(*28*). Finally, it will be interesting to ascertain whether the mechanism of removal of viral ligands by soluble forms of cell surface receptors is also operative for other viruses, such as for rhinoviruses treated with the soluble intercellular adhesion molecule–1 (ICAM-1) receptor (*29*).

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9. Chronically infected H9 cells were cultivated with uninfected H9 cells (1:4), and the medium was harvested 48 hours after infection. We had previously determined that this regimen was optimal for the production of virus with high infectious titers in which a large percentage of the gp120 and p24 in the cultures was virion bound (*12*). Samples of supernatant virus (100 μl) were fractionated by gel-exclusion chromatography under containment conditions on 2-ml columns of Sephacryl S-1000 (Pharmacia). Protein was eluted from the column with tris-buffered saline (TBS) (*10*), and fractions (two drops; average volume, 85 μl) were collected. The infectious HIV-1 content was assessed as described [J. A. McKeating *et al.*, *J. Gen. Virol.* **70**, 3327 (1989)]. We detected gp120 by twin-site ELISA (*10*). The capture antibody was D7324 (Aalto BioReagents, Dublin, Eire) to the conserved COOH-terminus of gp120, and bound gp120 was detected with a pool of HIV-1–positive human serum, an alkaline-phosphatase conjugated antibody to human immunoglobulin G (IgG) (SeraLab, Crawley, U.K.), and AMPAK (Novo Nordisk, Cambridge, U.K.) (*10*). The assays were calibrated with recombinant (CHO cell) gp120 (Celltech) [J. P. Moore *et al.*, *AIDS* **4**, 307 (1990)]. In some assays R1/87 rabbit antiserum (*10*) to recombinant gp120 ($HTLV\text{-}III_B$) was used instead of human HIV-1–positive serum, and similar results were obtained. Shed gp120 was shown to be present in a complex with sCD4 by detection of sCD4 bound onto antibody D7324–captured gp120 (*12, 13*); sCD4 does not interfere with the gp120 assays used. We measured p24 by twin-site ELISA with D7320 (Aalto BioReagents) as the capture antibody, alkaline-phosphatase conjugated MAb EH12E1 [R. B. Ferns *et al.*, *ibid.* **3**, 829 (1989)] as detection antibody, and AMPAK. The *gag* epitopes recognized by antibodies D7320 and EH12E1 are conserved between HIV-1 strains. The assay was calibrated with recombinant p24 (J. E. M. Gilmour *et al.*, *ibid.*, p. 717). The gp120 and p24 assays can detect approximately 3 pg of recombinant protein in 100 μl, and both are specific; recombinant gp120 is not detected in the p24 assay and vice versa. Furthermore, there was no ELISA signal when an irrelevant antibody [D7335 to the COOH-terminus of HIV type–2 (HIV-2) gp120] was substituted for the capture antibody in either assay.
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Contributions of Two Types of Calcium Channels to Synaptic Transmission and Plasticity

BRIAN EDMONDS,* MARC KLEIN,† NICHOLAS DALE,‡ ERIC R. KANDEL

In *Aplysia* sensory and motor neurons in culture, the contributions of the major classes of calcium current can be selectively examined while transmitter release and its modulation are examined. A slowly inactivating, dihydropyridine-sensitive calcium current does not contribute either to normal synaptic transmission or to any of three different forms of plasticity: presynaptic inhibition, homosynaptic depression, and presynaptic facilitation. This current does contribute, however, to a fourth form of plasticity—modulation of transmitter release by tonic depolarization of the sensory neuron. By contrast, a second calcium current, which is rapidly inactivating and dihydropyridine-insensitive, contributes to release elicited by the transient depolarization of an action potential and to the other three forms of plasticity.

CALCIUM HAS AN UNUSUAL ROLE IN nerve and muscle cells in that it is both a carrier of positive charge that contributes to excitability and an intracellular second messenger for a variety of cellular responses, including contraction and secretion (*1*). Calcium influx into most neurons occurs through at least two of three classes of voltage-dependent channels (*2–6*): an L-type channel that inactivates slowly (*7*) and is modulated by dihydropyridine compounds; and at least one of two dihydropyridine-insensitive channels, an N type that is activated by strong depolarization, or a T type activated by weak depolarization. What role does each of these channels have in the release of chemical transmitter and in synaptic plasticity?

In rat sympathetic neurons, the release of norepinephrine is primarily dependent on the N-type Ca^{2+} channel and does not appear to require the L-type channel (*8, 9*). By contrast, in dorsal root ganglion neurons from chicks, L-type channels contribute to the release of substance P (*8, 10*). However, in studies of vertebrate cells, transmitter release could not be initiated physiologically by action potentials, because dihydropyridine antagonists of L-type channels are only effective in tonically depolarized cells (*3, 10, 11*). As a result, release was elicited by prolonged depolarization of cells with a solution containing elevated concentrations of K^+. Moreover, release was monitored not by recording discrete postsynaptic potentials (PSPs) in a follower neuron but by determining the amount of labeled transmitter that accumulated in the bathing solution. As a result, the normal (fast) kinetics of release

Howard Hughes Medical Institute, Center for Neurobiology and Behavior, Columbia University, College of Physicians and Surgeons, New York, NY 10032.

