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11. The low-resolution EI mass spectrum (MS) measured on JEOL DX-303 HF exhibited the following peaks: EI/MS: mass-to-charge ratio *m/x* (%) 302 (100; M+), 284 (11; M - H_2O), 271 (23; M CH_2OH), 253 (2), 241 (4), 228 (4), 215 (6), 197 (6), 187 (9), 173 (10), 159 (15), 147 (17), 133 (15), 121 (23), 105 (20).
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Participation of Postsynaptic PKC in Cerebellar Long-Term Depression in Culture

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Long-term depression (LTD) in the intact cerebellum is a decrease in the efficacy of the parallel fiber-Purkinje neuron synapse induced by coactivation of climbing fiber and parallel fiber inputs. In cultured Purkinje neurons, a similar depression can be induced by iontophoretic glutamate pulses and Purkinje neuron depolarization. This form of LTD is expressed as a depression of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA)–mediated current, and its induction is dependent on activation of metabotropic quisqualate receptors. The effect of inhibitors of protein kinase C (PKC) on LTD induction was studied. Inhibitors of PKC blocked LTD induction, while phorbol-12,13-diacetate (PDA), a PKC activator, mimicked LTD. These results suggest that PKC activation is necessary for the induction of cerebellar LTD.

LTD IS INDUCED AFTER COACTIVATION of parallel fiber (PF) and climbing fiber (CF) inputs to a Purkinje neuron (PN) and is specific to those PF-PN synapses that are active during CF stimulation (*1*). Thus, cerebellar LTD is an anti-Hebbian process: synapses that are active during postsynaptic activation are weakened (*2*). As the PN is the sole output stage of the cerebellar cortex, LTD has been implicated in several forms of motor learning (*3*).

LTD is expressed as a depression of AMPA-mediated current. It is detected with pulses of glutamate, quisqualate, or AMPA but not with pulses of aspartate or NMDA (*4–6*). There is no evidence to suggest a presynaptic function in either the induction or the expression of cerebellar LTD. A quantal analysis has been reported that is consistent with postsynaptic expression (*7*).

Several principles have emerged regarding the induction and expression of cerebellar LTD. The aspect of CF activation that contributes to LTD induction seems to be a prolonged depolarization of the PN (*8*), which results in dendritic Ca^{2+} entry (*9*). As such, induction of LTD is blocked when PNs are electrically inhibited (*1, 6, 10, 11*) or loaded with a Ca^{2+} chelator (*12*), and LTD may be induced when depolarization sufficient to produce Ca^{2+} entry is substituted for CF activation (*5, 6, 10, 11*). Activation of PFs contributes to LTD induction by activating both AMPA (ionotropic quisqualate) receptors and metabotropic quisqualate receptors. Blockade of either of these receptors during conjunctive stimulation blocks induction of LTD (*6, 13*), and activation of both of these receptors substitutes for PF activation during induction of LTD (*5, 6, 14*). NMDA receptors do not seem to contribute to LTD induction (*6, 14*). Thus, three processes are necessary and sufficient for induction of cerebellar LTD, namely, PN depolarization sufficient to produce dendritic Ca^{2+} influx, activation of AMPA receptors, and activation of metabotropic quisqualate receptors.

The metabotropic quisqualate receptor is coupled to phospholipase C, which cleaves the membrane lipid phosphatidylinositol-bisphosphate to yield inositol-1,4,5,-trisphosphate and 1,2-diacylglycerol. The former releases Ca^{2+} from nonmitochondrial internal stores, and the latter is an activator of PKC (*15*). To investigate the possible role of PKC in the induction of cerebellar LTD, we applied PKC inhibitors and activators to PNs in culture to determine their effects on LTD induced by glutamate–depolarization conjunctive stimulation. As this protocol (*16, 17*) does not rely on synaptic stimulation to induce LTD, it allows unambiguous analysis of postsynaptic processes.

