused by Na^+ influx. Although the activation of I_{Ba} after TS tended to be slower than before (by about 10%; data not shown), the difference did not reach statistical significance.

To reduce $[Na^+]_i$, which we expected to reduce $[G\beta\gamma]$, we used a depletion protocol that included a 7 min incubation in a Na^+-free solution (Vorobiov *et al.* 1998). Depletion procedures of this kind are known to reduce $[Na^+]_i$. In neurones, 10 min incubation in Na^+-free solution leads to a decrease of $[Na^+]_i$ to undetectable levels (Rose & Ransom, 1997). The depletion procedure caused a gradual increase in I_{Ba} by 22.6 ± 3.0% ($n = 23$), and a parallel decrease in the extent of facilitation from

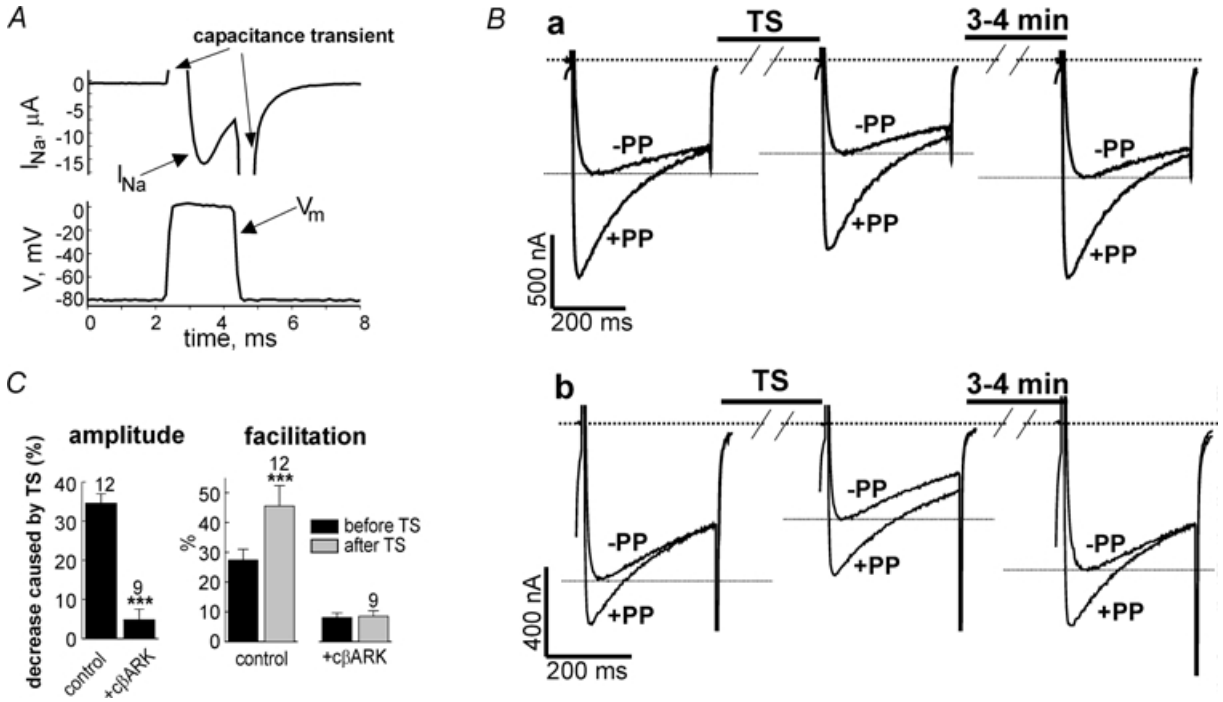

Figure 3. Inhibition of N-VDCCs expressed in *Xenopus* oocytes by sodium influx, and the effect of cβARK
A, quality of voltage control during a tetanic stimulation (TS) protocol. Membrane voltage (V_m) was measured using an additional electrode and a separate amplifier. Upper panel shows the sodium current measured in the high-Na^+ solution; lower panel shows the measured V_m. *B*, effect of TS-induced Na^+ entry in an oocyte that was not preincubated in Na^+-free solution (*a*) and in an oocyte that was incubated in Na^+-free solution for 7 min before the recording (*b*). I_{Ba} was recorded in Na^+-free solution, TS was applied in Ba^{2+}- and Ca^{2+}-free, high-Na^+ (ND96) solution. After TS, the oocytes were again kept in Na^+-free solution. *C*, summary of the effects of TS-induced Na^+ entry on amplitude and facilitation of I_{Ba}, and the effect of cβARK in oocytes that were exposed to Na^+-free solution for 7–10 min before the experiment. ***$P < 0.001$.

66.4 ± 6.3% to 48.2 ± 4.2% ($P = 0.02$; $n = 23$). Seven minutes incubation in control high-Na^+ solution did not cause any changes in I_{Ba} and facilitation (Fig. 4*A*; summarized in Fig. 4*C*). In support of a G$\beta\gamma$-dependent mechanism, m-cβARK strongly attenuated the changes caused by Na^+ depletion, both in I_{Ba} amplitude and in facilitation (Fig. 4*C*). Heavy overexpression of G$\beta\gamma$ abolished changes in amplitude and in facilitation caused by Na^+ depletion (Fig. 4*B* and *C*).