*Present address: Department of Pharmacology, University College of London, London LOCIE 6BT, United Kingdom.
†Present address: Clinical Research Institute of Montreal, Montreal, Quebec, Canada, H2W 1R7.
‡Present address: Department of Zoology, University of Bristol, Bristol BS8 1UG, United Kingdom.

could not be detected. Finally, since these cultured cells were plated alone, release might have occurred from sites other than those equivalent to mature synaptic structures.

We have been able to overcome these limitations in dissociated cell culture with single *Aplysia* sensory neurons that form effective synaptic connections with single identifiable motor neurons (*12*). We found that sensory neurons have at least two distinct classes of Ca^{2+} channels: a slowly inactivating channel (comparable to the L-type channel) that is blocked by the dihydropyridine antagonist nifedipine and a rapidly inactivating channel (comparable to the N-type channel) that is insensitive to nifedipine. In sensory cells, as in the bag cells of *Aplysia* (*13*), the pharmacological block produced by the dihydropyridine antagonist was not significantly voltage-dependent. As a result, we could examine the relative roles of the currents in transmitter release elicited with an action potential and monitored from the synaptic terminals by recording the PSP produced in the target motor neuron.

To characterize the Ca^{2+} current of *Aplysia* sensory neurons, we used the whole-cell voltage-clamp technique (*14*) on cultured neurons (*15*) and evoked Ca^{2+} currents (*16*) by stepping from a holding potential of −50 mV to various test potentials (Fig. 1A). Stepping to low voltages (−20 to −10 mV) produced an inward current that showed little inactivation during the test pulse. By contrast, steps to voltages higher than 0 mV elicited currents that had a rapid phase of inactivation early in the test pulse and a much slower phase of inactivation late in the pulse. The finding that, with strong depolarizations, the Ca^{2+} current acquires a rapid phase of inactivation superimposed on a slower phase suggested that a rapidly inactivating component of current may be recruited at the higher voltages.

To separate the components of Ca^{2+} current, we utilized the dihydropyridine antagonist nifedipine, which blocks the slowly inactivating L-type current in vertebrate sensory cells (*3, 10*). A saturating dose of nifedipine (10 μM) (*17*) reduced the total inward current at all test potentials (Fig. 1B, left), both at low voltages, where the total current inactivates slowly, and at high voltages, where the rapidly inactivating component is present (*18*). Subtraction of the current obtained in the presence of nifedipine from the control current revealed a difference current that is slowly inactivating (Fig. 1C, left), similar to the L-type current in vertebrate sensory neurons (*2–4, 10*). Even at higher voltages, the nifedipine difference current (Fig. 1C, left) does not manifest the rapid phase of inactivation observed in the total current (Fig. 1, A and B).

We next searched for a compound that could block the rapidly inactivating component recruited at high voltages. We found that the tetrapeptide FMRFamide (PheMet ArgPhe-amide), which enhances the S-type K^+ current and produces presynaptic inhibition in *Aplysia* sensory neurons (*19–21*), selectively depresses the rapidly inactivating Ca^{2+} current without affecting the slowly inactivating one (Fig. 1B, right) (*22–24*). Thus, FMRFamide has no detectable effect on the Ca^{2+} current at low voltages, where the rapid phase of inactivation is absent, but inhibits the current at higher voltages (0 mV and above), where the rapidly inactivating component emerges. The difference currents (Fig. 1C, right) indicate that the current inhibited by FMRFamide at high voltages is rapidly inactivating and is distinct from the current blocked by nifedipine (*25*).

The currents blocked by FMRFamide and nifedipine were independent of each other. The action of FMRFamide on the rapidly inactivating component is not diminished by pretreatment with nifedipine, which blocks the slowly inactivating component (Fig. 2A). Despite the fact that nifedipine (5 μM) produced a mean reduction of 20 ± 3% (SEM; n = 5) of the peak Ca^{2+} current (SEM, 3.0; n = 5), the effect of 10 μM FMRFamide in the presence of nifedipine was the same as in its absence [(FMRFamide difference in nifedipine)/(FMRFamide difference in control) = 112 ± 9.5% (SEM; not significant)] (*26*).

To obtain more direct evidence for two distinct types of Ca^{2+} channels, we carried out single-channel experiments (n = 8) (*27*). Selected traces (above) and an ensemble average (below), shown in Fig. 2B, were taken from a patch containing one or two types of channels that do not appear to inactivate. By contrast, records taken from a patch containing a distinct, low-amplitude channel that tended to inactivate during the

