Cellular/Molecular

Isoform Specificity of PKC Translocation in Living *Aplysia* Sensory Neurons and a Role for Ca^{2+}-Dependent PKC APL I in the Induction of Intermediate-Term Facilitation

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Protein kinase Cs (PKCs) are important effectors of synaptic plasticity. In *Aplysia*, there are two major phorbol ester-activated PKCs, Ca^{2+}-activated PKC Apl I and Ca^{2+}-independent PKC Apl II. Functional Apl II, but not Apl I, in sensory neurons is required for a form of short-term facilitation induced at sensorimotor synapses by the facilitatory transmitter serotonin (5-HT). Because PKCs are activated by translocating from the cytoplasm to the membrane, we used fluorescently tagged PKCs to determine the isoform and cell-type specificity of translocation in living *Aplysia* neurons. In Sf9 cells, low levels of diacylglycerol translocate Apl II, but not Apl I, which requires calcium for translocation at low concentrations of diacylglycerol. Accordingly, application of 5-HT to *Aplysia* sensory neurons in the absence of neuronal firing translocates Apl II, but not Apl I, consistent with the role of Apl II in short-term facilitation. This translocation is observed in sensory neurons, but not in motor neurons. Apl I translocates only if 5-HT is coupled to firing in the sensory neuron; firing alone is ineffective. Because combined 5-HT and firing are required for the induction of one type of intermediate-term facilitation at these synapses, we asked whether this form of synaptic plasticity involves activation of Apl I. We report here that dominant-negative Apl I, but not Apl II, blocks intermediate-term facilitation. Thus, different isoforms of PKC translocate under different conditions to mediate distinct types of synaptic plasticity: Ca^{2+}-independent Apl II is involved in short-term facilitation, and Ca^{2+}-dependent Apl I contributes to intermediate-term facilitation.

Key words: **synapses; synaptic transmission; synaptic plasticity; protein kinase C; *Aplysia*; learning**

Introduction

Protein kinase C (PKC) plays an important role in regulating synaptic strength by phosphorylating and regulating proteins such as ion channels, cytoskeletal proteins, and proteins involved in neurotransmitter release that, in turn, control synaptic strength (Keenan and Kelleher, 1998; Majewski and Iannazzo, 1998). Persistent changes in synaptic strength can depend on persistent activation of PKC, suggesting that regulation of PKC itself may be central in some memory systems (Malinow et al., 1988; Grunbaum and Muller, 1998; Sutton and Carew, 2000; Ling et al., 2002; Linden, 2003; Sutton et al., 2004). Thus, understanding how and when PKCs are activated is important for understanding diverse types of synaptic plasticity.

Behavioral sensitization in *Aplysia* is mediated in part by an increase in the strength of the connections between mechanoreceptor sensory neurons (SNs) and motor neurons (Kandel, 2001). Synaptic facilitation is mediated by the neurotransmitter serotonin (5-HT), which can induce facilitation in isolated ganglia and in cocultures of sensory neurons and motor neurons (Byrne et al., 1993; Byrne and Kandel, 1996). Interestingly, the mechanisms determining short-term facilitation (STF) depend on the state of the synapse. Facilitation at naive synapses requires activation of protein kinase A (PKA), whereas facilitation at synapses that have been depressed by previous activity depends on activation of PKC (Braha et al., 1990; Ghirardi et al., 1992; Klein, 1993). There is also a distinction between PKA and PKC in more persistent increases in synaptic strength [intermediate-term facilitation (ITF)] caused by 5-HT. Although ITF induced by spaced application of 5-HT depends on persistent activation of PKA, pairing 5-HT with firing in the sensory neuron leads to a form of ITF that depends on persistent activation of PKC (Sutton and Carew, 2000).

Sensitizing stimulation as well as 5-HT causes translocation of *Aplysia* PKC to neuronal membranes (Sacktor and Schwartz, 1990). Biochemical experiments indicate that in the *Aplysia* nervous system, there are only two major phorbol ester-activated PKCs, the Ca^{2+}-activated Apl I and the Ca^{2+}-independent Apl II (Kruger et al., 1991; Sossin et al., 1993). Biochemical analysis of PKC activation in pleural-pedal ganglia suggested that 5-HT activates Apl I but not Apl II (Sossin and Schwartz, 1992). More-

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over, characterization of purified enzymes also suggested that Apl I was easier to activate, even in the absence of calcium, than was Apl II (Pepio et al., 1998). However, 5-HT-mediated reversal of synaptic depression was blocked by dominant-negative forms of Apl II, but not Apl I, bringing these previous results into question (Manseau et al., 2001).

To address this issue, we measured PKC translocation in living cells using fluorescently tagged PKCs to examine the isoform specificity of enzyme translocation. These results are consistent with distinct physiological roles for the two different isoforms of PKC in sensory neurons, with Apl II responsible for one type of STF and Apl I necessary for a form of ITF.

Materials and Methods

Plasmid construction. The pNEX3 enhanced green fluorescent protein (eGFP) PKC Apl I and eGFP PKC Apl II as well as the dominant negatives have been described previously (Manseau et al., 2001). To construct monocistronic red fluorescent protein (mRFP) PKC Apl II, mRFP was amplified from an mRFP plasmid (Campbell et al., 2002) using overlap PCR such that the PCR piece had *Kpn*I and *Pvu*I sites at the ends and contained the linker between eGFP and Apl II at the end of mRFP so that the resulting mRFP-Apl II had the identical linker as eGFP-Apl II (Manseau et al., 2001). This piece was then inserted into pNEX3-eGFP-Apl II that had eGFP cut with *Kpn*I (Partial) and *Pvu*I.

Sf9 cell culture. The Sf9 cells were purchased from Sigma-Aldrich (Sigma-Aldrich, Oakville, Ontario, Canada). Sf9 cells were grown in Grace's medium (Invitrogen, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (Cansera, Etobicoke, Ontario, Canada) as a monolayer at 27°C. For transfection, cells were plated on MatTek glass bottom culture dishes (MatTek Corporation, Ashland, MA) with a glass surface of 14 mm and a coverslip thickness of 1.5 mm at a density of 0.11×10^6 cells/35 mm dish. Cells were transfected using the Cellfectin reagent (Invitrogen, Burlington, Ontario, Canada) following the recommendation of the manufacturer. Live-imaging on the confocal microscope was performed 48–72 h posttransfection. Cells were serum starved for 6 h before imaging sessions. 1,2-Dioctanoyl-*sn*-glycerol (DOG) (Avanti Polar Lipids, Alabaster, AL) and Ionomycin (Sigma-Aldrich) were dissolved in dimethyl sulfoxide and diluted to the final concentration with Grace's medium shortly before the experiment. During the experiment, the cells were not exposed to dimethyl sulfoxide concentrations >1%. All of the experiments were performed in a temperature-controlled chamber at 27°C and on at least three different occasions. In each experiment, recordings were obtained from two to six cells.