In most experiments Na^+ depletion did not cause a significant shift in the *I–V* curve, except one experiment in which the increase in I_{Ba} was exceptionally large (data not shown). The average $t_{90\%}$ in all cells did not significantly change after 7 min of Na^+ depletion (not

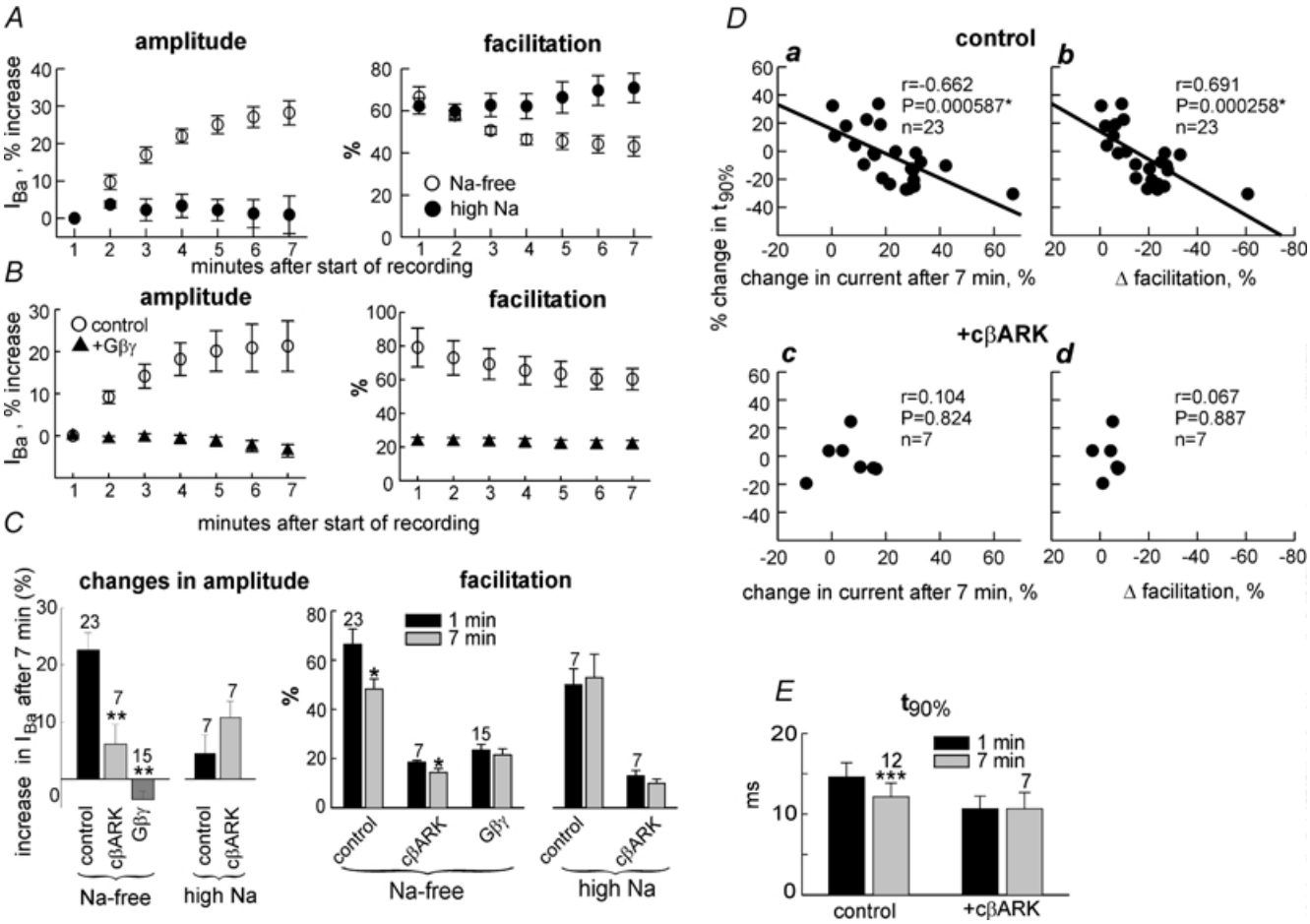

Figure 4. Modulation of N-VDCCs in *Xenopus* oocytes by Na^+ depletion and its G$\beta\gamma$ dependency
A, time course of changes in I_{Ba} (left panel) and in facilitation (right panel), caused by 7 min incubation in Na^+-free solution (○) or high-Na^+ solution (●), in a representative experiment. I_{Ba} was: in high-Na^+ solution, 849 ± 97 nA at $t = 1$ min and 862 ± 122 nA at $t = 7$ min ($n = 4$); in Na^+-free solution, 845 ± 98 nA at $t = 1$ min and 1075 ± 99 nA at $t = 7$ min ($n = 4$). In each cell, changes in I_{Ba} with time were monitored relative to I_{Ba} measured 1 min after the beginning of voltage clamp. *B*, the effect of Na^+ depletion on I_{Ba} amplitude (left panel) and facilitation (right panel) is altered by overexpression of G$\beta\gamma$ (▲; 5 ng Gβ RNA oocyte^{-1}). ○, channel expressed alone. n = 11–15 oocytes in each group. *C*, summary of the effects of 7 min incubation of oocytes in low- or high-Na^+ solutions, in oocytes that expressed N-VDCCs alone (control), with m-cβARK, or with G$\beta\gamma$. Left panel summarizes the changes in amplitude of I_{Ba}. $^{**}P < 0.01$ compared to control group. Right panel summarizes the changes in facilitation. $^{*}P < 0.05$ for the difference between 1st and 7th minute. *D*, $t_{90\%}$ of activation shows correlation with the change in I_{Ba} (*a*) and in $\Delta_{facilitation}$ (*b*) after 7 min of Na^+ depletion. This correlation is absent in oocytes that coexpress m-cβARK (*c* and *d*). The value of *P* was calculated using the Pearson correlation test. *E*, summary of the effects of depletion on $t_{90\%}$ of activation in oocytes that expressed N-VDCCs alone (control) or with m-cβARK. In the control group, only results from cells with more than a 20% increase in I_{Ba} after 7 min of Na^+ depletion (n = 12) are summarized. $^{***}P < 0.001$.