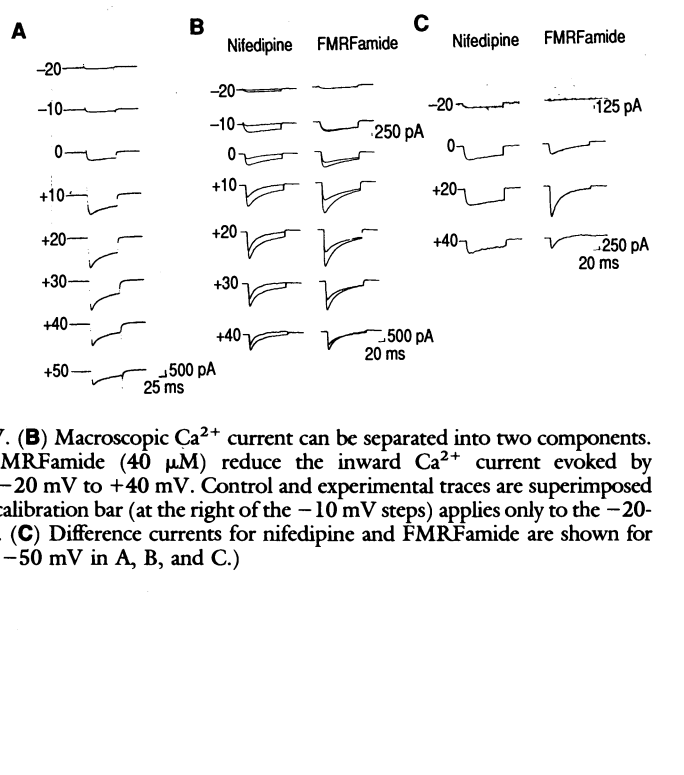


Fig. 2. (**A**) Inhibition of the Ca^{2+} current with FMRFamide is independent of the presence of the slowly inactivating, nifedipine-sensitive component. Effect of 10 μM FMRFamide alone on the Ca^{2+} current (left), and the effect of the same dose of FMRFamide after reducing the inward current with 5 μM nifedipine (right). (**B**) Single-channel data show at least two distinct classes of Ca^{2+} channels, recorded in a solution containing 110 mM Ba^{2+}. (B_1) Example of a cell-attached patch containing one and possibly two channels that contribute slowly inactivating current. Individual current traces (above) show openings (downward deflections) of two amplitudes that correspond to single-channel conductances of approximately 15 and 25 pS. An ensemble average of 34 sweeps is shown below. (B_2) Records taken from a different patch containing a very small amplitude channel for which the slope conductance could not be determined (above). The ensemble current from 22 sweeps is shown below. Membrane potentials are indicated relative to the resting potential, which is typically between −40 and −50 mV in sensory neurons.

Fig. 1. Macroscopic Ca^{2+} current of *Aplysia* sensory neurons. (**A**) Ca^{2+} currents for depolarizing voltage-clamp steps elicited from the holding potential (V_h) to test potentials ranging from −20 to +50 mV (shown at the left of each trace). The decay of the total inward current at these higher voltages could be fit, with the method of least squares, by the sum of two exponentials with time constants of approximately 18 ± 2 (SEM) and 180 ± 40 (SEM) ms, respectively (n = 4), at 15 mV. (**B**) Macroscopic Ca^{2+} current can be separated into two components. Nifedipine (10 μM) and FMRFamide (40 μM) reduce the inward Ca^{2+} current evoked by depolarizations ranging from −20 mV to +40 mV. Control and experimental traces are superimposed for each voltage. The 250-pA calibration bar (at the right of the −10 mV steps) applies only to the −20- and −10-mV current records. (**C**) Difference currents for nifedipine and FMRFamide are shown for selected test potentials (V_h = −50 mV in A, B, and C.)

voltage-clamp step are shown in Fig. $2B_2$. These data support the pharmacological and kinetic separation obtained for the macroscopic current.

Calcium influx is required for transmitter release (*28*). To determine the role of these two Ca^{2+} channels in transmitter release from sensory neurons, we cocultured one or more sensory neurons for 3 to 5 days with a motor neuron (*12*) and recorded from both the pre- and the postsynaptic cells with a microelectrode (*29*). The synaptic potential produced by an action potential in the sensory neuron was not significantly affected by either 5 or 10 μM nifedipine (Fig. 3A) (*30*). Since neither concentration of nifedipine significantly altered the mean amplitude (as percent of control) of the PSP (*31*), the data were pooled (Fig. $3A_2$) to give a value of 99.1 ± 7.9% (SEM; $t = 0.11$, not significant; $n = 9$). Thus, in these cells, the slowly inactivating current does not contribute significantly to normal transmitter release; these results are similar to those in sympathetic neurons (*9*).

Aplysia sensory neurons can undergo several forms of synaptic plasticity. We therefore next examined the role of the two components of Ca^{2+} current in three forms of plasticity: presynaptic inhibition, homosynaptic depression, and presynaptic facilitation.

Presynaptic inhibition by FMRFamide is expressed as a large reduction in the amplitude of the synaptic potential recorded in the motor neuron (Fig. 3B) (*19, 20*). The fact that FMRFamide selectively inhibits the rapidly inactivating Ca^{2+} current (Fig. 1B, right) is consistent with two ideas: (i) the rapidly inactivating component, unlike the nifedipine-sensitive component, contributes to transmitter release; and (ii) presynaptic inhibition of transmitter release from sensory neurons is at least partially mediated by modulation of this rapidly inactivating component. We cannot determine the relative importance of the inhibition of Ca^{2+} current to the inhibition of release in these experiments, because FMRFamide has multiple inhibitory actions on sensory neurons that include an enhancement of S-type K^+ current (*21*) and a direct effect on the transmitter release mechanism that is independent of changes in intracellular Ca^{2+} levels (*24, 32*). In testing whether the slowly inactivating component is required for the expression of presynaptic inhibition, we found that blocking the slowly inactivating current with nifedipine had essentially no effect on the ability of FMRFamide to inhibit transmitter release (Fig. 3C). Under normal conditions (Fig. 3B), 10 μM FMRFamide reduced the amplitude of the PSP to an average of 11 ± 7.2% of the average control value ($t = 12.4$, $P < 0.01$; $n = 5$). In the presence of 10 μM nifedipine (Fig. 3C), the same concentration of FMRFamide inhibited the PSP to an average of 6.25 ± 1.5% of the average control value ($t = 62.5$, $P < 0.01$; $n = 6$). The difference between the two groups was not statistically significant for either the average control amplitude or the average amplitude of the PSP in FMRFamide.