Confocal microscopy in SF9 cells. Cells expressing eGFP-Apl I and mRFP-Apl II were examined using a Zeiss laser scanning microscope (Zeiss, Oberkochen, Germany) with an Axiovert 200 and a 63× oil immersion objective. During imaging, DOG and/or Ionomycin were added to the dish at 30 s, and a series of 20 confocal images were recorded for each experiment at time intervals of 30 s.

Image analysis of SF9 cells. The time series was analyzed using NIH Image J software. An individual analysis of protein translocation for each cell was performed by tracing three rectangles at random locations at the plasma membrane and three rectangles at random locations in the cytosol. The relative increase in the amount of enzyme localized in the plasma membrane for each time point was calculated by using the ratio $r = \text{Average } (I_m)/\text{Average } (I_c)$ where Average (I_m) is the average fluorescence intensity at the plasma membrane and Average (I_c) is the average cytosolic fluorescence intensity. Mean values are given ± SE of the mean.

Aplysia *cell culture preparation.* Cultures were prepared following published procedures (Schacher and Proshansky, 1983; Klein, 1993). Adult *Aplysia californica* (76–100 gm; RSMAS University of Miami, Miami, FL; Alacrity Marine Biological Services, Redondo Beach, CA) were anesthetized by injection of 50–100 ml of 400 mM (isotonic) $MgCl_2$. Pleuropedal and abdominal ganglion were removed and digested in artificial seawater (ASW; see below for composition) containing 1% protease type IX (Sigma). Tail SNs and siphon (LFS) motor neurons were isolated and plated in L15 containing 10% or 50% *Aplysia* hemolymph on dishes (Glass Bottom Microwell Dish; MatTek Corporation) pretreated with poly-L-lysine (molecular weight, >300,000; Sigma). Dishes were prepared with isolated sensory neurons, isolated motor neurons, or cocultures of sensory neurons and motor neurons for synapse formation.

Microinjection of plasmid vectors. On day 1 after isolation, solutions of plasmids in distilled water containing 0.25% fast green were microinjected from back-filled glass micropipettes using a General Valve Driver (Parker Hannifin, Fairfield, NJ). The tip of the micropipette was inserted into the cell nucleus, and short pressure pulses (10–50 ms duration; 20 psi) were delivered until the nucleus became uniformly green. The cells were incubated overnight at room temperature for experiments on the following day. For experiments at later times, cultures were kept at 4°C until use.

Confocal microscopy and immunocytochemistry of Aplysia *neurons.* Live cell imaging: neurons expressing eGFP-PKC were imaged on a Zeiss Pascal scanning laser microscope using a 63× (1.2 numerical aperture) water immersion objective and 10% of a 25 mW argon laser. Optical sections (Z-stack interval of 0.8 μm) were acquired before and after incubation with 5-HT (10 μM) or phorbol dibutyrate (PDBu; 1 nM). Immunocytochemistry was as follows: cultures were incubated with ASW (control) or 5-HT (10 μM) for 1 or 5 min, fixed in 4% paraformaldehyde/30% sucrose/PBS for 10 min and then permeabilized with 0.3% Triton X-100 for 10 min. After quenching free aldehyde groups with 50 mM NH_4Cl for 15 min and blocking with 10% goat serum for 30 min at room temperature, cultures were incubated with Apl II antibody in 10% goat serum overnight at 4°C (1:1000) followed by Alexa Fluor 546-conjugated secondary antibodies (1:1000 in 10% goat serum) for 1 h at room temperature. Fluorescence was then visualized by confocal microscopy.

Fluorescence microscopy. Cultures were viewed at 20 or 40× with a Nikon (Tokyo, Japan) Diaphot microscope attached to a xenon lamp for fluorescence excitation. A combination of optical filters and dichroic mirror (Omega Optical) was used for excitation centered at 485 nm, and emission measurements were centered at 535 nm. Images were acquired with a cooled CCD camera (Retiga EXi) controlled by IPLab software (version 3.65; Scanalytics, Fairfax, VA). Sequences of 7–30 images of neuronal cell bodies taken at planes of focus separated by 1–3 μm were deconvolved using one of the haze-removing functions of IPLab (Nearest-Neighbor, 3D Inverse or 3D Blind algorithms) and stacks of one to five sections were analyzed for translocation.

Analysis of fluorescent images of neurons. Because the distribution of fluorescence on neuronal membranes was less uniform than that in Sf9 cells, a different quantitative technique was used that allowed unbiased measurement of the entire membrane (see Fig. 5*A*). For images of cell bodies acquired with either the confocal microscope or the Nikon Diaphot, background fluorescence was first subtracted, and then the fluorescence of concentric rings one pixel in width from the periphery to the center of the cell body was measured. Translocation resulted in an increase in the perimembrane fluorescence and a concomitant decrease in the cytoplasmic fluorescence. An illustration of the method as applied to translocation of Apl II by 5-HT is shown in Figure 5*A*. As seen in the figure, application of 5-HT results in an intersection of the fluorescence plot in the presence of 5-HT with the plots before 5-HT and after washout. We took the total fluorescence between the edge of the cell and the point of intersection as representing membrane-associated Apl II, and the fluorescence between the point of intersection and the nucleus, where the plots coincided, as representing cytoplasmic Apl II (see Fig. 5*A*). We then took the ratio between the membrane fluorescence and the cytoplasmic fluorescence as a measure of the distribution of the fluorescent PKC between the membrane and the cytoplasm. Translocation is expressed as the change in this ratio with the various treatments (see Figs. 5*C*, 6*B*). All measurements on the same neuron were done in exactly the same way to compare the distribution of fluorescence between the membrane and the cytoplasm under different experimental conditions. Because it is impossible to delineate exactly the boundary between the membrane and the cytoplasm, all of the measured changes are underestimates.