Nonpeptide, membrane-permeable PKC inhibitors with greatly improved specificity are now available. Two such compounds are calphostin C, which competes with phorbol esters at the regulatory site of PKC (*18*), and RO-31-8220, which competes with adenosine triphosphate (ATP) at the catalytic site (*19*). Both inhibitors blocked the induction of LTD when applied during glutamate–depolarization conjunction (Fig. 1A). After drug washout for 25 min, a second conjunctive stimulus induced LTD (t = 30 min). Glutamate–depolarization conjunction in the presence of RO-31-8220, but not calphostin C, produced a small potentiation of the glutamate current (RO-31-8220, 118 ± 5.5% of baseline; calphostin C, 104 ± 4.2% at t = 15 min, mean ± SEM, n = 5 per group). This potentiation is similar to that seen when glutamate–depolarization conjunction was applied in the presence of inhibitors of the metabotropic quisqualate receptor or when AMPA–depolarization conjunctive stimulation was applied (*6*). Application of PKC inhibitors after the induction of LTD (t = 10 to 60 min) produced no alteration of the depressed glutamate current (Fig. 1B), suggesting that either after induction, LTD does not require further PKC activity or that PKC activated by glutamate–depolarization conjunction is insensitive to these two PKC inhibitors.

We also applied a PKC inhibitory peptide, PKC(19-36), and a noninhibitory control peptide, [glu^{27}]PKC(19-36), (*20*) to the internal solution of conventional whole-cell patch electrodes (*21*). PNs dialyzed (with Ca^{2+} chelator free vehicle) for either 5 or 10 min before glutamate–depolarization conjunctive stimulation showed LTD (induced four out of four and five out of five times, respectively). However, LTD was

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observed in only two of five PNs dialyzed for 20 min before conjunctive stimulation. Therefore, longer dialysis interfered with some key step in the induction process. After 10 min of baseline recording, the PKC inhibitor peptide, but not the inactive control peptide or the vehicle, blocked LTD induced by glutamate–depolarization conjunction (Fig. 1C). Similar to the effect of RO-31-8220, glutamate–depolarization conjunctive stimulation in PNs loaded with PKC inhibitor peptide showed a small potentiation of glutamate current. Confirming previous findings (*12*), addition of the Ca^{2+} chelator BAPTA [bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid] (20 mM) to the internal saline was also effective in blocking LTD induction.

As the induction of LTD requires PN depolarization sufficient to cause dendritic Ca^{2+} influx (presumably via voltage-gated Ca^{2+} channels) and AMPA receptor activation, we sought to determine if RO-31-8220 or calphostin C might be reducing Ca^{2+} fluxes or AMPA currents to produce their effects on LTD induction. Neither RO-31-8220 nor calphostin C applied at the same concentrations that blocked LTD induction attenuated voltage-gated Ca^{2+} currents as measured with depolarizing steps from −80 mV in tetrodotoxin-tetraethylammonium (TTX-TEA) saline with cesium-filled perforated-patch electrodes (Fig. 2). Similarly, these inhibitors did not alter the resting free Ca^{2+} concentration as monitored by fura-2 imaging (*22*). We also observed no significant alteration of AMPA-mediated current as measured with AMPA test pulses applied at 0.05 Hz (RO-31-8220, 105 ± 3.7% of baseline; calphostin C, 101 ± 6.3% of baseline, measured 15 min after bath application, n = 5 per group). These results support the contention that RO-31-8220 and calphostin C affect LTD induction by inhibiting PKC.

We next tested the ability of an activator of PKC to induce LTD-like responses (Fig. 3A). Bath application of PDA (0.1 μM) induced depression of the PN response to AMPA but not to NMDA test pulses (*23*). Application of PDA in a flowing bath for 15 min induced a depression of the AMPA-mediated current that was evident at t = 10 min and that reached a stable amplitude (~50% of baseline) at t = 30 min. This selective depression of AMPA-mediated current induced by PDA was similar to LTD induced by quisqualate–depolarization conjunctive stimulation (Fig. 3B). The depression of AMPA current by both quisqualate–depolarization conjunctive stimulation and application of PDA was manifest as a simple reduction in current amplitude, without obvious alterations in the kinetics of onset or offset. That the response to AMPA but not NMDA was depressed after quisqualate–depolarization conjunctive stimulation or application of PDA argues that the depression induced by these treatments is not merely a generalized down-regulation of PN responsiveness. Our results confirm and extend a report that phorbol-12,13-dibutyrate induced a depression of PN responses to glutamate test pulses in the cerebellar slice (*5*).