shown). Furthermore, slight spontaneous slowing of I_{Ba} activation was often observed in the course of these long-lasting experiments under all conditions, such as high or low-Na^+ solutions, the presence of cβARK or G$\beta\gamma$, etc. (data not shown). The reason for this phenomenon is not known, but we suspect that it acted to offset the acceleration of activation and the decrease in $t_{90\%}$ that we expected to see as a result of Na^+ depletion. Indeed, the greater was the increase in I_{Ba} caused by the depletion procedure, the clearer became the decrease in $t_{90\%}$. Figure 4*Da* demonstrates a highly significant correlation ($P < 0.001$) between the change in $t_{90\%}$ and the change in I_{Ba} caused by the 7 min Na^+ depletion procedure in the same cell. Accordingly, the change in $t_{90\%}$ caused by Na^+ depletion was also correlated with the change in the extent of facilitation (Fig. 4*Db*). Both correlations were absent in the presence of cβARK (Fig. 4*Dc* and *d*), and no correlation of any kind between these parameters has been observed when oocytes were incubated in high-Na^+ solution for 7–10 min (data not shown).

We have calculated the change in $t_{90\%}$ in all cells that showed a 20% or more increase in current amplitude after Na^+ depletion (12 out of 23 oocytes). The decrease in $t_{90\%}$ in these cells was highly significant ($P < 0.001$, paired t test), and did not occur in oocytes that expressed cβARK (Fig. 4*E*). Though mild (17%), this change is comparable to that caused by G$\beta\gamma$ depletion by cβARK (Fig. 1*C*). Taken together, these results confirm that, in parallel with an increase in current amplitude and a decrease in the extent of tonic facilitation, the Na^+ depletion procedure causes an acceleration in activation kinetics of I_{Ba}, in line with the hypothesis that all these effects are caused by a decrease in [G$\beta\gamma$].

We stress that the effects of Na^+ in the depletion experiments were not due to changes in $[Na^+]_o$. The slow (minutes) increase in I_{Ba} that developed after shifting to Na^+-free solution could not be due to removal of a block caused by external Na^+ (Polo-Parada & Korn, 1997), because the latter effect is immediate (seconds). Also, the direct inhibitory effect of Na^+ on N-VDCCs is negligible (< 5%) at 20 mM Ba^{2+} used in these experiments (Polo-Parada & Korn, 1997).

Intracellular Na^+ causes VD facilitation of N-VDCCs in hippocampal neurones

To estimate the possible physiological impact of Na^+ regulation of N-VDCCs, the effects of intracellular Na^+ and of Na^+ influx on native neuronal N-VDCCs were studied in freshly dispersed hippocampal pyramidal neurones. I_{Ba} via the N-VDCCs was recorded using the whole-cell patch clamp technique, by a double-pulse protocol (Fig. 5*A*). Other Ca^{2+} channels were blocked by the appropriate inhibitors (see Methods).

First, we examined the effect of intracellular Na^+ on N-VDCCs in a Na^+-free extracellular solution. The pipettes were filled with a solution that contained either 10, 20 or 40 mM Na^+, or no Na^+ (replaced by tetraethylammonium). The currents were recorded 15–20 min after breaking into the cell, to let the intracellular Na^+ equilibrate with the pipette solution. The amplitudes of Ba^{2+} currents varied greatly between different neurones, making the comparison of current amplitudes among different groups meaningless. However, the VD facilitation changed very consistently depending on $[Na^+]_i$. In Na^+-free conditions, no VD facilitation was observed; in fact, the prepulse reduced I_{Ba} by 9% on average (Fig. 5*B* and *C*). This 'inhibition' probably reflected incomplete recovery from voltage-dependent inactivation caused by the prepulse. In contrast, already in 20 mM Na^+, a clear facilitation of 18.4 ± 4.9% was observed ($P < 0.001$). The extent of facilitation was stronger at negative than at positive potentials (compare the differences between −PP and +PP traces at −25 and at +25 mV in Fig. 5*A*). In fact, at +25 mV the prepulse did not increase the peak amplitude of I_{Ba}, but the differences in activation kinetics with and without the prepulse were still apparent (Fig. 5*A*, inset). This feature is typical for the classical voltage-dependent, G$\beta\gamma$-mediated process. The facilitation, used here as the most accurate measure of Na^+ effect, was dose dependent, reaching almost 40% at 40 mM Na^+ (Fig. 5*C*).

Other features of the Na^+ effect were also compatible with a voltage- and G$\beta\gamma$-dependent mechanism, and comparable with changes caused by a low dose of ACh in the oocyte experiments. The *I–V* curve showed a small shift of 3.0 ± 0.12 mV to positive potentials (Fig. 5*D*; $P < 0.05$; N-VDCCs activated at more negative potentials in the neurones than in the oocytes, probably due to the presence of the β subunit in these cells and the lower Ba^{2+} concentration in the external solution). The kinetics of activation of I_{Ba} were assessed from the time constant of activation τ_{act}. As also expected for a G$\beta\gamma$-dependent phenomenon, Na^+ slowed down the activation of I_{Ba} (Fig. 5*B* and *Ea*), and the kinetics of the facilitated I_{Ba} after the prepulse were faster than those of non-facilitated current in the presence of 20 mM Na^+, but not in Na^+-free solution (Fig. 5*B* and *Eb*).