Electrophysiology. An Axoclamp 2A amplifier (Molecular Devices, Palo Alto, CA) and glass micropipettes (tip resistance, 10–20 MΩ) filled with 0.5 M potassium chloride and 2 M potassium acetate were used for intra-

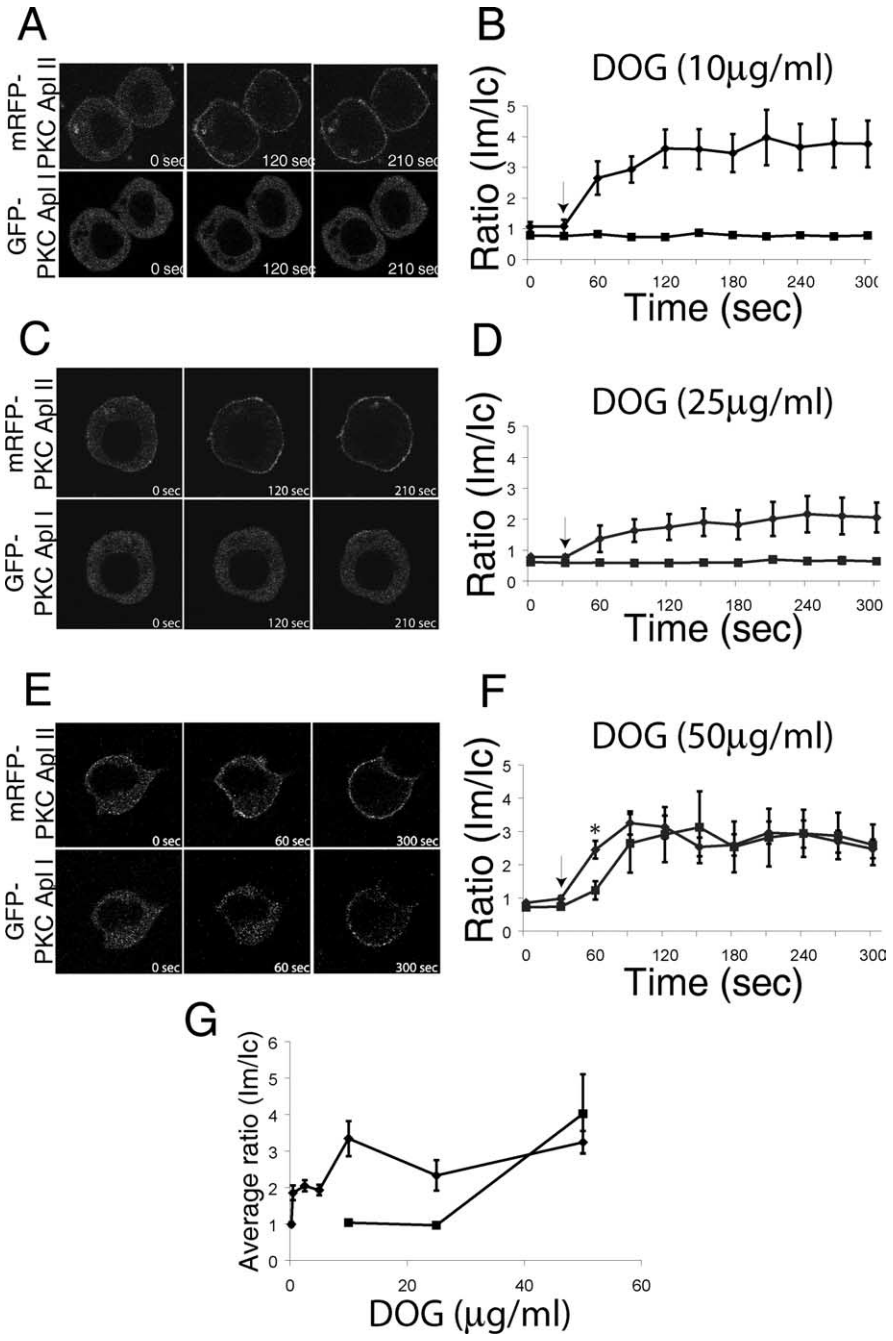

Figure 1. High amounts of DOG are required to translocate eGFP-PKC Apl I to the plasma membrane. ***A***, ***C***, ***E***, Confocal fluorescence images of Sf9 cells coexpressing eGFP-PKC Apl I (squares) and mRFP-PKC Apl II (diamonds) at different points of the time-lapse experiment. DOG (10, 25, and 50 μg/ml) was added to the dish after 30 s of recording in ***A***, ***C***, and ***E***, respectively. ***B***, ***D***, ***F***, Average of the time courses of plasma membrane localization of eGFP-PKC Apl I and mRFP-PKC Apl II under the conditions stated above. The arrow indicates the time point when DOG was added to the dish. The error bars are SEM. ***B***, $n = 5$; ***D***, $n = 5$; ***F***, $n = 9$. ***G***, Dose–response of mRFP PKC Apl II translocation and eGFP-PKC Apl I translocation at different concentrations of DOG ($n > 4$ for each time point); PKC Apl II is translocated at concentrations as low as 0.5 μg/ml, whereas eGFP-PKC Apl I is not translocated until 50 μg/ml.

cellular recordings. All recordings were done in ASW (in mM: 460 NaCl, 10 KCl, 11 CaCl2, 55 MgCl2, and 10 HEPES, pH 7.5). The membrane potential of sensory neurons or of isolated motor neurons was held at −55 mV. A train (10 Hz, 1–10 s) of short depolarizing pulses (5 ms) was delivered through the intracellular electrode to trigger action potentials where indicated. In cocultures, the motor neuron was impaled first, and its membrane potential was maintained at −80 mV. A supramaximal concentration of 5-HT (creatinine sulfate; Sigma) was delivered as a bolus of 100 μl (100 μM) or 20 μl (1 mM) in ASW by a hand-held pipette directly to the bath, ~5 mm from the cells.

In the experiments on intermediate-term facilitation, after eliciting a single test EPSP, 5-HT (10 μM) was added to the bath for a total of 5 min. Beginning at 1 min after 5-HT application, four trains of action potentials (10 Hz for 2 s) were triggered in the sensory neuron at 1 min intervals. The 5-HT was then washed out, and the EPSP was tested after 30 and 60 min. EPSP amplitude is expressed relative to the test EPSP. Control experiments included either 5-HT alone, stimulation alone, or neither (ASW alone).

All experiments were performed at room temperature (20–23°C).

Results

PKC Apl II can be translocated from cytoplasm to membrane by an analog of diacylglycerol alone, whereas the PKC Apl I requires a concomitant increase in intracellular calcium

SF9 cells were cotransfected with pNEX3-eGFP-Apl I and pNEX3-mRFP-Apl II plasmids and after 2–3 d, live translocation was measured following addition of DOG, a permeable analog of diacylglycerol (DAG). We noted that at low and intermediate concentrations of DOG (2.5–25 μg/ml), mRFP-Apl II translocated, but eGFP-Apl I did not (Fig. 1). This was not a result of the different fluorescent tags, because eGFP-Apl II translocated at similar concentrations of DOG (data not shown). There was also no competitive effect, because eGFP-Apl I did not translocate at these concentrations of DOG when transfected alone (data not shown). This was surprising, because kinase activity measurements on purified PKCs had indicated that Apl I was more easily activated than Apl II by low concentrations of DOG (Pepio et al., 1998; Manseau et al., 2001). At high concentrations of DOG (50 μg/ml), eGFP-Apl I translocated, although the translocation was significantly slower than that of mRFP-Apl II (Fig. 1). Because Apl I contains a C2 domain than can bind lipids in the presence of calcium (Pepio et al., 1998), we asked whether calcium could enhance translocation of Apl I. High concentrations of ionomycin (1 μM), a calcium ionophore, were sufficient to translocate eGFP-Apl I to membranes but did not translocate mRFP-Apl II (Fig. 2*A*). The failure of Apl II to translocate in response to increases in calcium concentration is expected, because the C2 domain of Apl II does not bind lipids in a calcium-dependent manner (Pepio et al., 1998). At lower concentrations of ionomycin (0.5 μM), translocation of eGFP-Apl I was small and transient (Fig. 2*B*). However, if this concentration of ionomycin was paired with a low concentration of DOG (5 μg/ml), eGFP-Apl I translocated robustly (Fig. 2*C*).

