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Modulation of Ca^{2+} Channels by PTX-Sensitive G-Proteins Is Blocked by *N*-Ethylmaleimide in Rat Sympathetic Neurons

Mark S. Shapiro, Lonnie P. Wollmuth,[a] and Bertil Hille

Department of Physiology and Biophysics, University of Washington School of Medicine, Seattle, Washington 98195

The actions of *N*-ethylmaleimide (NEM), a sulfhydryl alkylating agent, on G-protein–mediated inhibition of N-type Ca^{2+} channels in adult rat superior cervical ganglion (SCG) neurons were studied using whole-cell voltage clamp. In SCG neurons, inhibition of I_{Ca} occurs by at least three separable pathways: one pertussis toxin (PTX) sensitive and voltage dependent, and two PTX insensitive and voltage independent. NEM blocked PTX-sensitive inhibition nearly completely, with only small effects on PTX-insensitive inhibition. Somatostatin inhibition is completely PTX sensitive and was wholly blocked by a 120 sec exposure to 50 μM NEM, with shorter exposure times producing a less complete block. Inhibition of I_{Ca} by norepinephrine (NE) is approximately half PTX sensitive and was also approximately half NEM sensitive. One component of muscarinic inhibition is PTX insensitive, voltage independent, and mediated by a diffusible cytoplasmic messenger; this pathway was largely spared by NEM treatment. Another pathway is also PTX insensitive and voltage independent, used by substance P, and was also largely NEM insensitive. Hence, in SCG neurons, NEM selectively inactivates PTX-sensitive G-proteins. We also find evidence that the PTX-insensitive action of NE is distinct from the other PTX-insensitive pathways, and therefore assign it to a fourth signaling pathway.

[Key words: G-proteins, Ca^{2+} channels, N-ethylmaleimide, signal transduction, sympathetic neurons]

Heterotrimeric GTP-binding proteins (G-proteins) mediate many signaling pathways, transducing external signals into intracellular actions. Cloning has identified at least 15 different genes encoding G-protein α subunits (Simon et al., 1991), suggesting a multiplicity of signaling pathways. In neurons, many neurotransmitters modulate Ca^{2+} and K^+ channels via G-proteins acting either directly or through second messengers (for reviews, see Anwyl, 1991; Hille, 1992). One widely observed pathway uses G-proteins of the G_o/G_i class, which are sensitive to pertussis toxin (PTX) (Milligan et al., 1988). PTX-sensitive G-proteins are also uncoupled from receptors by *N*-ethylmaleimide (NEM), a sulfhydryl alkylating agent (Jakobs et al., 1982). In bullfrog atrial cells, for example, NEM blocks muscarinic actions that use PTX-sensitive G-proteins, while sparing β-adrenergic actions that use G_s (Nakajima et al., 1990). If NEM were a selective test for G_o/G_i involvement, the need for PTX treatment of cell cultures would be obviated, allowing rapid identification or disruption of G_o/G_i actions in acutely isolated cells. However, the specificity of NEM in targeting G_o/G_i is unknown since it has not been tested in a system with a large sample of PTX-sensitive and -insensitive signaling pathways.

Our laboratory and others have distinguished multiple G-protein–mediated pathways that influence the activity of Ca^{2+} channels in neurons of the rat superior cervical ganglion (SCG). Voltage-gated N-type Ca^{2+} channels in these noradrenergic sympathetic neurons (Plummer et al., 1989; Regan et al., 1991; Mintz et al., 1992; Boland et al., 1994) are inhibited by norepinephrine (Galvan and Adams, 1982; Lipscombe et al., 1989; Song et al., 1989), muscarinic agonists (Wanke et al., 1987; Song et al., 1989; Beech et al., 1991, 1992; Bernheim et al., 1991, 1992), somatostatin (Ikeda and Schofield, 1989; Beech et al., 1991; Shapiro and Hille, 1993), substance P (Shapiro and Hille, 1993), prostaglandin E_2 (Ikeda, 1992), adenosine (Zhu and Ikeda, 1993), neuropeptide Y (Plummer et al., 1991; Foucart et al., 1993), angiotensin II (Shapiro et al., 1994a), and pancreatic polypeptide (Foucart et al., 1993; Wollmuth et al., in press). Each transmitter uses one or two distinct intracellular signaling mechanisms. Altogether, three pathways have been defined so far using the criteria of PTX sensitivity, voltage dependence of the inhibition, requirement for a diffusible second messenger, and sensitivity to intracellular Ca^{2+} chelators (Beech et al., 1991, 1992; Bernheim et al., 1991; Shapiro and Hille, 1993). One pathway is PTX sensitive and voltage dependent, exemplified by actions of somatostatin. The two others are PTX insensitive and voltage independent. They are distinguished in that one, used by muscarinic agonists acting via M_1 receptors and angiotensin II (angioII), uses a diffusible second messenger and is inhibited by intracellular Ca^{2+} chelators such as 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA), while the other is membrane delimited, not BAPTA sensitive, and used by substance P (SP) and pancreatic polypeptide (PP). In this study, we also demonstrate that the PTX-insensitive component of adrenergic modulation is voltage dependent, indicating a fourth modulatory pathway. Because of this rich array of relatively well-defined and separable G-protein–mediated signaling, we were able to test NEM against four different G-protein pathways.

A preliminary account of this work has been presented in abstract form (Shapiro et al., 1994b).

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Correspondence should be addressed to Dr. Bertil Hille, Department of Physiology and Biophysics, SJ-40, University of Washington, G-424 HSB, Seattle, WA 98195.

[a]Present address: Max-Planck-Institut für Medizinische Forschung, Abteilung Zellphysiologie, Jahnstrasse 29, D-69028 Heidelberg, Germany.