Finally, we utilized a soluble G$\beta\gamma$ chelator, the QEHA peptide which, at 50–100 μM, blocks the effects of G$\beta\gamma$ on adenylyl cyclase and GIRK channels (Chen *et al.* 1995; Weng *et al.* 1996). A separate group of neurones was perfused with a pipette solution containing 20 mM Na^+

and 50 μM of QEHA. In these neurones, facilitation was absent ($-1 \pm 5.3\%$, $P < 0.05$ compared to 20 mM Na^+ without QEHA; Fig. 5*C*), supporting the involvement of $G\beta\gamma$ in Na^+-induced facilitation.

Na^+ influx inhibits N-VDCCs in hippocampal neurones

Initially, Na^+ influx was produced using a tetanic stimulation (TS) protocol similar to that employed in the oocytes, which was found to inhibit N-VDCCs and to cause the appearance of VD facilitation (data not shown). However, several lines of evidence suggested imperfect voltage control during TS, that could lead to stronger depolarization than desired, causing inactivation of N-VDCCs. We have therefore examined the effect of influx of Na^+ via NMDA receptor channels, probably the most relevant source of increased $[Na^+]_i$ during periods of electrical activity in neurones (Schiller *et al.* 2000; Rose & Konnerth, 2001). The membrane potential was kept constant, −100 mV, and NMDA was applied in the presence of Na^+ (100 mM) or in the absence of Na^+ (100 mM NMDG$^+$ substituted for Na^+) in the external solution. The external solution was Mg^{2+}- and Ca^{2+}-free and contained Ba^{2+} (10 mM), glycine and TTX. I_{Ba} was constantly stimulated and monitored. In this series of experiments a small tonic VD facilitation was usually observed, probably due to a different cell isolation procedure (see Fig. 6*Aa* and *D*, and Methods). Note that