We then examined a second form of plasticity, homosynaptic depression, which is characterized by a progressive reduction in the amount of transmitter released from sensory neurons with successive stimulation. Using nifedipine, we found that the homosynaptic depression achieved by stimulating the sensory neuron once every 40 s was not significantly affected by blockade of the slowly inactivating current ($n = 6$) (Fig. 3D).

A third form of plasticity, presynaptic facilitation, is expressed as a large increase in the amount of transmitter released from sensory neurons in response to the application of a facilitatory transmitter such as serotonin (5-HT). Neither 5 nor 10 μM nifedipine applied to a sensorimotor connection previously facilitated with 5-HT (Fig. 4A) altered the mean amplitude of the PSP expressed as percent of the control synaptic potential in 5-HT (*33*). Pooling the data gives a value of 102.4 ± 5.5% ($t = 0.44$, not significant, $n = 8$). Thus, the expression of presynaptic facilitation is not significantly affected by reducing the slowly inactivating current (*34*). To ensure that nifedipine was effectively blocking Ca^{2+} current, we tested the effects of nifedipine on transmitter release in the presence of 5-HT while simultaneously monitoring the Ca^{2+} current to verify the action of nifedipine (*35*). Nifedipine produced a substantial reduction in Ca^{2+} current, but transmitter release was essentially unaffected (Fig. 4B). Although nifedipine reduced the peak Ca^{2+} current by an average of 34 ± 6.4%, the mean amplitude of the postsynaptic potential in nifedipine as percent of control was 99 ± 3% ($t = 0.33$, not significant, $n = 12$). Our results support the conclusion that neither normal synaptic transmission nor any of the three forms of plasticity examined above require the dihydropyridine-sensitive, slowly inactivating current.

The fact that nifedipine had no significant effect on transmitter release under a variety of conditions suggests the possibility that the total amount of Ca^{2+} entering through

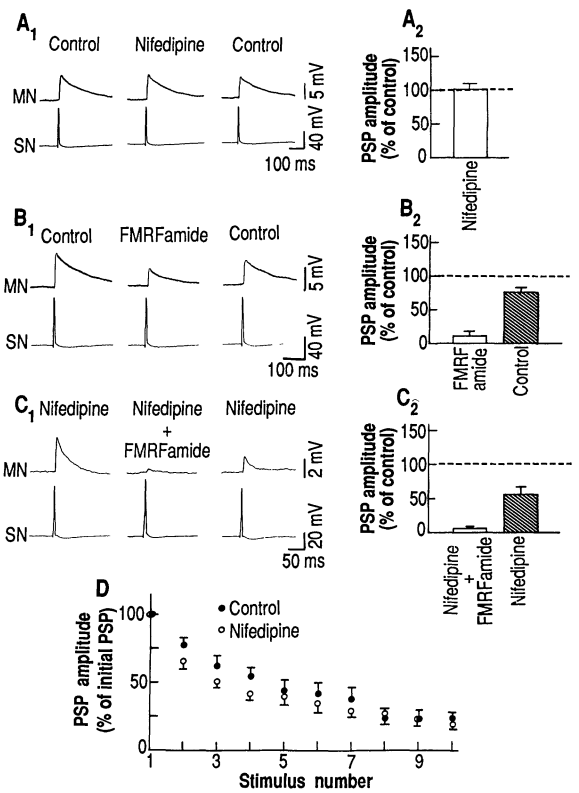


Fig. 3. (**A**) Nifedipine-sensitive component of Ca^{2+} current is not required for transmitter release. Representative records (A_1) show that 5 μM nifedipine has no significant effect on normal synaptic transmission. Pooled data for experiments with 5 and 10 μM nifedipine are shown in (A_2): the amplitude of the PSP in nifedipine is shown as a percent of the amplitude of the control PSP immediately preceding nifedipine application. (**B**) FMRFamide produces presynaptic inhibition in sensory neurons. Records illustrating the inhibitory action of FMRFamide (10 μM) are shown in (B_1). Pooled data are shown in (B_2). (**C**) The slowly inactivating Ca^{2+} current is not required for the expression of presynaptic inhibition. FMRFamide can inhibit transmitter release in the presence of nifedipine (C_1). Pooled data in (C_2) show that FMRFamide's inhibitory action is not altered by removal of the slowly inactivating Ca^{2+} current [compare with (B_2)]. [Error bars in (A), (B), and (C) denote SEMs.] The data in (B_2) and (C_2) are plotted as the average amplitude of the PSP in FMRFamide (open bars) and immediately after washout of FMRFamide (hatched bars) as a percent of the average control value preceding FMRFamide application. (**D**) Homosynaptic depression does not require the slowly inactivating Ca^{2+} current. The graph illustrates the effect of repetitive stimulation of the sensory neuron (one stimulus per 40 s) on the average PSP amplitude recorded in the follower neuron. Average amplitudes are plotted as a percentage of the average control value in the presence ($n = 6$) and absence ($n = 6$) of 10 μM nifedipine. Error bar, SEM. There was no statistically significant difference between the two groups for any of the stimulus numbers. (MN = motor neuron; SN = sensory neuron.)