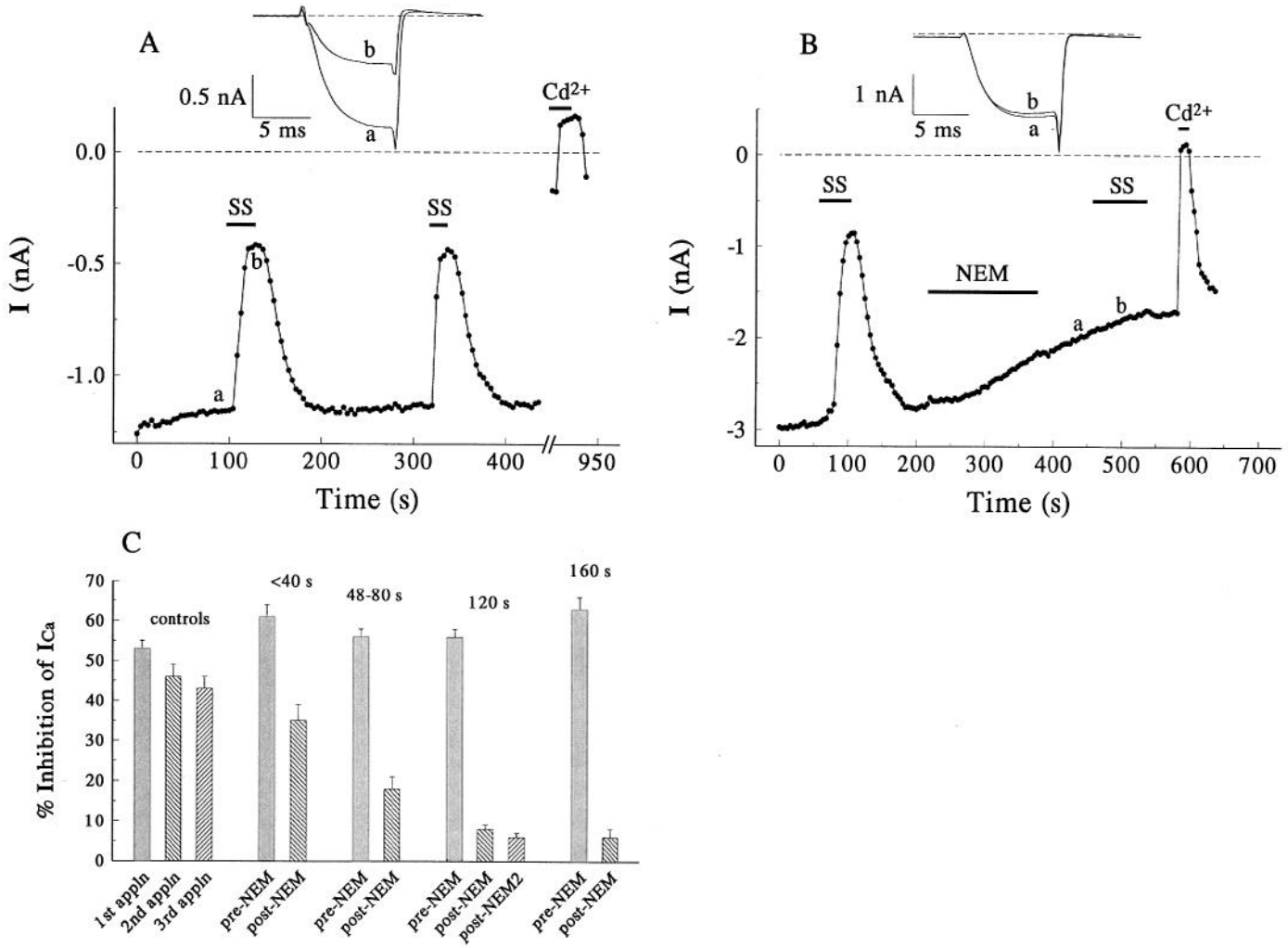

Figure 1. NEM treatment blocks inhibition of whole-cell I_{Ca} by SS. *A* and *B*, Peak current amplitudes measured near the end of a 12 msec step to 5 mV delivered every 4 sec for two different SCG neurons. *Insets* show current records recorded at the times indicated (each record is an average of three traces). Holding potential, −80 mV. Perfusion of bath solution was continuous and SS (250 nM), NEM (50 μM), and Cd^{2+} (100 μM) were added during times indicated by the *bars*. *A*, Series resistance (R_S), 2.5 MΩ; cell capacitance (C_M), 27 pF. *B*, R_S, 3.3 MΩ, C_M, 37 pF. In *A*, Cd^{2+} was applied at time 900 sec. *C*, Mean inhibition of I_{Ca} during paired applications of 250 nM SS with or without intervening exposure to 50 μM NEM for various lengths of time. The number of control cells was 30, with seven receiving a third SS application (without another NEM treatment). The average intervening time between washout of NEM and the third SS application was 11.7 min. The number of cells with NEM treatment were, *from left to right*, 7, 7, 29, 6.

Materials and Methods

Cells

Neurons were acutely dissociated from the SCG of 4–6 week, male Sprague–Dawley rats. Rats were anesthetized with methoxyflurane and decapitated. Ganglia were dissociated using methods of Bernheim et al. (1991), slightly modified by Shapiro and Hille (1993).

Solutions

For culture medium, L-15 medium was supplemented with 26 mM $NaHCO_3$, 30 mM glucose, 50 U/ml penicillin, and 50 μg/ml streptomycin, 5% fetal calf serum.

External. The Ringer solution used to record I_{Ca} was composed of (in mM) 160 NaCl, 2.5 KCl, 5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 8 glucose, 500 nM tetrodotoxin, pH adjusted to 7.4 with NaOH.

Internal. The pipette solution was composed of (in mM) 175 CsCl, 5 $MgCl_2$, 5 HEPES, 0.1 BAPTA, 3 Na_2ATP, 0.1 Na_2GTP, 0.08 leupeptin, pH adjusted to 7.4 with CsOH.

Reagents were obtained as follows: substance P and somatostatin, Peninsula; oxotremorine methiodide, Research Biochemicals Inc.; pertussis toxin, List; BAPTA, ATP, and GTP, Pharmacia LKB Biotechnology; papain, Worthington Biochemical Corporation; dispase (grade 2), collagenase (type 1), leupeptin, norepinephrine, and NEM, Sigma; and penicillin–streptomycin, L-15 medium, and fetal bovine serum, GIBCO. NEM was prepared as a stock solution in water at 50 mM and stored at −20°C.