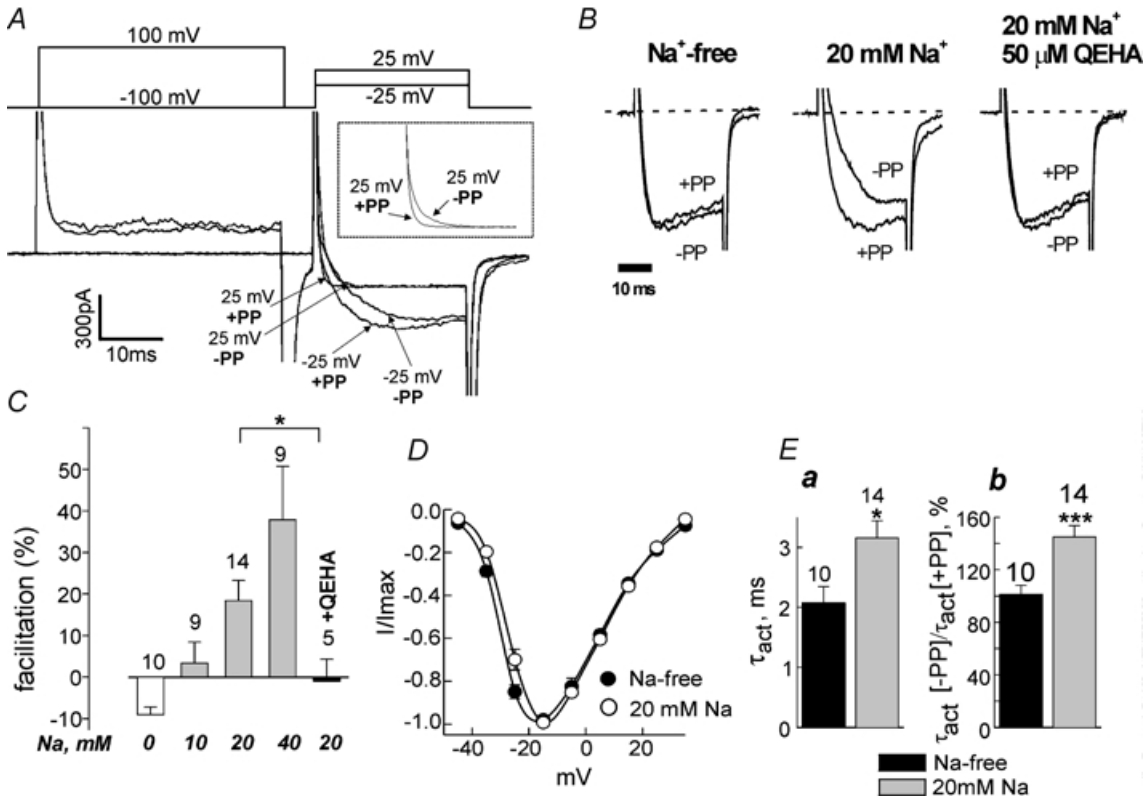

Figure 5. Intracellular Na^+ induces voltage-dependent facilitation of N-VDCCs in hippocampal neurones
A, I_{Ba} in the double pulse protocol. The first test pulse was from −100 to +25 mV or −25 mV without a prepulse (−PP). The second test pulse was applied after a 40 ms prepulse to +100 mV (+PP). Percentage facilitation was calculated as described in Fig. 1*A*. The inset shows on an expanded scale currents measured at +25 mV (see text). *B*, QEHA peptide prevents the Na^+-induced facilitation. Ba^{2+} currents measured at −25 mV in three representative neurones are shown. *C*, dose dependency of Na^+ effect on facilitation and summary of the effect of QEHA peptide at 20 mM Na^+. $^*P < 0.05$. *D*, *I–V* curves averaged from 6 neurones with 20 mM of Na^+ inside the cell (○) and 6 neurones with Na^+-free intracellular medium (●). Holding voltage was −100 mV. *E*, effect of intracellular sodium concentration on the activation kinetics of N-type calcium channels. The time constant of activation, τ_{act}, was calculated by fitting the rising phase of I_{Ba} to a single exponential equation. Calcium currents were elicited by depolarization from −100 to −25 mV. *Ea*, comparison of the absolute values of τ_{act} in neurones perfused with Na^+-free or Na^+ = 20 mM solutions. *Eb*, depolarizing prepulse accelerates the activation kinetics in the presence of 20 mM Na^+. *Y*-axis shows the ratio between τ_{act} after and before the prepulse. $^*P < 0.05$, $^{***}P < 0.001$.

measurement of N-VDCCs before and after Na^+ influx was done at constant $[Na^+]_o$.

Application of NMDA (300 μM) in the absence of Na^+ in extracellular solution caused a small inward current, most probably carried by Ba^{2+}, and induced no significant changes in the amplitude of I_{Ba} or in VD facilitation (Fig. 6*Aa*). Then the extracellular solution was exchanged to high-Na^+, and the same protocol was conducted. Addition of NMDA caused a large inward current, presumably carried largely by Na^+. After NMDA washout, I_{Ba} was reduced and the VD facilitation increased (Fig. 6*Ab*) in 7 out of 11 cells. The differences in facilitation are better illustrated in Fig. 6*Ac*, in which I_{Ba} values before and after NMDA are directly compared after scaling.

In four cells in which NMDA caused very small changes in I_{Ba} and facilitation, the NMDA currents themselves, measured in the high-Na^+ solution, were smaller (<0.8 nA) than in the other seven cells (> 1.2 nA). Figure 6*B* shows that both the decrease in I_{Ba} and the change in VD facilitation, caused by NMDA, strictly correlated with the amplitude of NMDA-induced inward current: larger NMDA-induced currents corresponded to greater changes in I_{Ba} and facilitation. This result, and the inability of NMDA to modulate I_{Ba} in the absence of extracellular Na^+, imply that the changes in I_{Ba} and in VD facilitation were caused by Na^+ influx caused by NMDA, rather than by the activation of the NMDA receptor itself.

Figure 6*C* and *D* summarizes the NMDA effects in the cells that showed NMDA currents above 1.2 nA. Figure 6*C* shows that the decrease in I_{Ba} caused by NMDA in high-Na^+ solution was 34.3 ± 4.4% (n = 7). This is significantly ($P < 0.001$) greater than in Na^+-free solution, where practically no change was observed (3.5 ± 4.1%, n = 6; in 1 out of the 7 cells, the records were done only in