To determine if the effect of PDA on the AMPA-mediated current was produced by PKC activation, we performed several control experiments. A phorbol ester inert with respect to PKC (4α-PDA) was applied with the same protocol used and did not alter the AMPA-mediated current (97 ± 4.0% of baseline at t = 15 min, 101 ± 3.5% at t = 30 min, n = 5). Subsequent application of PDA (from t = 30 to 45 min) produced depression of the AMPA-mediated current in these same cells (75 ± 6.1% of baseline at t = 45 min, 63 ± 6.8% at t = 60 min). When PDA was applied (t = 0 to 15 min, 0.1 μM) in the presence of calphostin C (2 μM, applied in the bath at t = −15 min and maintained throughout the experiment), no

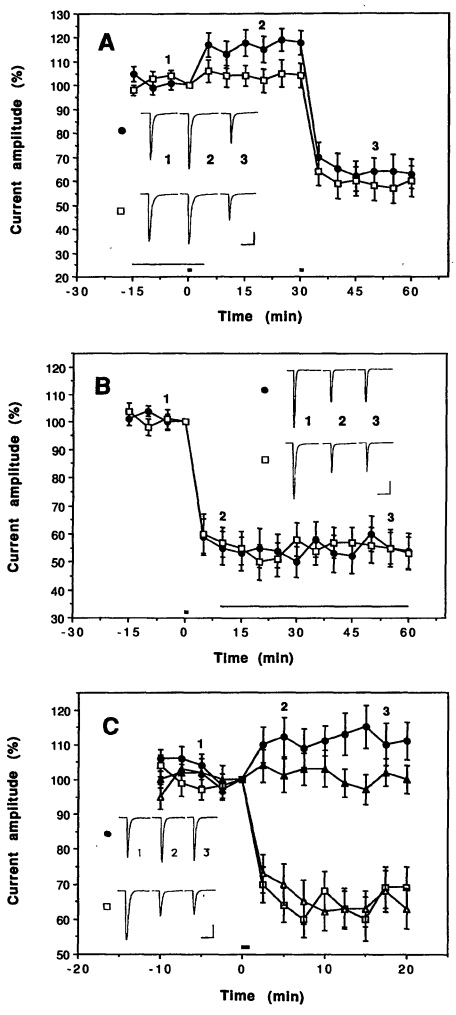

Fig. 1. Blockade of LTD induction by PKC inhibitors. (**A**) RO-31-8220 (●, 0.2 μM) or calphostin C (□, 2 μM) was applied in a flowing bath (from t = −15 to t = 5 min, indicated by bar on graph) during glutamate–depolarization conjunctive stimulation (indicated by short bar on graph at t = 0 min). After washout of the PKC inhibitors, a second conjunctive stimulation (t = 30 min) was applied. Graph points are the mean ± SEM of five separate experiments. Representative current traces are records from single PNs in this population, taken at the times 1, 2, and 3 indicated on the graph. Scale bars = 50 pA, 2 s. (**B**) PKC inhibitors were applied after induction of LTD by glutamate–depolarization conjunctive stimulation (t = 10 to 60 min). Scale bars = 50 pA, 2 s; n = 6 per group. (**C**) LTD induced by glutamate–depolarization conjunctive stimulation (t = 0 min) was monitored by means of conventional whole-cell recording in neurons with the following internal perfusates: vehicle (△), vehicle plus BAPTA (▲, 20 mM), vehicle plus PKC(19-36) (●, 10 μM), vehicle plus [glu^{27}]PKC(19-36) (□, 10 μM). Scale bars = 100 pA, 2 s; n = 5 per group.