Whole-cell recording

The whole-cell version of the patch-clamp technique (Hamill et al., 1981) was used to voltage clamp and dialyze cells at room temperature (20–26°C). Electrodes were pulled from glass hematocrit tubes (VWR Scientific Corp., Seattle, WA) and had resistances of 1–2 MΩ. Whole-cell membrane current was recorded using a List EPC-7 patch clamp. Partial series resistance compensation was employed and currents low-pass filtered at 2 kHz and stored and analyzed on an IBM-compatible computer using the BASIC-FASTLAB software and hardware package (INDEC Systems Inc., Capitola, CA). Liquid-junction potentials measured using a Beckman ceramic-junction, saturated-KCl electrode were corrected during data analysis. The junction potential between the Ringer and the standard pipette solution was −2 mV (pipette negative).

The recording chamber consisted of three connected wells cut out from a layer of Sylgard at the bottom of a 35 mm Petri dish. Cell suspensions were pipetted into the center and largest well (100–200 μl).

The two end chambers were the inflow and outflow for superfusion. Solution flow was continuous at 1–2 ml/min. Inflow to the chamber was by gravity from several reservoirs, selectable by activation of solenoid valves (Lee, Westbrook, CT). Solution exchange was complete in 20 sec or less.

The amplitude of the whole-cell I_{Ca} near 5 mV was defined as that sensitive to block by 100 μM Cd^{2+}. Results are reported as mean ± SEM. In some instances, a *t* test was used to test for statistical significance of the blocking action of NEM. Significance was assumed if $p < 0.05$.

Results

NEM blocks somatostatin inhibition of I_{Ca}

In SCG neurons, Ca^{2+} currents (I_{Ca}) reach a maximum near 5 mV with 5 mM Ca^{2+} in the bath. We measured modulation by transmitters using brief depolarizations to near 5 mV applied from a holding potential (V_{hold}) of −80 mV every 4 sec. I_{Ca} is robustly suppressed by somatostatin (SS) via a PTX-sensitive G-protein (Ikeda and Schofield, 1989; Shapiro and Hille, 1993). To test the effect of NEM on SS inhibition, we briefly applied SS twice, separated by application of either NEM or only Ringer. We almost always obtained full reversal of SS inhibition after each washout of the peptide. In Figure 1*A*, SS (250 nM) is applied twice, suppressing I_{Ca} by 57% and 55%. Although SS inhibition does desensitize (Ikeda and Schofield, 1989; Shapiro and Hille, 1993), there is hardly any reduction of inhibition for the second application due to the brief, yet maximally inhibiting, applications of SS that we used. Later in the experiment, 100 μM Cd^{2+} is added to the bath, which totally blocks I_{Ca}. In Figure 1*B*, the two applications are separated by a 160 sec exposure of the cell to 50 μM NEM. The initial application of 250 nM SS inhibited I_{Ca} by 67%, but the post-NEM SS application produced no discernable inhibition. Thus, NEM blocks SS inhibition of I_{Ca} in the SCG. As in this experiment, exposure of cells to NEM often increased the rate of "rundown" of I_{Ca}.

We wanted to find an exposure time to NEM that would optimize its effect on the pathway mediating SS inhibition, without causing excessive rundown of I_{Ca}. Figure 1*C* summarizes the relationship between exposure time to NEM and block of SS inhibition. In all experiments, we used the two-application protocol as in Figure 1*B*. We found that by 120 sec, NEM block of SS inhibition was maximal, and all subsequent NEM treatments were 120 sec in duration. We also found that a further post-NEM application of SS after a longer time did not show relief of the NEM block of SS inhibition of I_{Ca} (bar labeled "post-NEM2" in Fig. 1*C*), indicating that the actions of NEM do not reverse on this time scale.

NEM partly attenuates NE inhibition of I_{Ca}

In SCG neurons, norepinephrine (NE) inhibits I_{Ca} via two G-protein pathways, one of which is PTX sensitive (Beech et al., 1992). We found that NE-induced inhibition was usually fully reversible and so tested the effects of NEM treatment using the same pre- and post-NEM application protocol as for SS. In Figure 2*A*, initial, brief applications of SS and NE inhibited I_{Ca} by 63% and 79%, respectively. Following our standard NEM treatment (50 μM for 120 sec), SS inhibition is nearly abolished (6%). In contrast, the inhibition by NE, while attenuated, remains significant (38%). Results from many cells are summarized in Figure 2*B*. Repeated applications of NE without intervening NEM treatment produced nearly identical inhibitions, 55 ± 4% compared to 50 ± 4%. The inhibition produced by the post-NEM application of NE was about half that produced by the pre-NEM application, 24 ± 2% compared to 54 ± 3%.