Thus, Apl I translocated only when DOG was paired with calcium influx, whereas DOG alone was sufficient to translocate PKC Apl II.

Visualization of 5-HT-induced translocation of PKC Apl II from cytoplasm to membrane in *Aplysia* sensory neurons

Biochemical experiments failed to demonstrate translocation of Apl II from the soluble to the membrane fraction of homogenates of *Aplysia* ganglia after exposure to the facilitatory transmitter 5-HT (Sossin and Schwartz, 1992; Sossin et al., 1994). However, in subsequent physiological experiments, overexpression of a dominant-negative version of Apl II in *Aplysia* sensory neurons blocked the facilitation of synaptic transmission by 5-HT at synapses that had undergone previous homosynaptic depression (Manseau et al., 2001), suggesting that Apl II is activated by 5-HT in sensory neurons. Biochemical experiments lack the sensitivity to detect restricted translocation in specific cells or specific compartments. We therefore examined the translocation of Apl II using immunocytochemistry of sensorimotor cocultures either in the presence or in the absence of 5-HT. Our results indicated that 5-HT induced translocation of Apl II to the plasma membrane in sensory neurons but not in motor neurons (Fig. 3).

Immunocytochemical experiments do not allow before and after comparison of individual cells, and there was considerable variability in the amount of Apl II membrane staining. Therefore, to more quantitatively characterize the translocation of Apl II by 5-HT in sensory neurons, we used live imaging of sensory neurons expressing eGFP-Apl II. Consistent with the immunocytochemistry results, Apl II visibly translocated to membranes in sensory neurons (Fig. 4). Translocation of Apl II by 5-HT did not require the presence of a motor neuron in the culture and was observed in both the cell body and in the neurites of isolated sensory neurons (Fig. 4*A*). Moreover, we could also detect translocation in sensory neuron varicosities adjoining neurites of motor neurons in cocultures where synaptic connections were verified with electrophysiological recording (Fig. 4*B*). Thus, Apl II appears to undergo translocation by 5-HT at putative synaptic sites, consistent with the role of Apl II in short-term reversal of synaptic depression (Manseau et al., 2001).

Although detectable translocation was observed in 20 of 24 isolated sensory neurons and in 37 of 42 sensory neurons in cocultures, no translocation was seen in experiments with seven motor neurons in isolation or five motor neurons in cocultures (Fig. 4*C*). Application of PDBu, a nonspecific activator of PKCs, caused translocation of Apl II in seven of seven motor neurons (Fig. 4*C*) as well as in sensory neurons (data not shown), indicating that the differential translocation of Apl II by 5-HT was not

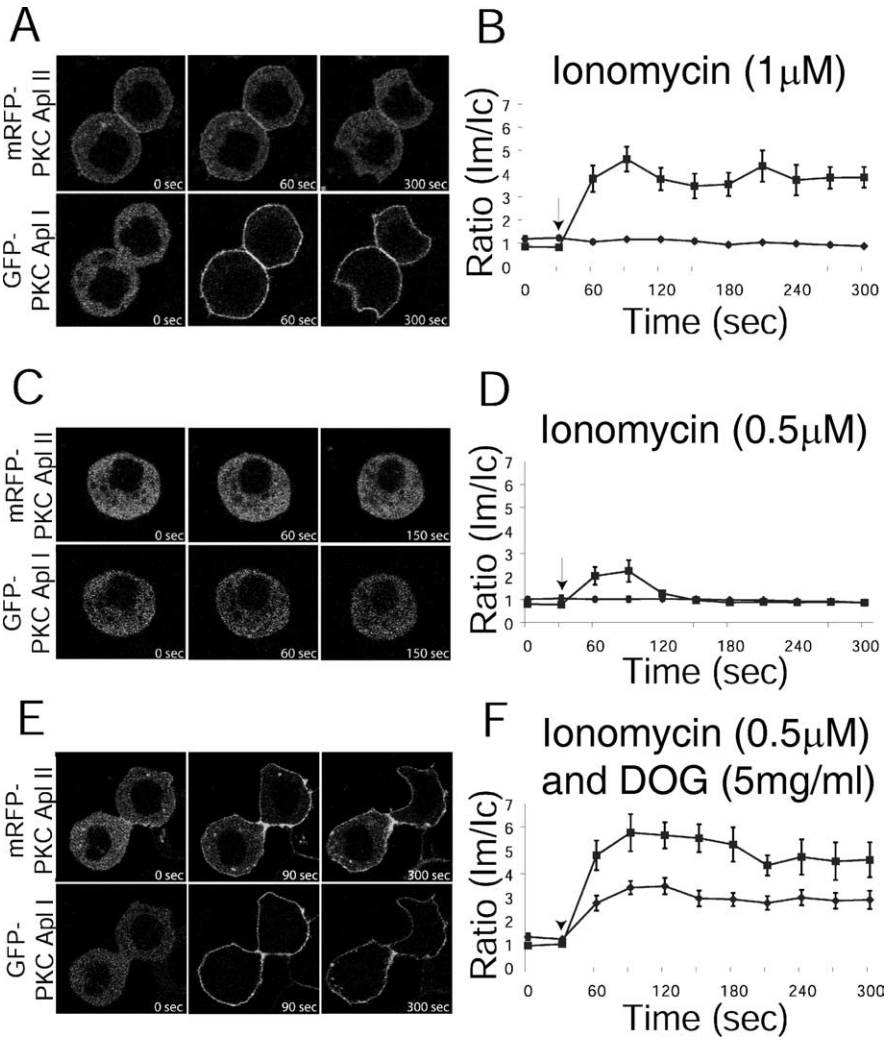

Figure 2. Increase in the concentration of intracellular calcium combined with low amounts of DOG lead to translocation of eGFP-PKC Apl I to the plasma membrane. ***A***, ***C***, ***E***, Confocal fluorescence images of Sf9 cells coexpressing eGFP-PKC Apl I (squares) and mRFP-PKC Apl II (diamonds) at different points of the time-lapse experiment. Ionomycin (1 μM; 0.5 μM) and a combination of Ionomycin (0.5 μM) and DOG (5 μg/ml) was added to the dish after 30 s of recording in ***A***, ***C***, and ***E***, respectively. ***B***, ***D***, ***F***, Average of the time courses of plasma membrane localization of eGFP-PKC Apl I (■) and mRFP-PKC Apl II (◆) under the conditions stated above. The arrow indicates the time point when DOG was added to the dish. The error bars are SEM. ***B***, $n = 5$; ***D***, ***F***, $n = 9$.