voltage-dependent channels is normally large enough to saturate the release process. Thus, producing only a partial reduction in the Ca^{2+} current, with nifedipine or any other Ca^{2+} channel blocker, might not sufficiently decrease Ca^{2+} influx to a nonsaturating level. To address this problem, we produced a similar reduction in the Ca^{2+} current with the relatively nonselective blocker Cd^{2+} (*36*) and examined the effect on synaptic transmission in 5-HT (*37*). A dose of 10 μM Cd^{2+} blocked the peak Ca^{2+} current to about the same level as 10 μM nifedipine (Fig. $4C_1$). However, unlike nifedipine, 10 μM Cd^{2+} produced a 35 ± 6.9% ($t = 5.07$, $P < 0.01$, $n = 7$) reduction in the amplitude of the PSP (Fig. $4C_2$). Because other experiments, in which we monitored spontaneous miniature potentials produced by sensory neurons, indicate that Cd^{2+} acts presynaptically (*38*), these results strengthen the conclusion that the slowly inactivating current is not required to evoke transmitter release under the conditions described above.

We next investigated the role of the slowly inactivating Ca^{2+} current in the enhancement of transmitter release by tonic depolarization. Evoked transmitter release from the *Aplysia* neuron L10 can be modulated by varying the membrane potential (*39*). Tonic depolarization of L10 produces an enhancement of release, which is partially due to an increase in steady-state Ca^{2+} influx during the depolarization (*39*). This type of transmitter modulation occurs in sensory neurons and is manifest both as a change in evoked transmitter release (*40*) and as an alteration in spontaneous release—a change in the frequency of spontaneous miniature potentials (*32*). Depolarizing the sensory cell to voltages where the slowly inactivating current activates (approximately −30 mV) causes an increase in the frequency of occurrence of spontaneous miniature potentials (the mean time interval decreases), and this increase is dependent on extracellular Ca^{2+} (*32*). We suspected that the slowly inactivating Ca^{2+} current might contribute to this enhancement of release. As predicted, nifedipine reduced the increase in spontaneous release produced by depolarization. Depolarization to voltages between −30 and 0 mV reduced the mean time interval between consecutive spontaneous miniature potentials recorded in a follower neuron to an average of 35 ± 6.2% ($n = 5$) of the average control value at the resting potential (between −40 and −50 mV) (Fig. 5A), whereas nifedipine reversed this effect of depolarization to 67 ± 11% of control. The increase in the mean time interval between miniature potentials produced by nifedipine during depolarization was significantly different from the mean time interval observed during depolarization alone ($t = 4.64$, $P < 0.01$). Moreover, nifedipine did not affect the enhancement of spontaneous release by 5-HT at the resting potential (*41*), suggesting that the effect of nifedipine was specific to the change in miniature potential frequency produced by depolarization.

We also tested the role of the slowly inactivating current in depolarization-induced changes in evoked release. Depolarization of the sensory neuron to −30 mV, a potential at which the slowly inactivating current is selectively activated, was sufficient to cause an enhancement of transmitter release (Fig. $5B_2$). Moreover, this effect of

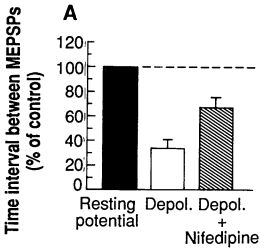
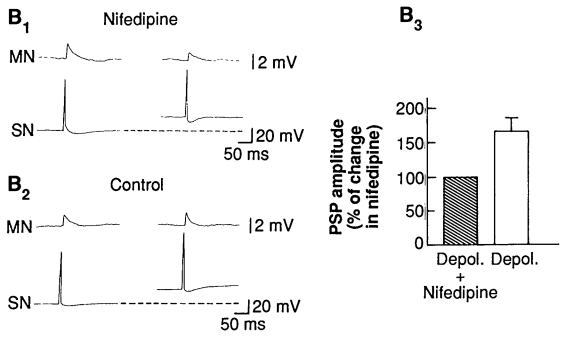


Fig. 5. The slowly inactivating current is important for the modulation of transmitter release in response to sensory neuron depolarization. (**A**) Averaged data illustrates that the reduction of the mean time interval between spontaneous miniature postsynaptic potentials (MEPSPs) with depolarization to voltages between −30 and 0 mV (open bar) as compared to control at resting potential (solid bar) is partially reversed by the application of 10 μM nifedipine (hatched bar). (**B**) The slowly inactivating current is important for depolarization-induced changes in evoked transmitter release. Presynaptic depolarization to −30 mV for 5 to 8 s before eliciting an action potential is ineffective in enhancing release in the presence of nifedipine (B_1); however, after washing (B_2), depolarization enhances release. (B_3) The average depolarization-induced change in the PSP in the absence of nifedipine as a percent of the average change in the presence of 10 μM nifedipine. (Error bar denotes SEM.)