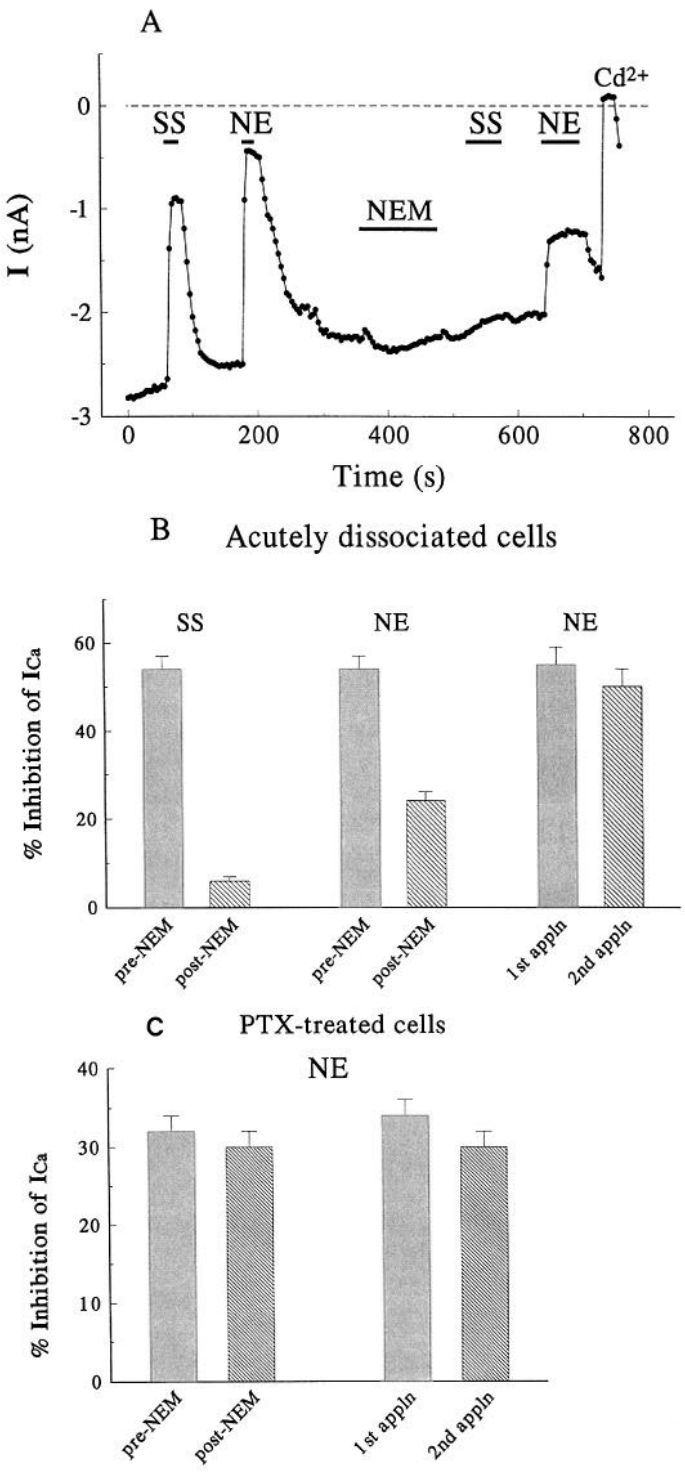

Figure 2. NEM treatment blocks one pathway of NE inhibition. *A*, Peak I_{Ca} amplitudes measured as in Figure 1. Concentrations used: SS, 250 nM; NE, 10 μM; NEM, 50 μM; and Cd^{2+}, 100 μM. NEM was bath applied for 120 sec. R_S, 3.6 MΩ, C_M, 48 pF. *B*, Average inhibition by repeated applications of SS (250 nM) or NE (10 μM) on acutely dissociated SCG neurons. The number of neurons tested was, for NEM-treated SS, 11; NEM-treated NE, 11; and control NE, 9. *C*, Average inhibition by paired applications of NE (10 μM) on SCG neurons cultured overnight with 500 ng/ml PTX. The number of neurons tested was, for control NE, 10; NEM-treated NE, 12.

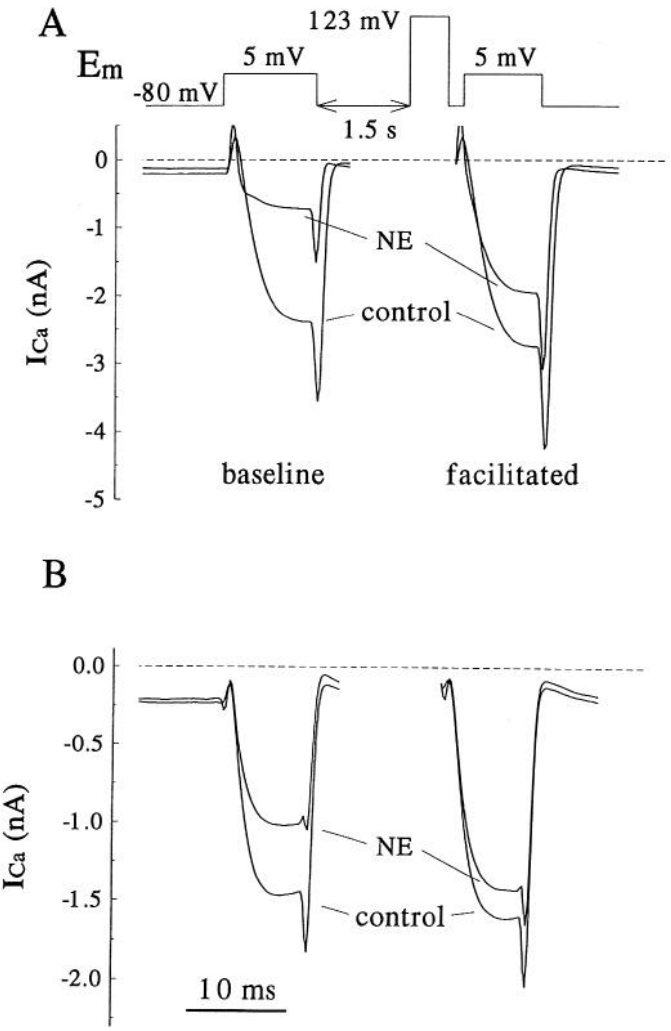

Figure 3. NEM-sensitive inhibition of I_{Ca} by NE is voltage dependent: superimposed current traces for the same cell before (*A*) and after (*B*) exposure to 50 μM NEM for 120 sec. Currents were produced by a double-pulse voltage protocol (not drawn temporally to scale); every 4 sec, an 8 msec depolarization to 5 mV was applied, followed by a 1.5 sec waiting period at −80 mV, a 24 msec prepulse to 123 mV, a 5 msec repolarization to −80 mV to close Ca^{2+} channels, and then another 8 msec depolarization to 5 mV. Superimposed currents, shown only for the two steps to 5 mV, were recorded in the absence (*control*) or presence of 10 μM NE. Currents were subtracted by those in the presence of 100 μM Cd^{2+}. Filter 2 kHz, sample interval 200 μsec. R_S, 5.2 MΩ, C_M, 28 pF.

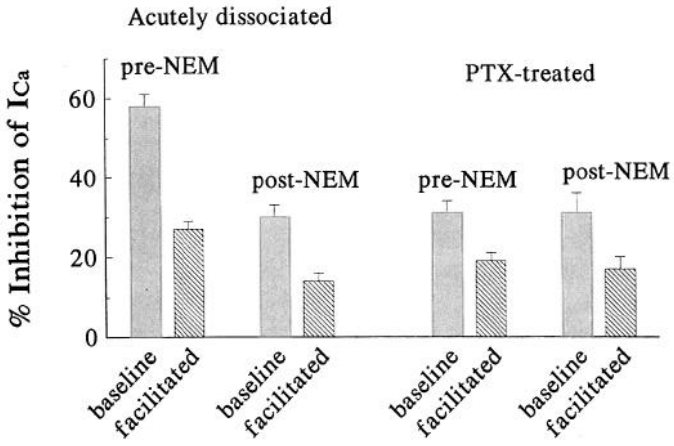

Figure 4. The NEM- and PTX-insensitive inhibition by NE is voltage dependent. *Bars* are percentage inhibition of I_{Ca} by 10 μM NE before and after exposure to NEM in acutely dissociated SCG neurons or SCG neurons cultured overnight with 500 ng/ml PTX. The number of neurons were, for acutely dissociated pre-NEM, 13; post-NEM, 11; and PTX-treated pre- and post-NEM, 7.