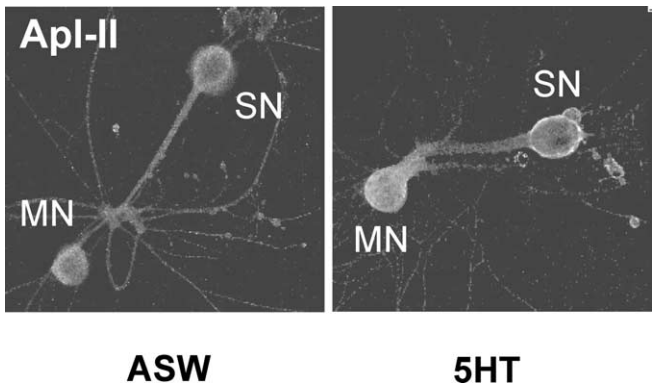

Figure 3. Endogenous calcium-independent PKC Apl II translocates from the cytoplasm to the membrane of sensory neurons but not motor neurons. Each image is of a coculture of an *Aplysia* SN and a motor neuron (MN) stained with an antibody to PKC Apl II. Cultures exposed to 5-HT (right) show a concentration of immunofluorescence in the membrane of sensory neurons compared with controls (left), but this shift in immunofluorescence is not apparent in motor neurons.

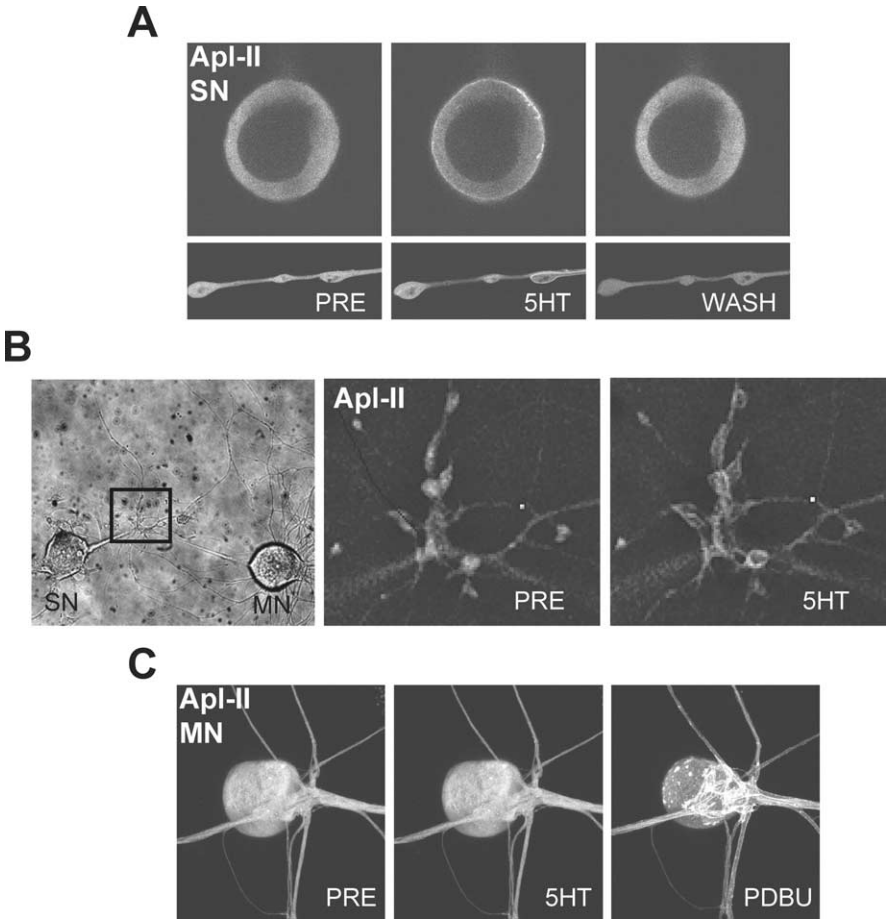

Figure 4. Translocation by 5-HT of eGFP-Apl II in living sensory neurons but not in motor neurons. ***A***, Application of 5-HT to an SN results in a shift of fluorescence from the cytoplasm to the membrane of both the cell body (top) and the neurites (bottom); this translocation reverts to the control when the 5-HT is removed (WASH). ***B***, Translocation of eGFP-Apl II in synaptic regions. Left, Bright-field image of a coculture of a sensory neuron expressing eGFP-Apl II and a motor neuron (MN) from which a synaptic potential was recorded. Right, Detail of fluorescence images before (PRE) and after application of 5-HT. Fluorescence in the presynaptic varicosities shifts from being uniformly distributed before 5-HT to being relatively concentrated at the membrane afterward. ***C***, 5-HT fails to cause translocation in a motor neuron (MN), whereas subsequent application of phorbol dibutyrate leads to a redistribution of fluorescence (PDBu). Projection of a Z-stack of confocal images of the motor neuron.

the result of a deficiency in the ability of eGFP-Apl II to translocate in the motor neuron or of insufficient sensitivity of our detection methods. Interestingly, translocation by PDBu was not solely to the plasma membrane but also to what appear to be cytoskeletal compartments (Fig. 4*C*). This is consistent with previous biochemical experiments, demonstrating that PDBu translocates PKC to both actin filaments and microtubules (Nakhost et al., 1998, 2002; Kabir et al., 2001). In contrast, in sensory neurons, 5-HT mainly translocated Apl II to the plasma membrane.

We quantified Apl II translocation by expressing it as a change in the ratio between the fluorescence associated with the membrane and the fluorescence in the cytoplasm (see Materials and Methods). When the fluorescence intensity is plotted as a function of distance from the edge of the neuron, translocation appears as an increase in the intensity nearest the edge accompanied by a decrease in the fluorescence further into the cell (Fig. 5*A*). Consistent with the absence of PKC in the nucleus, the fluorescence of the nucleus does not change.

Using this method of quantifying translocation, we examined the time course of translocation of Apl II in response to 5-HT (Fig. 5*B*). As seen in the summary plot (Fig. 5*C*), translocation could be detected by 30 s, the earliest time point we examined, and was maintained in the presence of 5-HT for up to 5 min, the latest time we examined in this set of experiments. Washing out the 5-HT resulted in reversal of translocation, which could be induced again by subsequent application of 5-HT (data not shown).