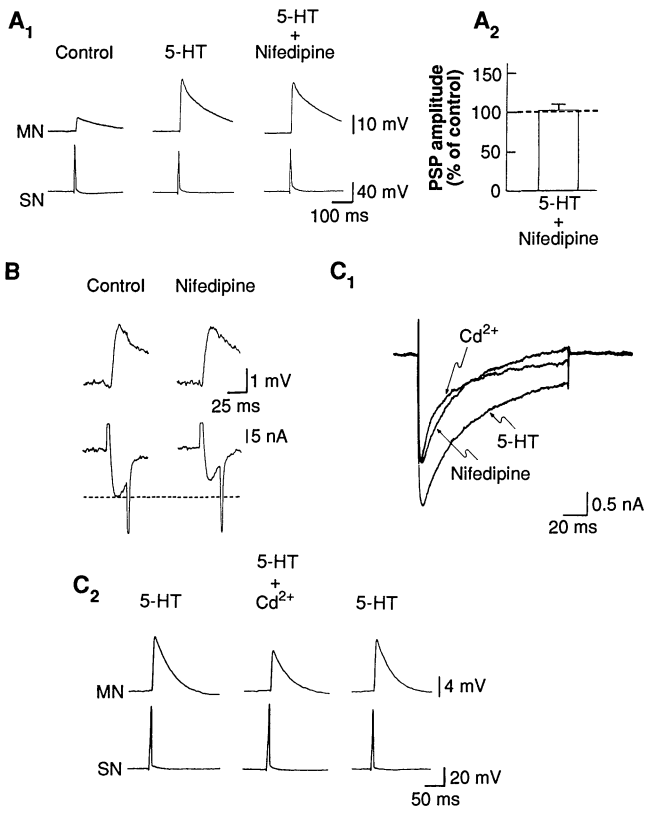


Fig. 4. (**A**) Presynaptic facilitation with 5-HT does not require the slowly inactivating Ca^{2+} current. (A_1) Blocking the slowly inactivating current with 5 μM nifedipine does not affect the amplitude of a PSP previously facilitated with 10 μM 5-HT. (A_2) Pooled data for 5 and 10 μM nifedipine show that nifedipine has no significant effect on the amplitude of the PSP, as compared to the amplitude of the PSP previously facilitated with 5-HT. (Error bar denotes SEM.) (**B**) Transmitter release from sensory neurons was elicited with depolarizing voltage-clamp steps from a V_h of −50 mV to +15 mV in the continued presence of 5-HT. Both the PSP (above; ac-coupled) and the Ca^{2+} current (below) were recorded directly. Although 10 μM nifedipine reduced the Ca^{2+} current, transmitter release was unaffected. (**C**) A partial reduction in the Ca^{2+} current with a low dose of Cd^{2+} reduces the amplitude of the PSP. Holding potential, −50 mV; test potential, +15 mV. (C_1) In the continued presence of 10 μM 5-HT, 10 μM Cd^{2+} reduces the inward Ca^{2+} current to approximately the same level as a saturating dose of nifedipine (10 μM). (C_2) Unlike nifedipine, 10 μM Cd^{2+} reduces the amplitude of the PSP. These experiments were conducted on facilitated sensorimotor connections in 5-HT.

depolarization was blocked in the presence of 10 μM nifedipine (Fig. $5B_1$). The average percent change in the amplitude of the PSP upon depolarization to −30 mV was 67% larger in control than the average percent change in 10 μM nifedipine ($t = 3.35$, $P < 0.05$, $n = 5$) (Fig. $5B_3$). Taken together, these data suggest that certain forms of plasticity may utilize a class of Ca^{2+} channels that, under other conditions, does not participate in the release process.

We find that the dihydropyridine nifedipine produces a substantial reduction in the total Ca^{2+} current; however, removal of this major component of Ca^{2+} influx has no significant effect on synaptic transmission or on three major forms of synaptic plasticity: presynaptic inhibition, homosynaptic depression, and presynaptic facilitation. In addition, 5-HT, which produces presynaptic facilitation, selectively enhances the slowly inactivating current (*34*), yet this current is not required for the expression of presynaptic facilitation. The data suggest that it is not possible to implicate modulation of a macroscopic Ca^{2+} current in an alteration of transmitter release without first identifying the different components of Ca^{2+} current and characterizing their functional roles. Any increase in Ca^{2+} influx important for presynaptic facilitation in the sensory neurons is likely to be indirectly mediated, occurring via depression of K^+ currents, which prolongs activation of the rapidly inactivating Ca^{2+} current by causing spike broadening (*42, 43*).

Why do the channels that contribute slowly inactivating current not contribute to routine release and to most forms of plasticity? These channels could be remote from the terminals so that they are unable to contribute Ca^{2+} to the release sites. However, with steady-state depolarization, the channels do contribute Ca^{2+} to effect release, suggesting either that the slowly inactivating channels are not very distant from release sites, or that prolonged Ca^{2+} influx occurring due to tonic Ca^{2+} channel activation effectively saturates local intracellular Ca^{2+} buffers, thereby allowing Ca^{2+} to diffuse over much greater distances. During an action potential, the channels simply may not remain open long enough to allow Ca^{2+} to diffuse to release sites. Regardless of the mechanism, the rapidly inactivating Ca^{2+} channels contribute to release controlled by the action potential and its modulation, whereas the slowly inactivating channels may contribute to release only when the membrane is depolarized for longer times.