As a control for the efficacy of the NEM treatment, tests with SS in the same cells showed that NEM nearly abolished the inhibition produced by SS (54 ± 3% vs 6 ± 1%). Thus, in contrast to SS inhibition, which is wholly NEM sensitive, NE inhibition is about half NEM sensitive.

The fraction of NE-induced inhibition that is insensitive to NEM is similar to that which is insensitive to PTX (Beech et al., 1992). To test whether NEM affects the PTX-insensitive component of NE inhibition, we cultured SCG neurons overnight with 500 ng/ml PTX. Confirming previous work, we found the PTX-insensitive component to be substantial, a suppression of I_{Ca} of 34 ± 3% (Fig. 2*C*), about half the total. This PTX-insensitive NE component, like the full NE inhibition, does not desensitize, and a second NE application suppresses the current nearly as much as the first, 30 ± 3%. Our standard NEM treatment, 50 μM for 120 sec, does not significantly reduce this PTX-insensitive inhibition, 32 ± 3% (pre-NEM) versus 30 ± 3% (post-NEM). Thus, NEM does not block PTX-insensitive NE inhibition. These results strongly suggest that the component of NE inhibition blocked by NEM is that mediated by G-proteins of the G_o/G_i class, and that the NEM- and PTX-sensitive pathways of NE modulation are the same.

The NEM- and PTX-insensitive NE pathways are voltage dependent

In SCG neurons, a major pathway for muscarinic, somatostatin, adrenergic, and other agonist modulation of Ca^{2+} channels is characterized by a strong voltage dependence of inhibition (Bean, 1989; Ikeda and Schofield, 1989; Beech et al., 1992; Shapiro and Hille, 1993). For example, SS suppresses I_{Ca} by 57% at 0 mV, but by only 18% at 120 mV (Shapiro and Hille, 1993). These observations have been modeled as a G-protein–induced shift of channels from an unmodulated to a modulated gating mode, where modulated channels open at much more positive potentials (Bean, 1989; Elmslie et al., 1990; Kasai, 1992). Furthermore, voltage-dependent inhibition of this type has been closely associated with PTX-sensitive G-proteins (for a review, see Anwyl, 1991). Beech et al. (1992) found that NE inhibition of I_{Ca} in the SCG is about half voltage dependent. As NE inhibition is also half sensitive to PTX, these data suggested that the pathway mediated by PTX-sensitive G-proteins may be responsible for the voltage-dependent component. We tested this idea directly using both NEM and PTX treatments to define the PTX-insensitive component.

To determine the voltage dependence of inhibition of I_{Ca}, our experiments measured the number of Ca^{2+} channels available to open from either V_{hold} = −80 mV or very soon after a step to a strongly depolarized potential. We used a double-pulse protocol (Ikeda, 1991; Shapiro et al., 1994a), in which I_{Ca} was elicited by a test pulse to near 5 mV either without or almost directly following a prepulse to 123 mV. Figure 3 shows such a test for NE inhibition, both before and after NEM treatment. In Figure 3*A*, inhibition by 10 μM NE was 62% for the first test pulse and 26% for the second test pulse. Hence, in this cell the

voltage-dependent fraction is 0.58. Surprisingly, after NEM treatment NE inhibition remains voltage dependent, with an inhibition of 28% for the first test pulse and 12% for the second test pulse. Thus, even with the NEM-sensitive G-proteins inactivated, the voltage-dependent fraction is 0.57, indistinguishable from the pre-NEM value. The left side of Figure 4 summarizes these data from many cells. In acutely dissociated neurons without NEM treatment, the baseline (first pulse) current was inhibited by 58 ± 3%, while the facilitated (second pulse) current was inhibited by 27 ± 2% (voltage-dependent fraction 0.54 ± 0.05), and after NEM treatment they were inhibited by 30 ± 3% and 14 ± 2%, respectively (voltage-dependent fraction 0.52 ± 0.09). We must therefore conclude that the inhibition of NE mediated by NEM-insensitive G-proteins is as voltage dependent as that mediated by NEM-sensitive ones.

Because of this surprising answer, we also tested whether the PTX-insensitive fraction of NE inhibition is also voltage dependent. These measurements were made using the same protocol as in Figure 3. As before, SCG neurons were incubated overnight with 500 ng/ml PTX. The right side of Figure 4 summarizes these results, all from PTX-treated cells. Inhibition of I_{Ca} by NE before NEM treatment is partly voltage dependent (31 ± 3% to 19 ± 2%, fraction 0.39 ± 0.06), a fractional voltage dependence only slightly less than for non-PTX-treated (acutely dissociated) cells. NEM treatment affects neither the inhibition of the baseline (first pulse) or facilitated (second pulse) current and the inhibition is still voltage dependent (31 ± 5% to 17 ± 3%, fraction 0.45 ± 0.11). By comparison, we did not observe significant voltage dependence for other PTX- and NEM-insensitive transmitter-induced inhibition of I_{Ca} (see below). We also used idazoxan, an α_2-adrenergic antagonist, to verify that all NE-induced inhibition in the SCG is indeed via adrenergic, not other, receptors, or by some other mechanism (e.g., channel block). In these experiments, inhibition of I_{Ca} by 10 μM NE was 48 ± 6%, but only 8 ± 1% when coapplied with 10 μM idazoxan (data not shown). Thus, like earlier work in SCG neurons (Song et al., 1989; Schofield, 1990), we find that nearly all the observed NE-induced inhibition of I_{Ca} uses α_2-adrenergic receptors.