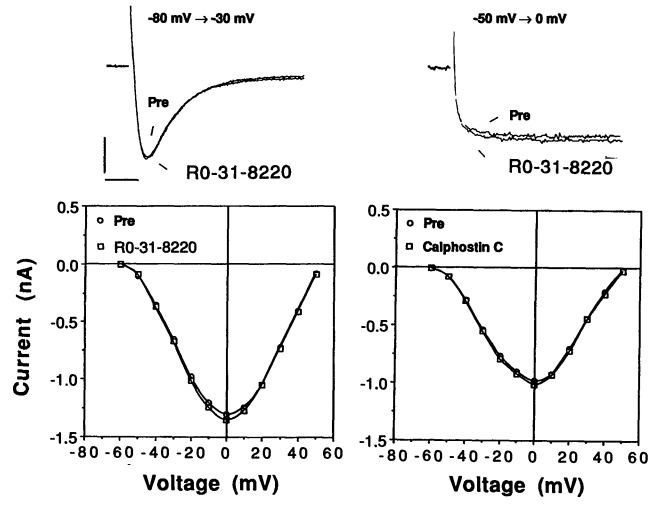

Fig. 2. No effect of PKC inhibitors on voltage-gated Ca^{2+} currents in cultured PNs. PKC inhibitors were applied in the same manner in which they blocked LTD induction (15-min exposure, RO-31-8220 = 0.2 μM, calphostin C = 2.0 μM). Use of the perforated-patch recording technique prevented the rundown that would be expected with conventional whole-cell recording over this time period. The current-voltage relations are derived from the peaks of currents evoked by depolarizing steps from a holding potential of −80 mV. The current traces and the current-voltage relations are records from single PNs. Scale bars = 8 ms, 200 pA for the −80 mV to −30 mV step and 100 pA for the −50 mV to 0 mV step; n = 4.

depression of AMPA-mediated current was induced (93 ± 4.7% of baseline at t = 15 min, 100 ± 5.2% at t = 30 min, n = 5). Subsequent quisqualate–depolarization conjunction (at t = 35 min) in these same cells also failed to induce LTD (110 ± 6.1% of baseline at t = 45 min, 108 ± 6.0% at t = 60 min). In addition, application of PDA (0.1 μM for 15 min) did not alter resting concentrations of free Ca^{2+} as measured by fura-2 imaging (*22*).

If the depression of AMPA-mediated current produced by PDA and quisqualate–depolarization conjunction were acting through similar mechanisms, then the induction of one should occlude the effect of the other (Fig. 4). Quisqualate–depolarization conjunction produced a depression of the response to AMPA test pulses that was not further altered by bath application of PDA (0.1 μM; t = 10 to 25 min, or 1 μM; t = 35 to 50 min). Similarly, depression of AMPA-mediated currents produced by PDA (0.1 μM; t = 0 to 15 min) occluded the effect of subsequent quisqualate–depolarization conjunctive stimulation applied at either the normal duration (6 stimuli, t = 30 min) or double the normal duration (12 stimuli, t = 45 min).

Application of PDA together with AMPA pulses produced a depression of AMPA-mediated current (Fig. 3A). It is not surprising that this depression can occur in the absence of PN depolarization, as PDA activates PKC by increasing its affinity for Ca^{2+} (*24*). It is not clear if AMPA pulses are required during PDA application to produce depression of AMPA currents. Application of PDA without concomitant AMPA pulses produced a highly variable response; of the PNs tested, ~70% failed to show a depression of AMPA currents when AMPA pulses were resumed after 20 min of PDA washout. This result is difficult to interpret, as it is not clear if PDA washout was complete when AMPA pulses were resumed.

The present results suggest that activation of PKC is necessary for the induction of cerebellar LTD in culture. However, it is not clear if PKC activation is required for the continued expression of LTD. It is possible that LTD induction alters PKC such that it is no longer sensitive to the inhibitors used in this study. As RO-31-8220 is a catalytic site inhibitor, LTD induction is unlikely to be mediated by proteolytic cleavage of PKC into separate regulatory and catalytic domains (*25*), as has been suggested for hippocampal long-term potentiation (*26*). The ability of PDA to produce a depression of AMPA responses that outlasts its duration of application also suggests that continued PKC activation is not required for expression of LTD (Fig. 3A). However, washout of PDA from these cultures is probably slow. Consequently, one cannot conclude that the depressed response to AMPA persists in the absence of PDA.