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15. Pleural-pedal ganglia freshly dissected from *Aplysia californica* (50 to 100 g) were incubated at 34°C for approximately 2.5 hours in 1% protease (Sigma, Type IX) in L-15 medium (Flow Laboratories, McLean, VA) plus appropriate salts (*12*). Ganglia were then desheathed in a high-Mg^{2+} seawater solution (50% artificial seawater, 50% isotonic $MgCl_2$), and sensory cells were removed individually by suction [I. D. Dietzel, P. Drapeau, J. G. Nicholls, *J. Physiol. (London)* **372**, 191 (1986)]. After isolation, cells were transferred to plastic culture dishes (Falcon, 1006) containing modified L-15 medium without glutamine and kept at 4°C for up to 4 days until used. This protocol effectively prevented process outgrowth.
16. Calcium currents were recorded in an extracellular solution containing 460 mM tetraethylammonium chloride (TEA-Cl), 55 mM $MgCl_2$, 11 mM $CaCl_2$, 10 mM CsCl, 10 mM Hepes, 0.1 mM 3,4-diaminopyridine, pH 7.6. The patch pipette contained 444 mM CsCl, 30 mM CsOH, 6.8 mM $MgCl_2$, 2.8 mM $CaCl_2$, 10 mM bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (Bapta), 100 mM Hepes, 5 mM adenosine-5′-triphosphate (ATP), 0.1 mM guanosine-5′-triphosphate (GTP), 10 mM glutathione (reduced), 5.6 mM glucose, pH 7.2. This gives an intracellular free Ca^{2+} concentration of approximately 100 nM. Confirmation that adequate blockade of K^+ channels was achieved came from the observations that the outward tail currents (at −50 mV) observed in seawater were eliminated with these solutions and that the application of 1 mM Cd^{2+} yielded flat current records for depolarizing steps as high as +100 mV.
17. Half-maximal block of the slowly inactivating current was achieved with about 1.2 μm nifedipine, and 10 μM nifedipine reduced the current by about 95% (B. Edmonds and E. R. Kandel, unpublished data).
18. All drugs were applied with a multibarreled superfusion pipette [W. Boll and H. D. Lux, *Neurosci. Lett.* **56**, 335 (1985)], which was placed 50 to 100 μM from the cell (or pair of cells). During the recording period, the cell was superfused continuously with either the control solution (*16*) or the test solution. Both control and test solutions contained 0.1% ethanol (a vehicle for nifedipine) and 0.025% fast green (to confirm that the solutions flowed onto the cell). Neither the ethanol nor the fast green at these concentrations had obvious effects on either membrane currents or transmitter release. Test solutions contained the following compounds as indicated: nifedipine (Sigma), made from a 10 mM stock solution in absolute ethanol; FMRFamide (Peninsula); 5-HT (Sigma); $CdCl_2$.
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25. This selective inhibition of the rapidly inactivating Ca^{2+} current by FMRFamide is analogous to the relatively selective effect of norepinephrine (NE) on the N-type channel in sympathetic neurons in which NE also inhibits transmitter release [D. Lipscombe, S. Kongsamut, R. W. Tsien, *Nature* **340**, 639 (1989); M. R. Plummer, M. Kanevsky, P. Hess, *Biophys. Soc. Annu. Meet Abstr.* **57**, 302a (1990)].
26. The experiment in Fig. 2A was also performed two times with a higher concentration of FMRFamide (40 μM), which produces a larger reduction in the rapidly inactivating current. The results of these experiments were qualitatively identical to those with the lower dose.
27. Single-channel experiments were performed in the cell-attached configuration. The pipette solution contained 410 mM TEA-Cl, 110 mM $BaCl_2$, 10 mM Hepes, 0.5 mM 3,4-diaminopyridine, pH 7.6. Recording protocol was the same as for the whole-cell experiments (*14*), except that pipette resistances were higher (5 to 8 megohms), and current traces were filtered at 1.5 kHz (at −3 dB).
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29. Electrophysiological recordings were made with standard techniques. All data were stored on FM tape and analyzed off-line. Microelectrodes were filled with 2.5 M KCl and had resistances ranging from 10 to 15 megohms. The recording solution consisted of artificial seawater (460 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, and 55 mM $MgCl_2$, buffered to pH 7.6 with 10 mM Hepes) supplemented with glucose, amino acids and vitamins [M. Eisenstadt *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3371 (1973)]. For all transmitter release experiments, action potentials were elicited once every 40 s in the sensory neuron.
30. We failed to observe any changes in action potential duration with nifedipine; however, occasionally we found that nifedipine produced a small (0.25 to 2

mV) reduction in the afterhyperpolarization (AHP). Using a low concentration of TEA (1 mM), which preferentially blocks the Ca^{2+}-dependent K^+ current in *Aplysia* sensory neurons, we could mimic the change in the AHP without any observable effect on transmitter release ($n = 6$).
31. In 10 μM nifedipine, the average amplitude of the PSP was 89.1 ± 9.0%, not significantly different from the average control value ($t = 1.21$; $n = 5$). When pooled with the data obtained from experiments with 5 μM nifedipine, the results were still not significant (see text).
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33. In the presence of 10 μM 5-HT, application of 10 μM nifedipine did not significantly alter the average amplitude of the synaptic potential as percent of the average control value in 5-HT (98.0 ± 3.2%; $t = 0.63$; $n = 4$). Moreover, when pooled with the data from experiments with 5 μM nifedipine, the result was still not significant (see text).
34. Because FMRFamide, an agent that causes presynaptic inhibition, reduces Ca^{2+} current, we might suspect that 5-HT, which causes presynaptic facilitation, would enhance this current. Although modulation of Ca^{2+} current by 5-HT was not detected previously (*42*), the isolation of the Ca^{2+} current with the whole-cell recording technique (*14*) allowed us to detect a modulation of the Ca^{2+} current by 5-HT. Contrary to expectation, 5-HT does not modulate the rapidly inactivating current. Rather, it selectively enhances a current that is similar and possibly identical to the nifedipine-sensitive, slowly inactivating component of current (B. Edmonds, unpublished data). Despite this enhancement by 5-HT, blockage of this current with nifedipine does not significantly affect the expression of presynaptic facilitation. Therefore, modulation of a given species of Ca^{2+} current does not necessarily imply that modulation of that particular current is important for transmitter release.
35. The sensory neuron was voltage-clamped with a single microelectrode, and release was monitored with an electrode in the follower cell. We unmasked the Ca^{2+} current of the presynaptic cell by perfusing with a TEA-containing, Na^+-free solution (Figs. 1 and 2A), to which we added 100 mM Na^+ to carry the postsynaptic current.
36. Although we did not rigorously test the selectivity of Cd^{2+} as a Ca^{2+} channel-blocker in sensory neurons, it was clear that both of the components that we had identified were at least partially blocked by 10 μm Cd^{2+}. For example, Fig. $4C_1$ shows that the sustained current at the end of the pulse is almost completely blocked by nifedipine, but is only partially reduced by Cd^{2+}. By contrast, both Cd^{2+} and nifedipine inhibited the peak current to the same extent, suggesting that Cd^{2+} also reduced the rapidly inactivating component.
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Autonomous Developmental Control of Human Embryonic Globin Gene Switching in Transgenic Mice