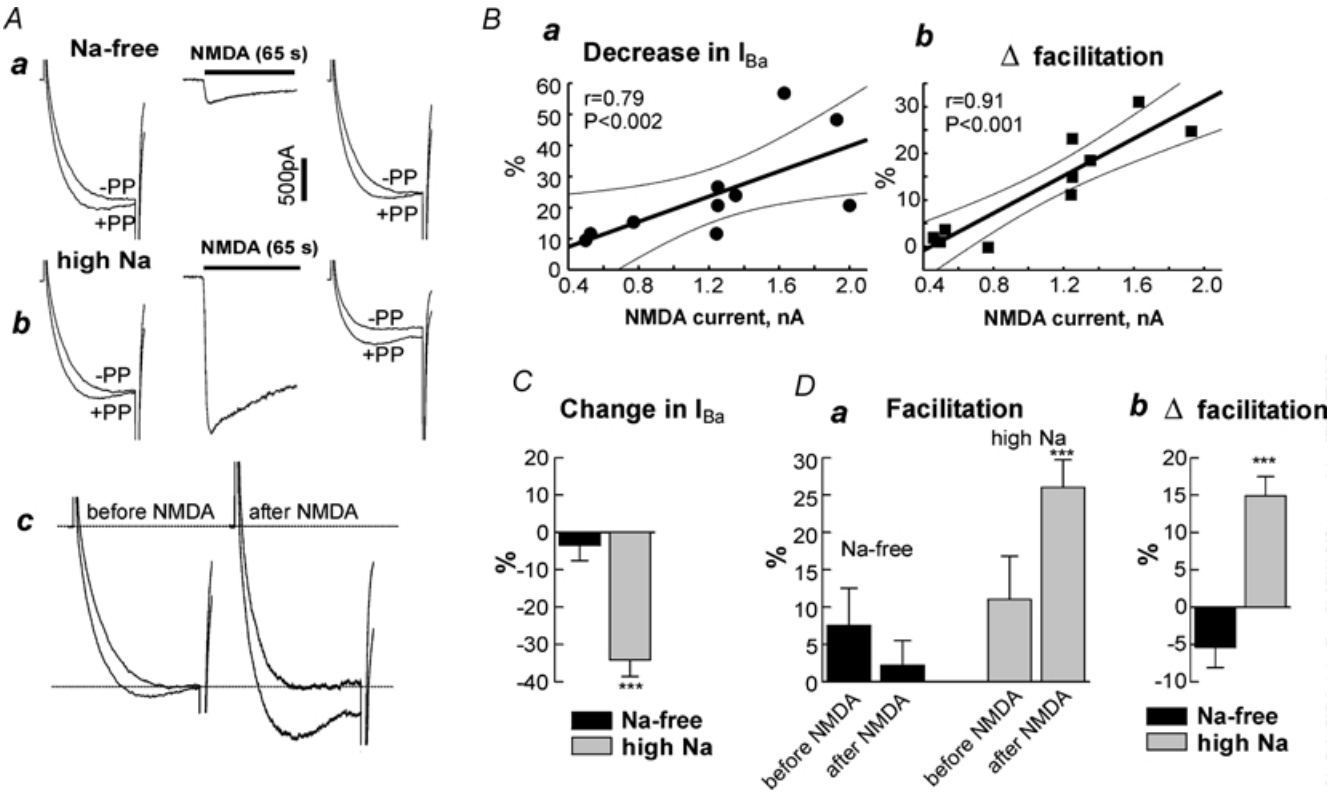

Figure 6. Na^+ influx through NMDA receptor channels inhibits N-VDCCs in hippocampal neurones
A, effect of Na^+ entry through NMDA receptor channels. *Aa*, I_{Ba} was measured in a Na^+-free solution. The double pulse protocol was as in Fig. 5. NMDA (100 μM) was applied in the Na^+-free solution for 65 s, washed out, and immediately I_{Ba} was measured again. Only the first second of the NMDA record is shown. *Ab*, continuation of the experiment in the same cell, after shifting to high Na^+-solution. The currents measured before and after NMDA are shown again in *Ac* after the scaling up of I_{Ba} recorded after NMDA to that recorded before NMDA. *B*, correlation of changes in I_{Ba} (*a*) and in facilitation (*b*) induced by Na^+ influx through NMDA receptors with the amplitude of NMDA responses. The straight line is a least squares fit, the dashed lines show 95% confidence intervals. *C* and *D*, summary of the effects of Na^+ entry through NMDA receptors on amplitude (*C*) and facilitation (*D*) of I_{Ba}. n = 6 in Na^+-free solution and n = 7 in high-Na^+ solution. ***$P < 0.001$.

high-Na^+ solution). Five to ten minutes after the washout of NMDA, I_{Ba} partially recovered to 85.7 ± 5.3% of its initial amplitude. The absolute values of percentage facilitation under different conditions are summarized in Fig. 6*Da*, and $\Delta_{facilitation}$ is shown in Fig. 6*Db*. The tonic facilitation observed in Na^+-free solution was actually slightly reduced by NMDA from 7.5% to 2.2% ($P = 0.1$). Incubation in high-Na^+ solution by itself appeared to increase the facilitation slightly, to 11.2%, but this change did not reach statistical significance ($P = 0.24$ compared to Na^+-free solution before NMDA, but $P = 0.016$ compared to Na^+-free solution after NMDA). Most importantly, in high-Na^+ solution, after NMDA the facilitation increased from 11.2 ± 3.7% to 26.1 ± 5.8% ($P < 0.001$). Kinetic slowing was observed in 6 out of 7 cells; the increase in τ_{act} was only 11% (from 2.84 ± 0.57 ms to 3.13 ± 0.56 ms), but highly significant ($P = 0.005$ by paired t test). Again, the depolarizing prepulse did not fully relieve the NMDA-induced inhibition of I_{Ba}; the facilitated I_{Ba} was still reduced by 24.7 ± 4.5% after NMDA application. This corresponds to a 28% relief of inhibition by the prepulse.

Discussion

Our findings demonstrate a previously unknown modulation of neuronal Ca^{2+} channels: inhibition by intracellular Na^+. It occurs within the physiological range of Na^+ concentrations, and exhibits hallmarks of a $G\beta\gamma$-mediated process: it is accompanied by voltage-dependent facilitation and is attenuated by scavengers of $G\beta\gamma$ and by depolarization. Our results are in line with the hypothesis that the molecular mechanism of this modulation is the regulation, by Na^+ ions, of the dynamic equilibrium between $G\alpha$-bound and free $G\beta\gamma$.

Intracellular Na^+ inhibits N-VDCCs

Our results suggest that increased $[Na^+]_i$ inhibits N-VDCCs. First, inhibition of I_{Ba} by Na^+ influx caused by depolarizing tetanic stimulation (TS) in oocytes or activation of NMDA channels in neurones, did not take place when Na^+ influx was eliminated. Second, *depletion* of Na^+ in the oocytes *increased* I_{Ba}. Third, intracellular perfusion of hippocampal neurones with Na^+ was accompanied by the appearance of VD facilitation, which was dose dependent and absent at $[Na^+]_i = 0$. Although direct comparison of amplitudes of I_{Ba} with and without Na^+ was impossible, the VD facilitation, which was always highly correlated with inhibition of I_{Ba} throughout this study, attests to an inhibitory effect of Na^+.