Thus, the experiments using either PTX or NEM and NE give the same unexpected answer: the PTX- and NEM-insensitive modulatory pathway is almost as voltage dependent as that which is sensitive to PTX and NEM.

The PTX-insensitive muscarinic modulation of I_{Ca} is mostly NEM insensitive

In SCG neurons, muscarinic inhibition of I_{Ca} is mediated by dual G-protein pathways. The first is PTX sensitive and voltage dependent and is also used wholly by SS as well as by other transmitters in the SCG. A second muscarinic pathway uses an as yet unidentified diffusible second messenger and is PTX insensitive and voltage independent. To ascertain the effects of NEM on the latter pathway, we tested PTX-treated neurons, which will have the first pathway knocked out. Because the actions of muscarinic agonists are sometimes not fully reversible in the dialyzed whole-cell configuration, we switched from the pre- and post-NEM protocol used for tests on SS and NE to a population study in which alternate cells were treated with either NEM or only Ringer for 120 sec before applying oxotremorine-M (oxo-M), a muscarinic agonist. We also measured the voltage dependence of the inhibition by oxo-M, for cells with and without NEM treatment.

The results of these experiments are presented in Figure 5. In

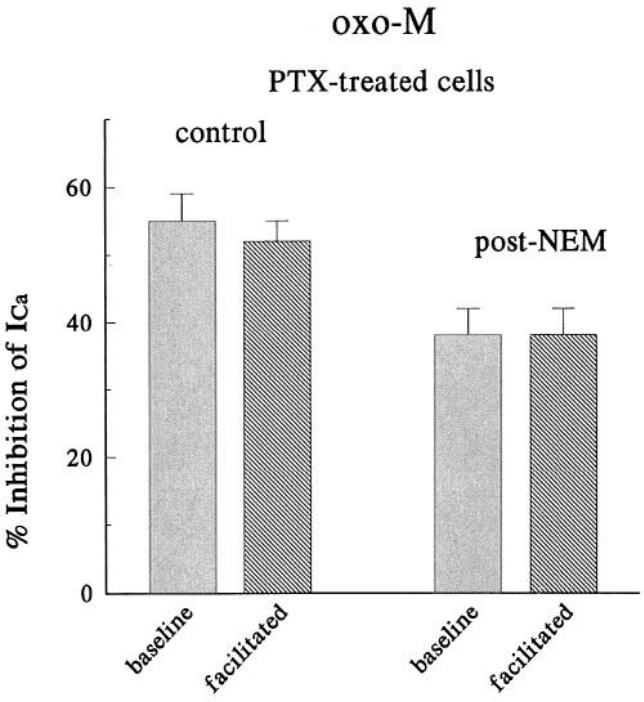

Figure 5. The PTX-insensitive muscarinic modulation of I_{Ca} is mostly NEM insensitive and not voltage dependent. *Bars* are percentage inhibition of I_{Ca} by 10 μM oxo-M in SCG neurons cultured overnight with 500 ng/ml PTX. The control and post-NEM records were obtained from different cell populations and were alternated to avoid systematic bias. The number of neurons were, for control, 8; and post-NEM, 10.

agreement with previous work (Beech et al., 1992), the PTX-insensitive component of muscarinic inhibition in control cells is all voltage independent (baseline 55 ± 4%, facilitated 52 ± 3%). In NEM-treated cells, the inhibition is partly attenuated but still fully voltage independent (baseline 38 ± 4%, facilitated 38 ± 4%). This result with oxo-M contrasts with that for NE (Fig. 4), which still showed a voltage dependence after NEM. The partial reduction by NEM of muscarinic inhibition in PTX-treated cells shows that NEM can depress signaling in more ways than its action on PTX-sensitive G-proteins.

Beech et al. (1991) found that the PTX-insensitive muscarinic pathway is blocked by intracellular Ca^{2+} chelators. Thus, with 20 mM BAPTA instead of our usual 0.1 mM in the whole-cell pipette, muscarinic inhibition is mostly voltage dependent and nearly all PTX sensitive, suggesting that only G_o/G_i-mediated muscarinic signaling can still operate. With 20 mM BAPTA in the pipette, muscarinic inhibition is typically 57 ± 8% (Shapiro et al., 1994a). When NEM was applied to 20 mM BAPTA-loaded neurons in this study, inhibition by oxo-M was only 10 ± 1% (n = 5), indicating, as expected, that NEM nearly abolishes BAPTA-insensitive muscarinic inhibition of I_{Ca}.

Inhibition of I_{Ca} by SP is NEM insensitive

SP also inhibits I_{Ca} in SCG neurons via PTX-insensitive G-proteins, but uses a distinct intracellular pathway that is completely voltage independent and does not involve a diffusible second messenger (Shapiro and Hille, 1993). We tested the effects of NEM on SP inhibition using a population study in which alternate cells were NEM treated. Figure 6 summarizes these experiments. We also tested SS in the same cells to verify the efficacy of NEM. In control, SP inhibited I_{Ca} by 27 ± 5%, where-

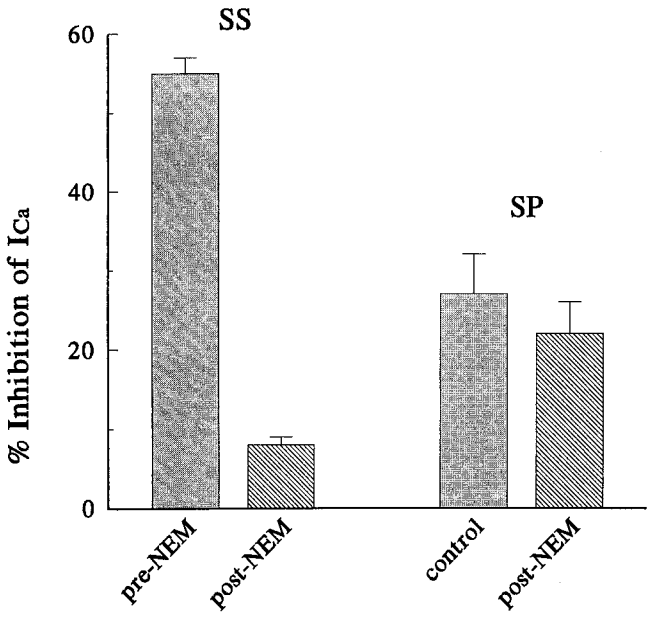

Figure 6. Inhibition by SP is NEM insensitive. Mean inhibition of I_{Ca} by 500 nM SP in acutely dissociated SCG neurons. The control and post-NEM records were obtained from different cell populations and were alternated to avoid systematic bias. The number of neurons were, for pre- and post-NEM SS, 18; control SP, 24; and post-NEM SP, 24.