Translocation of calcium-dependent PKC isoform Apl I requires both 5-HT and concurrent action potential firing in the sensory neuron

Translocation of eGFP-Apl I in Sf9 cells requires both DOG and a rise in intracellular calcium concentration (Fig. 2). In contrast, previous biochemical results indicated that 5-HT alone could increase Apl I activity on membranes in pleural-pedal ganglia (Sossin and Schwartz, 1992; Sossin et al., 1994). However, in these dissected ganglia, there may have been considerable calcium entry caused by firing and/or mechanical injury.

To determine the requirements for Apl I translocation in sensory neurons, we examined translocation of eGFP-Apl I (Fig. 6). Although high concentrations of calcium could translocate eGFP-Apl I in Sf9 cells (Fig. 2), impalement of a sensory neuron with a microelectrode (data not shown), which would increase intracellular calcium at least transiently, or electrical stimulation of the neuron, which also results in a significant increase in the intracellular calcium concentration (Boyle et al., 1984), had little effect on the subcellular localization of Apl I (Fig. 6*A*,*B*), indicating that these levels of calcium entry were not sufficient to translocate the enzyme. Likewise, application of 5-HT alone did not result in translocation. However, electrical stimulation of the sensory neuron in the presence of 5-HT resulted in a shift of fluorescence from the cytoplasm to the membrane (Fig. 6). In nine experiments, firing of the sensory neuron in the presence of 5-HT resulted in an increase of 30 $\pm$ 8.6% (SEM) in the translocation index (calculated as in Fig. 5*A*) compared with 5-HT alone ($p < 0.01$) (Fig. 6*B*). When stimulation ceased, even in the continued presence of 5-HT, the translocation reversed. Translocation could be induced a second time by a subsequent train of action potentials in the presence of 5-HT, and this too reversed when the stimulation ended (data not shown). Thus, eGFP-Apl I can be translocated by physiological levels of calcium when 5-HT is present, suggesting that activation of Apl I in sensory neurons could act as a detector of coincident 5-HT and activity.

The same protocol of 5-HT application and electrical stimulation of the motor neuron resulted in a much smaller, but nonetheless significant, translocation of fluorescent eGFP-Apl I (Fig. 6*B*). In 10 experiments on motor neurons, the translocation index increased by 4.2 $\pm$ 1.7% ($p < 0.05$) with firing in the presence of 5-HT, compared with an increase of 30% in sensory neurons ($p < 0.02$). In contrast, application of PDBu to motor neurons in two experiments resulted in increases of 27 and 31%

in the translocation index ($p < 0.02$ compared with 5-HT), which is comparable to the translocation induced by concurrent 5-HT and firing in sensory neurons.

Overexpression of a dominant-negative form of Apl-I in sensory neurons blocks a form of intermediate-term synaptic facilitation

Sensorimotor synapses of *Aplysia* display several different forms of facilitation, which can be distinguished by the conditions required for their induction, by their requirement for transcription or translation and by their time course (Montarolo et al., 1986; Ghirardi et al., 1995; Sutton and Carew, 2000; Sharma et al., 2003). In contrast to the short-term synaptic facilitation that is induced by 5-HT alone, repeated stimulation of the sensory neuron in the presence of 5-HT leads to an ITF that can last several hours (Bailey et al., 2000; Sutton and Carew, 2000; Sutton et al., 2004). The induction of ITF can be blocked by inhibitors of PKC (Sutton and Carew, 2000; Sutton et al., 2004), indicating that PKC activity contributes to this form of synaptic plasticity. However, these experiments did not reveal which isoform of PKC is involved or whether it acts in the presynaptic or the postsynaptic neuron. Given the requirement for both 5-HT and electrical stimulation for the translocation of Apl I in sensory neurons, it seemed likely that this isoform of PKC might contribute to ITF at the sensorimotor synapses. Because introduction of a dominant-negative form of Apl II into the sensory neuron was shown to be effective in blocking facilitation of depressed synapses by 5-HT alone (Manseau et al., 2001), we tested whether ITF could similarly be blocked by overexpressing a dominant-negative version of Apl I in sensory neurons.

PKC-dependent ITF was demonstrated in intact ganglia (Sutton and Carew, 2000). In cultures, a form of ITF dependent on 5-HT and activity has also been described (Bailey et al., 2000), but using a different protocol. First, we determined that we could induce ITF in sensorimotor cocultures with a protocol similar to the one used to induce PKC-dependent ITF in ganglia. Four trains of high-frequency firing in the sensory neuron in the presence of 5-HT resulted in an increase in the postsynaptic potential that lasted for at least 1 h after the 5-HT was removed, characteristic of ITF (Fig. 7). In contrast, the STF induced by application of 5-HT alone decayed by 30 min, and firing the sensory neuron in the absence of 5-HT also did not lead to ITF (Fig. 7).

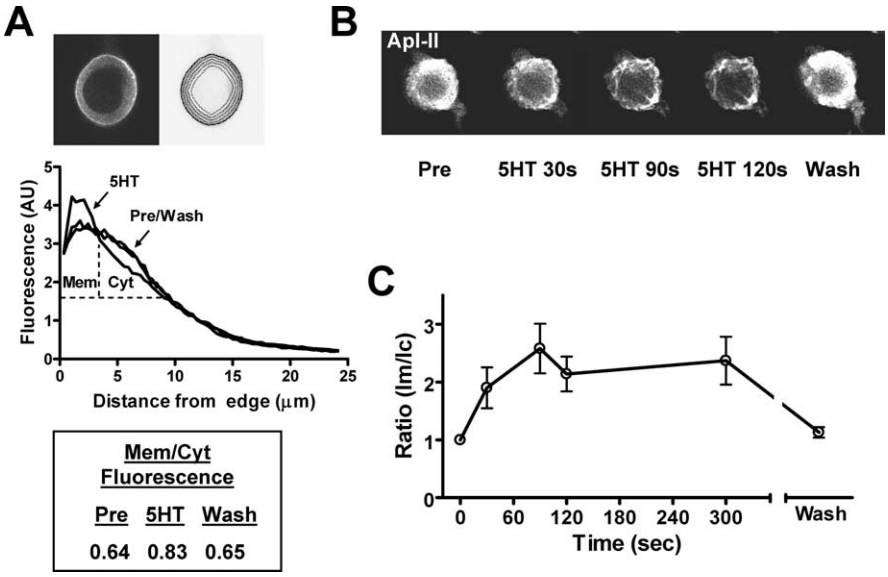

Figure 5. Time course of translocation of eGFP-Apl II. ***A***, Quantification of translocation. The fluorescence of concentric shells one pixel in thickness was measured beginning at the periphery of a sensory neuron cell body (top). The fluorescence of concentric shells is plotted against distance from the edge of the neuron before (Pre) and during application of 5-HT and after washout (middle). The total fluorescence in the outermost 3–4 μm (Mem) increases with 5-HT application, the fluorescence of the next 6–7 μm (Cyt) decreases, and the fluorescence of the innermost ~15 μm, representing the nucleus, does not change. The ratios of the total fluorescence associated with the membrane to the total fluorescence associated with the cytoplasm are given in the box at the bottom. ***B***, Projections of a sensory neuron expressing eGFP-Apl II before, during, and after application of 5-HT. ***C***, Summary plot showing ratio of fluorescence intensity of the membrane to that of the cytoplasm in three to seven separate experiments; the ratio is expressed relative to the baseline value before 5-HT application (time 0). Error bars represent SEM.