Molecular mechanism

We believe that the mechanisms of Na^+ effects on N-VDCCs were identical in oocytes and in neurones, because the effects of lowering $[Na^+]$ (by depletion in oocytes, by perfusion with Na^+-free solution in neurones), and of increasing $[Na^+]_i$ by Na^+ influx, caused qualitatively identical changes in both cell types. Na^+-induced changes in I_{Ba} were paralleled by changes in VD facilitation and in activation kinetics. The extent and time course of changes in these three parameters were strongly correlated (Figs 4 and 6), suggesting a connection between these phenomena. All changes caused by increased or decreased $[Na^+]_i$ were in the direction predicted by the hypothesis that Na^+ regulates N-VDCCs via changes in $[G\beta\gamma]$. Additional lines of evidence suggest that a large part of Na^+-induced inhibition, and all of Na^+-induced VD facilitation, were $G\beta\gamma$ dependent. First and foremost, all changes in I_{Ba} and VD facilitation caused by both influx and depletion of Na^+ in oocytes, and the VD facilitation caused by 20 mM $[Na^+]_i$ in hippocampal neurones, were blocked by $G\beta\gamma$ scavengers. Second, the changes in I_{Ba} and facilitation caused by Na^+ depletion in the oocytes were fully blocked by overexpressed $G\beta\gamma$. The latter maximally activates $G\beta\gamma$-mediated processes, occluding additional modulations via this pathway (Herlitze *et al.* 1996; Ikeda, 1996).

Hence, our results suggest that Na^+ regulation of N-VDCCs is $G\beta\gamma$ dependent; in the simplest case, it may be mediated by changes in $[G\beta\gamma]$. However, more complex schemes are possible. It is notable that the consequences of Na^+- and GPCR-induced activation of G proteins are similar only in terms of changes in $[G\beta\gamma]$, but not $G\alpha$. Whereas activation of GPCRs results in the appearance of free $G\alpha_{GTP}$ (Gilman, 1987), Na^+ appears to facilitate the dissociation of $G\alpha_{GDP}$ from $G\beta\gamma$ (Rishal *et al.* 2003). The effects of $G\alpha$ on N-VDCCs are not well understood although they are clearly less robust than those caused by $G\beta\gamma$ (Herlitze *et al.* 1996; Ikeda, 1996); we cannot exclude the possibility that $G\alpha_{GDP}$ or $G\alpha_{GTP}$ may additionally regulate the channel. A synergistic effect of $G\beta\gamma$ and some unknown direct action of Na^+ on N-VDCCs also cannot be excluded at present.

Changes in $[G\beta\gamma]$ caused by changes in $[Na^+]_i$ are probably very mild. Biochemical measurements suggest that Na^+ causes only a 2- to 3-fold decrease in the affinity of binding between $G\alpha$ and $G\beta\gamma$ (Rishal *et al.* 2003). Kinetic modelling based on Na^+-induced activation of GIRK channels suggests that the Na^+-induced rise in $[G\beta\gamma]$ is about 15–30% over the basal level, much less than that typically induced by activation of GPCRs (data not

shown). While using N-VDCCs as 'biosensors' of [G$\beta\gamma$], we found that current amplitude and VD facilitation are more sensitive indicators of moderate changes in [G$\beta\gamma$] than the kinetic slowing and the shift in the *I–V* curve (Figs 1 and 2). One nanomolar ACh caused a 40% inhibition of I_{Ba}, correlated with a substantial change in facilitation ($\Delta_{facilitation}$ of ~20%), whereas the kinetic slowing and the shift in the *I–V* curve, though measurable, were on the verge of detection. Low doses of coexpressed G$\beta\gamma$, which caused an increase in facilitation of 20–22%, also caused only slight acceleration of activation of I_{Ba}. These phenomena are qualitatively and quantitatively similar to those caused by increased $[Na^+]_i$. The effects of depletion of intracellular G$\beta\gamma$ by cβARK on facilitation and $t_{90\%}$ in oocytes were also comparable to those induced by depletion of Na^+. We propose that Na^+ entry causes only small changes in free [G$\beta\gamma$] which cause little kinetic slowing or shift in *I–V* curve, but already suffice to cause a meaningful inhibition of N-VDCCs, by 20–40% in *Xenopus* oocytes and in hippocampal neurones. A parallel clear change in the extent of VD facilitation ($\Delta_{facilitation}$ of 15–30%) is always observed.

If all the Na^+-induced inhibition of N-VDCCs in oocytes is mediated by G$\beta\gamma$, should not the G$\beta\gamma$-induced inhibition be fully removed by depolarizing prepulses? Not necessarily. The extent of removal of inhibition depends on the parameters of depolarizing prepulse and interpulse interval, and on the concentration of G$\beta\gamma$ (Zamponi & Snutch, 1998*b*; Herlitze *et al.* 2001). Incomplete relief of G_o-coupled, GPCR-induced inhibition of N-VDCCs by prepulses is often observed both in neurones and in *Xenopus* oocytes (Ikeda, 1991; Canti *et al.* 2000). In the oocytes, the depolarizing prepulse relieved only 25–40% of inhibition caused by ACh. Very similar relief of inhibition, of 25–33%, was attained by depolarizing prepulses after TS-induced Na^+ influx in oocytes, and after NMDA-induced Na^+ influx in neurones (Figs 3 and 6). These results further support the hypothesis that a similar mechanism (i.e. an increase in [G$\beta\gamma$]) underlies Na^+- and GPCR-induced inhibition of N-VDCCs.