Our experiments do not address the question of whether PKC activation is sufficient for the induction of LTD. As the three processes necessary for cerebellar LTD induction (metabotropic quisqualate receptor activation, PN depolarization, and AMPA receptor activation) produce divergent biochemical signals, it is difficult to determine if PKC activation is a final common pathway for these processes. Ca^{2+} influx as a consequence of PN depolarization could activate a wide range of Ca^{2+}-activated second messenger systems.

In conclusion, these results suggest that postsynaptic PKC activation is necessary for induction of LTD in cultured PNs. It is likely that PKC activation is not required for continued LTD expression. It is not clear if PKC activation is sufficient for LTD induction or if other processes, possibly mediated by the AMPA receptor, are required.

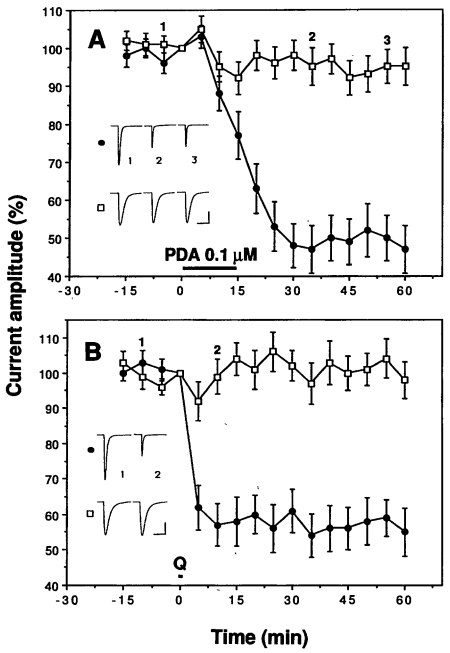

Fig. 3. A similar depression of AMPA-mediated current produced by quisqualate–depolarization conjunctive stimulation and application of the PKC activator PDA. The holding potential was set at −65 mV rather than −80 mV to partially relieve the voltage-dependent blockade of the NMDA-associated channel by Mg^{2+}. This voltage shift combined with longer pulses produced an NMDA current of comparable amplitude to the AMPA current. AMPA (●) and NMDA (□) test pulses were delivered alternately at 0.05 Hz from two separate iontophoretic electrodes aimed at the same site on the PN. Quisqualate, given together with depolarization to induce LTD, was applied through a third iontophoretic electrode aimed at the same site as the test pulses. (**A**) PDA (0.1 μM) was applied in the bath from t = 0 to 15 min. Scale bars = 50 pA, 2 s; n = 6. (**B**) Quisqualate–depolarization conjunction was applied at t = 0 min (Q on graph). Scale bars = 50 pA, 2 s; n = 5.