NATACHA RAICH, TARIQ ENVER, BETTY NAKAMOTO, BETTY JOSEPHSON, THALIA PAPAYANNOPOULOU, GEORGE STAMATOYANNOPOULOS*

The mechanisms by which expression of the β-like globin genes are developmentally regulated are under intense investigation. The temporal control of human embryonic (ε) globin expression was analyzed. A 3.7-kilobase (kb) fragment that contained the entire human ε-globin gene was linked to a 2.5-kb cassette of the locus control region (LCR), and the developmental time of expression of this construct was studied in transgenic mice. The human ε-globin transgene was expressed in yolk sac–derived primitive erythroid cells, but not in fetal liver or bone marrow–derived definitive erythroid cells. The absence of ε gene expression in definitive erythroid cells suggests that the developmental regulation of the ε-globin gene depends only on the presence of the LCR and the ε-globin gene itself (that is, an autonomous negative control mechanism). The autonomy of ε-globin gene developmental control distinguishes it from the competitive mechanism of regulation of γ and β-globin genes, and therefore, suggests that at least two distinct mechanisms function in human hemoglobin switching.

IN HUMANS, PRIMITIVE ERYTHROPOIESIS takes place in the blood islands of the embryonic yolk sac. Definitive erythropoiesis originates in the fetal liver and shifts to the bone marrow at around the time of birth. At different stages of ontogeny, human erythroid cells contain different β-globin chains, ε chain synthesis is restricted to embryonic cells, $^{G}\gamma$ and $^{A}\gamma$ chains predominate in the fetal stage, and the δ and β chains are maximally produced in the adult. The corresponding genes are arranged in a single locus in the order that they are expressed during ontogeny: 5′-ε, $^{G}\gamma$, $^{A}\gamma$, δ, β-3′.

Division of Medical Genetics, University of Washington, Seattle, WA 98195.

*To whom correspondence should be addressed.

The activation and high-level transcription of the β-globin locus are thought to be dependent on the locus control region (LCR). This segment of DNA, which is located 6 to 20 kb upstream of the ε gene, is characterized by a series of erythroid-specific hypersensitive sites (*1*). Reverse genetics has shown that the LCR confers high-level, position-independent, copy number–dependent expression on a linked β-globin gene in transgenic mice (*2*). The LCR effect appears to be dominant, as it confers high-level, erythroid-specific expression on a number of cis-linked heterologous erythroid (*3*), housekeeping (*4*), and nonerythroid (*5*) genes. Experiments in transgenic mice show that linkage of the LCR to the individual human fetal (γ) or adult (β) globin genes results in their expression at all stages of mouse development (*6, 7*). Correct developmental regulation is restored in constructs that contain both the γ and β genes. This suggests that the fetal-to-adult globin gene switch is mediated by a reciprocal mechanism in which the genes compete for the influence of the LCR (*7*).

To investigate the nature of the developmental control of the human embryonic globin gene, we linked the human ε-globin gene to the LCR and analyzed its stage-specific expression in transgenic mice. A 3.7-kb Eco RI fragment that contained the entire human ε-globin gene (*8*) was linked to a 2.5-kb cassette (*9*) that contained hypersensitive sites I to IV of the human

Table 1. Human embryonic gene expression in the blood of transgenic mice. Human ε expression could not be quantitated in the liver since it could not be detected in either long exposure or by scintillation counting. The absence of human ε expression in definitive erythroid cells was further confirmed by RNase protection analysis of the blood of adult μLAR transgenic ε mice. In three experiments ε expression could not be detected even when ten times the normal amount of RNA was used in the analyses. The change in expression from 29% at d11 to 0.28% at d14 represents a reduction in human ε expression by 100-fold during development. As shown in Fig. 4, similar reductions are seen in the endogenous mouse embryonic globin genes (ζ, ϵ^{y}, βh1).

Blood sample date	Gene expression (cpm)		Gene expression human ε/(mouse α + ζ)	
	Human ε	Mouse α + ζ	Percent	Corrected for gene copies
d11	3,605	24,691	14.6	29.2
d14	65	45,727	0.142	0.284