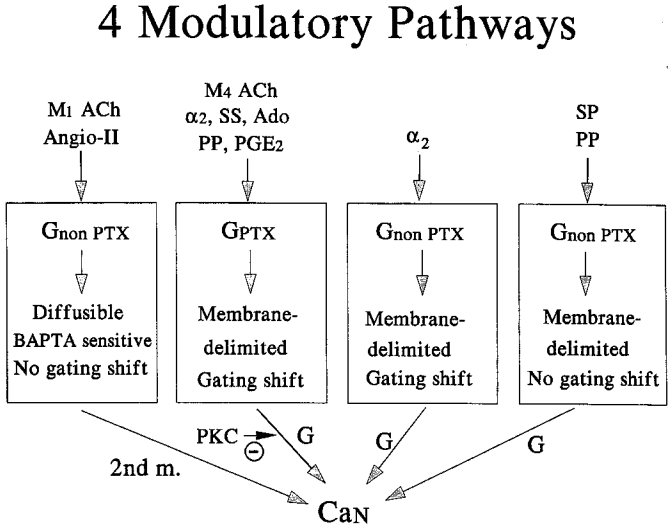

Figure 7. Schematic diagram of four modulatory pathways that target N-type Ca^{2+} channels in the SCG. Each pathway uses a G-protein and suppresses Ca^{2+} current. The pathway on the *left* uses a diffusible cytoplasmic messenger, whereas the others predict a direct interaction between a G-protein and the channel protein. *M_1 ACh,* M_1 muscarinic receptor; *M_4 ACh,* M_4 muscarinic receptor; *α_2,* α_2-adrenergic receptor; *Ado,* adenosine; *PGE_2,* prostaglandin E_2; *Ca_N,* N-type Ca^{2+} channel; *PKC,* protein kinase C. Gating shift refers to voltage dependence of the inhibition. Modified from Hille (1992). For citations, see introductory remarks.

as following NEM treatment, SP inhibited I_{Ca} by 22 ± 4%. This difference was not statistically significant at the $p = 0.05$ level. In the same cells, SS inhibition was, however, nearly abolished by NEM, 55 ± 2% versus 8 ± 1%. Thus, the signaling pathway used by SP, which is PTX insensitive, is largely spared by NEM.

Discussion

A major aim of this study was to characterize the specificity of NEM actions in a system with several different G-protein–coupled signaling pathways (Fig. 7). We find that NEM reliably blocks Ca^{2+} channel modulation by PTX-sensitive G-proteins. Inhibition of I_{Ca} by SS is wholly PTX sensitive (Ikeda and Schofield, 1989; Shapiro and Hille, 1993) and was nearly abolished by NEM (Fig. 1). Inhibition by NE is about half PTX sensitive (Beech et al., 1992) and was reduced by about half after NEM treatment. Furthermore, for NE the PTX-insensitive component was not affected by NEM (Figs. 2–4). The PTX-sensitive component of muscarinic modulation was also blocked by NEM. On the other hand, SP inhibition is wholly PTX insensitive (Shapiro and Hille, 1993) and was largely unaffected by NEM treatment (Fig. 6). The only lack of specificity for G_o/G_i over other G-proteins was the partial attenuation by NEM of the PTX-insensitive component of muscarinic inhibition. Perhaps further refinement of the NEM exposure protocol could reduce both this side effect and the acceleration of I_{Ca} rundown. A significant advantage of NEM is the rapidity of its action (2 min), which makes it possible for each cell to serve as its own control in pre- and post-NEM experiments for transmitters whose effects are reversible and repeatable.

Our data are in accord with the conclusion that NEM disrupts signaling mediated by G_i and G_o. NEM has been shown to alkylate several cysteines on G_i and G_o, which blocks coupling to receptors (Winslow et al., 1987). Interestingly, although the cysteine that is the target of ADP ribosylation by PTX is not among those NEM alkylates, NEM also prevents subsequent ADP ribosylation by PTX (Winslow et al., 1987; Shinoda et al., 1990). NEM has been used in other systems to disrupt PTX-sensitive ion channel modulation. In N18 neuroblastoma cells, NEM blocks PTX-sensitive cannabinoid inhibition of N-type calcium channels. In addition, "prepulse facilitation," which presumably results from relief of tonic inhibition by PTX-sensitive G-proteins, is reduced by >80% by NEM in these cells (Mackie et al., 1993). In SCG neurons, PP also inhibits I_{Ca} via parallel PTX-sensitive and -insensitive pathways. For PP as well, NEM treatment abolishes the former pathway, and spares the latter one (Wollmuth et al., in press). NEM also blocks muscarinic activation of the G_k-activated K^+ channel in heart using a protocol similar to ours (Nakajima et al., 1990; Ito et al., 1991). Likewise, NEM occludes the activation (presumably via G_i) of an inwardly rectifying K^+ channel by muscarinic and cannabimimetic agonists in AtT20 cells (K. Mackie, personal communication).

Binding studies have shown that NEM uncouples a number of G-protein–coupled receptors from G_o/G_i at roughly similar concentrations to ours, with higher concentrations sometimes also disrupting agonist–receptor binding. NEM at 5 μM (15 min, 4°C) uncouples μ-opioid receptors from G_o/G_i, and millimolar NEM concentrations disrupt agonist–receptor binding (Larsen et al., 1981; Ueda et al., 1990). α_2-Adrenergic receptors are uncoupled from G_o/G_i by NEM at 1 mM (25°C, 5 min; Jakobs et al., 1982). Kitamura and Nomura (1987) found that 50 μM NEM (4°C, 30 min) selectively uncouples these receptors from G_o/G_i, while 500 μM NEM disrupts agonist–receptor binding. NEM uncouples dopamine receptors from G-proteins with an EC_{50} of 7 μM, and higher concentrations (IC_{50} = 1.2 mM) disrupt agonist–receptor binding (Kilpatrick et al., 1982). Similarly, NEM (100 μM, 4°C, 30 min) uncouples $GABA_B$ receptors (Asano and

Ogasawara, 1986) and muscarinic receptors (60 μM, 4°C, 30 min; Harden et al., 1982) from G-proteins.