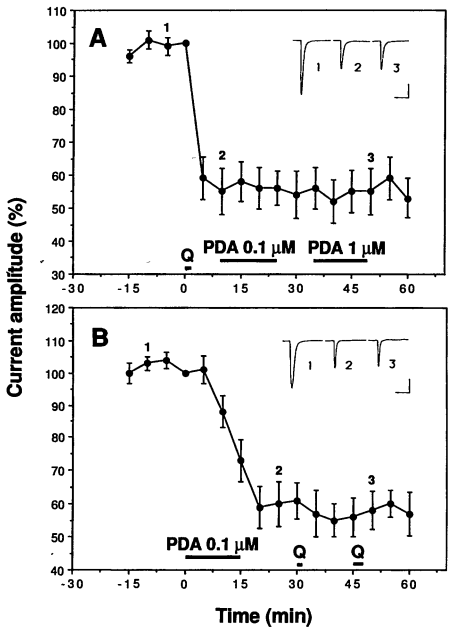

Fig. 4. Nonadditive depression of AMPA-mediated current by quisqualate–depolarization conjunctive stimulation and PDA. (**A**) Induction of LTD by quisqualate–depolarization conjunctive stimulation (t = 0 min, Q on graph) occludes further alterations of AMPA-mediated current produced by bath application of PDA at a concentration of 0.1 μM (t = 10 to 25 min) or 1 μM (t = 35 to 50 min). Scale bars = 50 pA, 2 s; n = 6. (**B**) Bath application of PDA (0.1 μM, t = 0 to 15 min) induced a stable depression of AMPA-mediated current that prevented further alteration by quisqualate–depolarization conjunctive stimulation applied with either 6 (t = 30 min, Q on graph) or 12 (t = 45 min, Q on graph) conjunctive stimuli. Scale bars = 50 pA, 2 s; n = 6.

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16. Mouse cerebellar cultures were prepared and maintained by the method of K. Schilling, M. Dickinson, J. Connor, and J. Morgan (*Neuron*, in press) as previously described (*6*). Cultures used for recording had been maintained in vitro for 12 to 24 days. PNs were chosen for recording by distinct morphological criteria (large round soma, prominent dendritic arbor) as verified by staining with the PN-specific immunochemical markers PEP-19 [L. Sangameswaran, J. Hempstead, J. Morgan, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5651 (1989)] and calbindin-D_{28K} [M. Thomasset *et al.*, *Dev. Pharmacol. Ther.* 7, 6 (1984)]. Nystatin patch electrodes [R. Horn and A. Marty, *J. Gen. Physiol.* **92**, 145 (1988)] were attached to PN somata and were used to apply a holding potential of −80 mV. Iontophoresis electrodes (tip diameter, <0.5 μm) were filled with excitatory amino acid receptor agonists and were positioned ~20 μm away from a large-caliber dendrite 10 to 20 μm from the soma. Pulses of agonists (30 to 100 ms duration) were applied at a frequency of 0.05 Hz. After acquisition of 15 min of baseline responses, we induced LTD by pairing six successive glutamate test pulses with six 4-s depolarization steps to −10 mV, timed so that the depolarization onset preceded the glutamate pulse by 500 ms. This treatment reliably induced a stable depression of the glutamate-mediated inward current (typically 50 to 65% of its baseline value) that was independent of alterations in input resistance or resting potential (*6*; D. J. Linden and J. A. Connor, in preparation). Cells were bathed in a solution (adjusted to pH 7.35 with NaOH) that contained NaCl (150 mM), KCl (5 mM), $CaCl_2$ (2 mM), $MgCl_2$ (0.8 mM), Hepes (10 mM), glucose (10 mM), tetrodotoxin (0.005 mM), and picrotoxin (0.1 mM). Tetrodotoxin and picrotoxin were added to the external saline to improve the resolution of the voltage clamp and to block spontaneous synaptic potentials, but the addition of these compounds was not required for the induction of LTD. For recordings of voltage-gated Ca^{2+} currents (as in Fig. 1C), 10 mM TEA-Cl was added. The internal saline (adjusted to pH 7.35 with KOH) consisted of K_2SO_4 (95 mM), KCl (15 mM), $MgCl_2$ (8 mM), and Hepes (10 mM). For Ca^{2+} current recording, Cs_2SO_4 and CsCl were substituted for K_2SO_4 and KCl, respectively, in an equimolar fashion. Patch electrodes were pulled from N51A glass (Garner Glass Co., Claremont, CA) and polished on a microforge to yield a resistance of 1 to 3 megohms when measured with normal internal and external salines. Nystatin (250 μg/ml) was added to the electrode filling saline. This concentration gave access resistance to the cell that was typically <10 megohms. Only cells with stable resting potentials of > −45 mV were used. Membrane currents induced by application of excitatory amino acids were stored on a chart recorder that imposed a 0- to 30-Hz bandpass. Membrane currents in response to voltage steps were filtered at 3 kHz, digitized with a 12-bit analog-to-digital converter operating at a sampling frequency of 20 kHz, and stored on the hard disk of an INDEC-LSI-11/23 computer. Leakage current was determined from 10-mV hyperpolarizing pulses and was digitally subtracted from records of voltage-gated currents. Experiments were conducted at 22° to 24°C. Imaging of intracellular free Ca^{2+} with the indicator fura-2/AM was performed as described [J. A. Connor, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6179 (1986)].