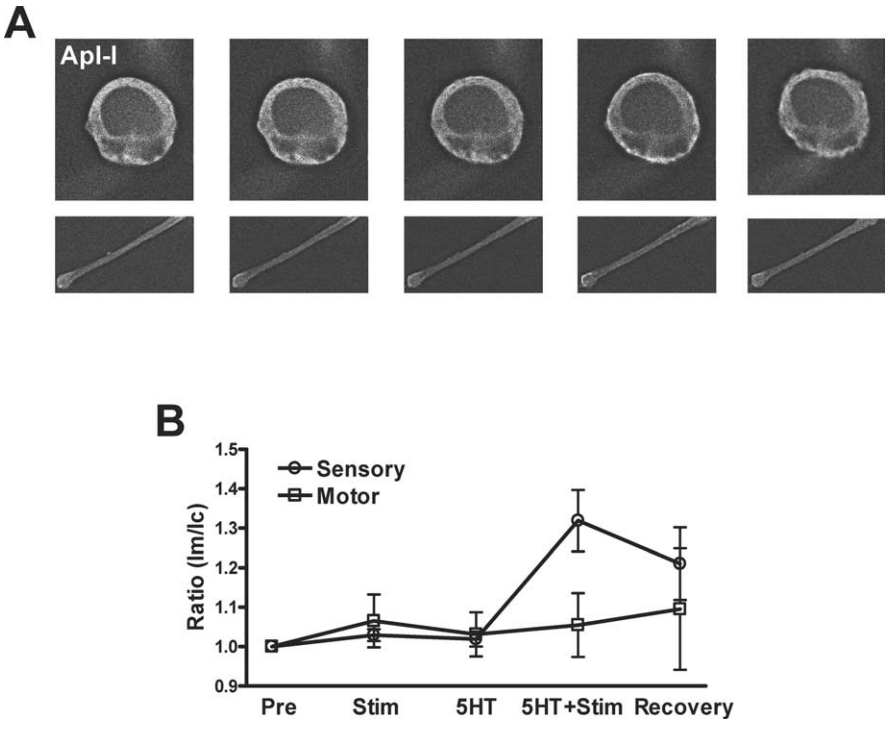

Figure 6. Translocation of calcium-dependent Apl I requires sensory neuron firing concurrent with 5-HT application. ***A***, Cell body (top) and neurite (bottom) of a sensory neuron expressing eGFP-Apl I in control condition (Pre), with stimulation of the sensory neuron at 10 Hz (Stim), subsequent application of 5-HT, stimulation in the presence of 5-HT (5-HT+Stim), and after cessation of stimulation, still in the presence of 5-HT (Recovery). Translocation of fluorescence is clear only with conjoint 5-HT and firing and recovers when the stimulation is stopped. ***B***, Ratio of membrane fluorescence to cytoplasmic fluorescence (calculated as in Fig. 5*A*) in three to nine experiments on sensory neurons and two to five experiments on motor neurons. Translocation in the sensory neurons is significantly greater than that in the motor neurons ($p < 0.01$; two-way ANOVA).

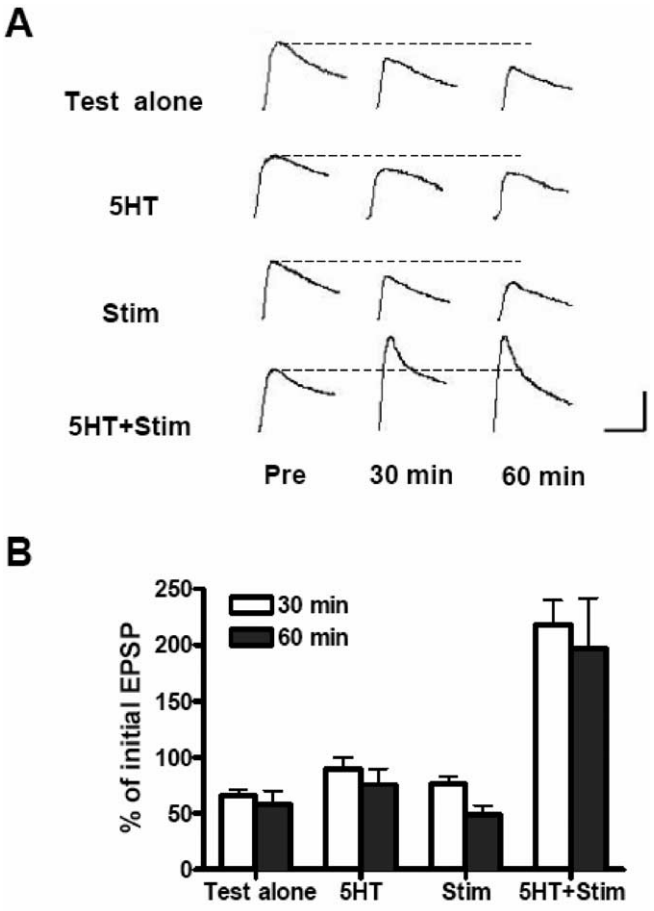

Figure 7. Intermediate-term facilitation induced by combined 5-HT and sensory neuron firing. ***A***, Postsynaptic potentials in motor neurons elicited with single action potentials in sensory neurons in cocultures were compared with baseline EPSPs (Pre) 30 or 60 min after no treatment (Test alone), after a 5 min application of 5-HT alone or four trains of action potentials in the sensory neuron in the absence of 5-HT (Stim), or after combined 5-HT and stimulation of the sensory neuron. Only combined 5-HT and firing resulted in significant facilitation of the EPSP. Calibration: 20 mV, 25 ms. ***B***, Summary of experiments like those in ***A***, showing that only the combination of 5-HT and firing in the sensory neurons gives rise to intermediate-term facilitation at 30 and 60 min time points ($p < 0.001$ for both times; two-way ANOVA).