Thus, since NEM may act on cysteines of receptors and other signaling enzymes at somewhat higher concentrations and longer exposures than we used here, some caution is warranted in using NEM. The slight attenuation of angioII (Shapiro et al., 1994a) and PTX-insensitive muscarinic inhibition by NEM (Fig. 5) may be due to such a reduction in functional angioII and M_1 receptors. Nevertheless, we do find significant selectivity, since the occluding actions of NEM on signaling by SS, NE (present results), and PP (Wollmuth et al., in press) closely parallel those of PTX in SCG neurons.

An unexpected result of this work is the voltage dependence of the PTX- and NEM-insensitive component of adrenergic inhibition of I_{Ca}. We measured voltage dependence as the relief of inhibition by a strongly depolarizing prepulse. For NE, the fractional relief was only slightly less for the PTX-insensitive, and not less for the NEM-insensitive, inhibition than for the PTX- and NEM-sensitive inhibitions, respectively. This was a surprising result since the literature associates voltage-dependent inhibition of Ca^{2+} channels exclusively with PTX-sensitive G-proteins. Like adrenergic inhibition, muscarinic inhibition is about half voltage dependent and half PTX sensitive, but its PTX-insensitive component is wholly voltage independent (Beech et al., 1992). Similarly for PP, the PTX- and NEM-sensitive component is responsible for all the voltage dependence, and the PTX- and NEM-insensitive fraction is wholly voltage independent (Wollmuth et al., in press). Thus, NE modulation stands out among the others in this regard.

What possible interpretations are there of our NE data? The most intriguing conclusion is that a PTX-insensitive G-protein, that is, one not in the G_o/G_i family, also exerts a voltage-dependent inhibitory action on the N-type Ca^{2+} channel. This would be novel but not so unlikely given the proliferating variants of G-protein subunits currently being identified and the high degree of sequence similarity between them. The α_{2A}-adrenergic receptor can activate as many as four G-protein α subunits from three different subfamilies, and domain models suggest that the region on $G\alpha_{o,i}$ subunits that is acted on by PTX and interacts with receptors differs from that which interacts with effectors (for a review, see Conklin and Bourne, 1993). Furthermore, it may be that the action on Ca^{2+} channels is mediated by $\beta\gamma$ subunits. In that case, our data could be simply explained by PTX- and NEM-sensitive and -insensitive α subunits sharing a common $\beta\gamma$. Thus, there is no a priori reason why a non-G_o/G_i protein could not also activate a voltage-dependent mechanism of Ca^{2+} channel inhibition.

Another interpretation of our results is that the "PTX- and NEM-insensitive" component of NE modulation uses a subtype of the G_o/G_i family of G-proteins that is relatively insensitive to ADP ribosylation or NEM alkylation. There are at least five different species of heterotrimeric G-protein α subunits in this family (Simon et al., 1991; Clapham, 1994), and one could be less sensitive to these reagents than the others. We do feel, however, that our PTX and NEM treatments were robust and ample to block widely studied PTX-sensitive G-protein–mediated actions. For example, SS inhibition, which is very reliable and strong in the SCG, was practically abolished using our PTX and NEM protocols in nearly every cell. Thus, the species of G-protein responsible, insensitive to PTX and NEM, yet voltage dependent, seems unique.

Figure 7 summarizes the four modulatory pathways we have found to target N-type Ca^{2+} channels in large SCG neurons. The first and second pathways are the best described. The second is used partially or wholly by many transmitters in these neurons, and is very similar to that described in a variety of neurons for both N-type and P-type Ca^{2+} channels. It is mediated by G_o/G_i class G-proteins in a membrane-delimited fashion, which probably involves a direct interaction between activated G-proteins and the channel protein. This pathway is not affected by intracellular Ca^{2+} chelators (Beech et al., 1991; Shapiro and Hille, 1993), for NE completes its action in less than 600 msec (Beech et al., 1992), and is voltage dependent. This voltage dependence has been modeled as due to G-protein–induced changes in gating (Elmslie et al., 1990; Kasai, 1991; Boland and Bean, 1993) and permeation of the channel (Kuo and Bean, 1993). NEM is very effective at blocking this pathway.

The left-hand pathway in Figure 7 is used by muscarinic agonists (M_1 receptors) and angioII in the SCG. It uses PTX-insensitive G-proteins, is not voltage dependent, and is blocked by intracellular Ca^{2+} chelators. Experiments with cell-attached patches indicate that a diffusible cytoplasmic messenger is involved, but the messenger is, as yet, unidentified (see Hille, 1992). Compared to the other pathways, this one is somewhat slower in action for muscarinic agonists, and much slower for angioII. With our exposure protocol, NEM produces a small attenuation of this pathway for both agonists. Muscarinic agonists and angioII also inhibit a K^+ current, the M current, in SCG cells via a very similar signaling pathway (Beech et al., 1991; Shapiro et al., 1994a).

The right-hand pathway is not sensitive to NEM at all. Used by SP and PP in the SCG, it is also PTX insensitive and voltage independent, but does not require a second messenger and is not blocked by intracellular Ca^{2+} chelators.

The PTX- and NEM-insensitive component of adrenergic inhibition seems unique, so we propose to assign it a separate pathway. It is membrane delimited and insensitive to Ca^{2+} chelators, but though PTX and NEM insensitive it nevertheless is voltage dependent. The action of this component is also complete in less than 600 msec (Beech et al., 1992). Since the primary sympathetic neurons we study are noradrenergic, modulation of cellular function by NE is probably important at many levels; for example, secretion of transmitter, rhythmic firing, catecholamine synthesis, and gene transcription. Thus, seemingly parallel inputs into a neural system may satisfy the need for versatility in determining how to alter the pattern of synaptic activity. Further study on a molecular level may identify the G-protein and receptor subtypes mediating these signals.

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