17. Excitory amino acid receptor agonists were delivered by iontophoresis and were prepared in 10 mM Hepes as follows: 10 mM glutamate (pH 7.1); 10 mM quisqualate (pH 7.2); 10 mM AMPA (pH 7.1); 100 mM NMDA (pH 7.3). These compounds were delivered through polished patch pipettes (resistance, 4 to 6 megohms when filled with normal internal saline) with negative current pulses. Braking currents were applied to prevent drug leakage.

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23. Studies with exogenous agonists have shown a developmental decrease in the response of PNs to NMDA, resulting in a complete lack of sensitivity to this compound in the adult [J. L. Dupont, G. Gardette, F. Crepel, *Dev. Brain Res.* **34**, 59 (1987); G. Garthwaite, B. Yamani, J. Garthwaite, *ibid.* **36**, 288 (1987); E. Audinat, T. Knopfel, B. H. Gahwiler, *J. Physiol. (London)* **430**, 297 (1990)]. Our cultures of embryonic PNs showed NMDA responses typical of those seen in other neuronal types: that is, they were attenuated by DL-2-amino-5-phosphonopentanoic acid (AP5) and exhibited a Mg^{2+} and voltage-dependent block. When the membrane potential was clamped to −80 mV, the NMDA response became very small (<10% of the glutamate current was blocked by 20 μM AP5 at this potential). To produce NMDA currents of easily measurable amplitude (as in Fig. 3), membrane potential was clamped to −65 mV, and NMDA pulses were typically applied for durations two to three times that of AMPA.

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27. We thank K. Schilling, who developed the cell culture technique used in this study; M. Smeyne and R. Sun for technical assistance; W. Muller, M. Dickinson, and J. Petrozzino for helpful discussion; and G. Lawton (Roche Welwyn, United Kingdom) for providing RO-31-8220.

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Targeting of the Master Receptor MOM19 to Mitochondria

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The targeting of proteins to mitochondria involves the recognition of the precursor proteins by receptors on the mitochondrial surface followed by insertion of the precursors into the outer membrane at the general insertion site GIP. Most mitochondrial proteins analyzed so far use a mitochondrial outer membrane protein of 19 kilodaltons (MOM19) as an import receptor. The gene encoding MOM19 has now been isolated. The deduced amino acid sequence predicts that MOM19 is anchored in the outer membrane by an NH_2-terminal hydrophobic sequence, while the rest of the protein forms a hydrophilic domain exposed to the cytosol. MOM19 was targeted to the mitochondria via a pathway that is independent of protease-accessible surface receptors and controlled by direct assembly of the MOM19 precursor with GIP.

THE BIOGENESIS OF MITOCHONDRIA involves the translocation of cytosolically synthesized precursor proteins into or across the organellar membranes (*1, 2*). In *Neurospora crassa*, two mitochondrial outer membrane proteins (MOMs) were identified as import receptors for precursor proteins. The 19-kD protein MOM19 functions as a receptor for most precursors analyzed, including all precursors that carry an NH_2-terminal signal sequence (*3–5*), and is referred to as the master receptor. The 72-kD protein MOM72 is a receptor for the precursor of the adenosine diphosphate–adenosine triphosphate (ADP-ATP) carrier, which contains internal targeting signals (*4, 6*). Precursor proteins interact with these receptors and are inserted into the outer membrane at a common site, termed the general insertion site (general insertion protein = GIP) (*7, 8*). The 38-kD protein MOM38 forms part of the GIP and is assembled with MOM19 and MOM72 in the mitochondrial receptor complex (*9*). To determine the mechanism by which the master receptor is targeted to mitochondria, we investigated the biogenesis of MOM19.

A full-length cDNA clone that encodes MOM19 from *N. crassa* was isolated (*10*) and used to obtain the complete MOM19 gene (*11*). The nucleotide sequences of both

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