Potential physiological significance

The physiological impact of Na^+-induced modulation of N-VDCCs will crucially depend on two parameters: the dynamic range of $[Na^+]_i$ needed for regulation of N-VDCCs, and the time course of modulation. In hippocampal neurones, the VD facilitation, absent at 0 mM $[Na^+]_i$, was well developed at 20 mM $[Na^+]_i$. The extent of facilitation, ~18%, was close to that caused by NMDA (~14%). It appears that in *Xenopus* oocytes, the pathway that leads to inhibition of N-VDCCs is already operational at resting $[Na^+]_i$ because: (1) depletion of resting $[Na^+]_i$ affected N-VDCCs, and (2) the TS-induced Na^+ influx caused a significantly stronger inhibition of I_{Ba} when Na^+ was previously depleted from the cell, than on the background of resting $[Na^+]_i$. Therefore, we estimate that the resting $[Na^+]_i$, ~10 mM, lies close to the midpoint of the dynamic range of $[Na^+]_i$ that inhibits N-VDCCs. The dose dependency of the Na^+ effect in perfused neurones (Fig. 5*C*) supports this notion; the net change in facilitation in 10 mM Na^+ is above 10% (compared to a 'negative' facilitation in 0 mM Na^+) and becomes highly significant at 20 mM Na^+. The Na^+-dependent dissociation of G$\alpha\beta\gamma$ and the slow Na^+ regulation of GIRK channels both occur in the same range, between 5 and 40 mM $[Na^+]_i$ (Rishal *et al.* 2003).

Such changes in $[Na^+]_i$ are common in neurones. After short periods of synaptic activity (stimulation at 50–100 Hz for 0.1–1 s), $[Na^+]_i$ reached ~30 mM in most dendritic spines. In apical dendrites of CA1 hippocampal pyramidal cells, a train of three to five *postsynaptic* action potentials led to an elevation of 8–13 mM above basal $[Na^+]_i$ (Rose & Konnerth, 2001), doubling the resting $[Na^+]_i$. These relatively mild changes are well within the dynamic range of Na^+ concentrations that regulate N-VDCCs. Less information is available regarding changes in presynaptic $[Na^+]_i$. However, measurable increases in Na^+ levels have been reported in nerve termini of cerebellar granule cells, following short periods of activity, although the absolute values of $[Na^+]_i$ have not been estimated (Regehr, 1997).

The regulation of G$\alpha\beta\gamma$ dissociation by Na^+ is much slower than receptor-induced dissociation, taking several tens of seconds (Rishal *et al.* 2003). Such long-lasting changes in $[Na^+]_i$ most probably do take place in neurones. In presynaptic nerve termini, following 5–40 action potentials (at 100 Hz), increased $[Na^+]_i$ slowly decayed to resting levels, with two time constants of 6–7 s and 2–3 min (Regehr, 1997). Dendritic $[Na^+]_i$ transients caused by a mere 200 ms train of five postsynaptic action potentials last for 5–10 s, far longer than the electrical activity itself (Rose & Konnerth, 2001). The presence of glutamate in the synaptic cleft following periods of extensive activity may last for tens of seconds; Na^+ influx via glutamate-gated channels and neurotransmitter transporters is expected to last at least as long as the transmitter is present. Recent work shows that changes in $[Na^+]_i$ caused by trains of presynaptic action potentials in dendrites of cerebellar Purkinje neurones last for tens of seconds and spread over substantial areas (Kuruma *et al.* 2003). We conclude that

both the magnitude and the duration of changes in $[Na^+]_i$, caused by electrical activity or by certain neurotransmitters in neurones, are sufficient to modulate N-VDCCs via the $G\beta\gamma$-dependent mechanism. Since this regulation is slow, it is probably not involved in robust signal transduction, but in the fine-tuning of cellular processes. It is tempting to speculate that the Na^+-induced changes in $[G\beta\gamma]$ might affect long-lasting changes in excitability, or those forms of neuronal plasticity that are crucially dependent on postsynaptic spikes, especially paired with activation of NMDA receptors (reviewed by Linden, 1999).

Many effectors of Na^+ (Na^+-coupled transporters and pumps, NMDA receptor channels, voltage- and Na^+-activated K^+ channels, GIRK channels by the direct Na^+-binding pathway) show little response to resting levels of Na^+, because the EC_{50} for Na^+ is in the range of 30–40 mM. In these cases, Na^+ may be acting as a second messenger: its concentration, tightly controlled in the resting cell by the Na^+–K^+-ATPase, transiently rises upon the appearance of a specific signal (excitatory neuronal activity), to regulate such effectors. In comparison, the regulation of N-VDCCs described here, and the slow, $G\beta\gamma$-mediated activation of GIRK channels (Rishal *et al.* 2003), may already be operational at resting $[Na^+]_i$. Other examples of similar 'high affinity' modulations by Na^+ (EC_{50} of about 10 mM) are inhibition of nucleoside diphosphate kinase (Marshall *et al.* 1999) and inhibition of the epithelial amyloride-sensitive Na^+ channels by a G protein- and ubiquitin-dependent mechanism (Ishibashi *et al.* 1999). Such reactions may operate in servo-type regimes, that sensitively respond to bidirectional changes in intracellular Na^+ concentration.

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Acknowledgements

We thank the colleagues who kindly provided the materials and the original cDNA constructs: Ravi Iyengar (QEHA), Y. Mori (α_{1B}), E. Reuveny (cβARK), M. I. Simon (G protein subunits), H. A. Lester (NaIIA-α) and E. Liman (pGEM-HE). We are grateful to Ilana Lotan, Bernard Attali, Wolfgang Schreibmayer, Cristine Rose and Eitan Reuveny for helpful suggestions. This work was supported by grants from Israel Basic Research Fund and the US-Israel Binational Science Foundation to N.D.

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