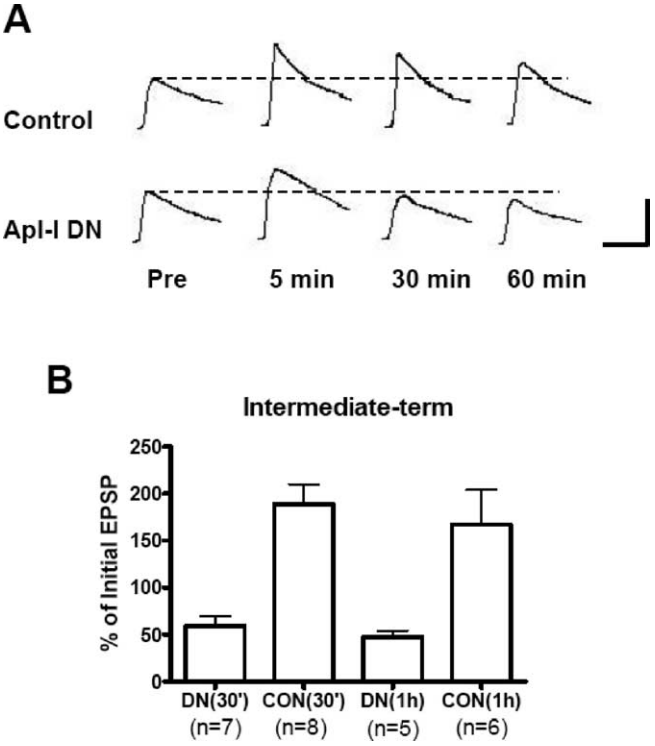

Figure 8. Overexpression of a dominant-negative version of Apl I in the sensory neuron blocks ITF. ***A***, Sample traces from experiments in which the ITF induction protocol was applied to a control culture and to a culture in which the sensory neuron expressed a dominant-negative (DN) form of Apl I. Both cultures show STF after a 5 min exposure to 5-HT, but only the control shows ITF at 30 and 60 min. Calibration: 20 mV, 25 ms. ***B***, ITF was blocked in cocultures that expressed dominant-negative Apl I ($p < 0.001$ at 30 min, $p < 0.01$ at 60 min; two-way ANOVA). The controls in ***B*** consisted of cultures in which the sensory neuron was not injected with any plasmid or cultures in which the sensory neuron expressed a wild-type eGFP-Apl I. The two types of control were not different from each other ($p > 0.05$) and were therefore pooled.

Overexpression in the sensory neuron of an altered form of eGFP-Apl I in which the catalytic lysine is converted to arginine and the autophosphorylation sites required for correct folding are converted to glutamic acid (Manseau et al., 2001) completely blocked the facilitation of the postsynaptic potential at 30 min and 1 h (Fig. 8) but had no effect on STF caused by 5-HT alone ($p = 0.4$; t test). Overexpression of wild-type Apl I-eGFP had no effect on the ITF induced by pairing of firing with 5-HT at any time point (Fig. 8*B*). Similarly, overexpression of dominant-negative Apl II in the sensory neurons also did not reduce ITF (Fig. 9) in contrast to the inhibition by this construct of short-term reversal of depression caused by 5-HT alone. These experiments thus demonstrate that a functional calcium-dependent Apl I in the sensory neuron is necessary for the ITF induced by the pairing of 5-HT with sensory neuron firing.

Discussion

Properties of PKC isoforms determine different requirements for induction of synaptic plasticity

The induction of different forms of synaptic plasticity and their behavioral consequences varies widely depending on the stimulation protocol, neuronal activity, and the time elapsed since the presentation of the stimulus (Neveu and Zucker, 1996; Nguyen and Kandel, 1996; Wang and Kelly, 1996; Huang, 1998; Zucker, 1999; Lee et al., 2000; Luscher et al., 2000; Soderling and Derkach, 2000; Yasuda et al., 2003). Different kinds of plasticity are distinguished by their dependence on transient covalent modifications, persistent enzyme activity, or the synthesis of new RNA or protein. The sensorimotor synapses that mediate defensive withdrawal in *Aplysia* display diverse types of short-term, intermediate-term, and long-term facilitation, depending on whether the facilitatory neurotransmitter is presented once or repeatedly, and whether or not the presence of the transmitter is accompanied by firing in the sensory neuron. All of these types of plasticity involve the activity of protein kinases, as well as the actions of other enzymes such as phosphatases and proteases.

At sensorimotor synapses of *Aplysia*, 5-HT acting through PKC is involved in two types of synaptic facilitation, STF of depressed synapses, and ITF resulting from concurrent 5-HT and sensory neuron firing. We now show how each of these forms of facilitation relies on the respective properties of two isoforms of PKC. Unlike biochemical assays, these imaging experiments demonstrate activation of PKCs under physiological conditions in individual living neurons and in real time.

STF of depressed synapses is mediated by the calcium-independent PKC Apl II in the sensory neurons (Manseau et al., 2001). We show by direct visualization of Apl II in living sensory neurons that its activation requires only the presence of 5-HT,

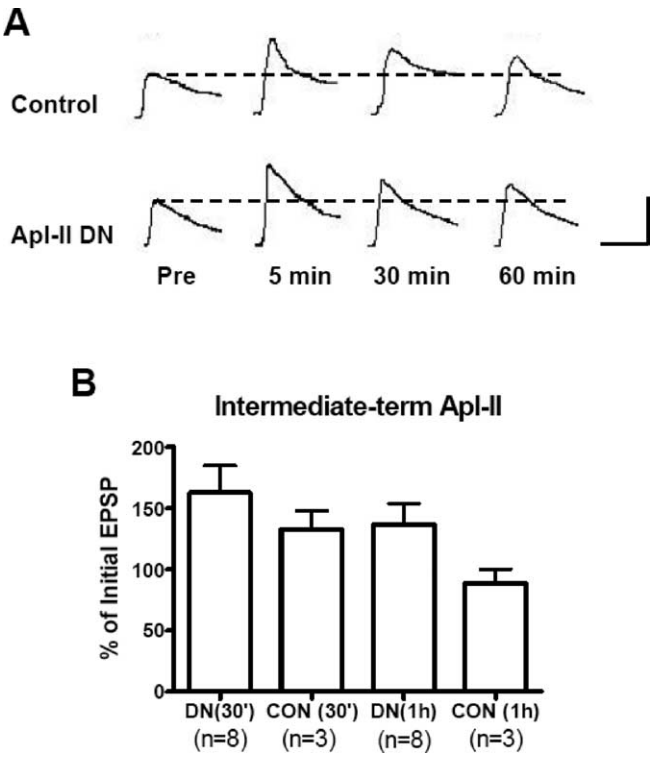

Figure 9. Overexpression of a dominant-negative version of Apl II in the sensory neuron does not reduce ITF. ***A***, Sample traces from experiments in which the ITF induction protocol was applied to a control culture and to a culture in which the sensory neuron expressed a dominant-negative (DN) form of Apl II. Both cultures exhibit normal STF and ITF. Calibration: 20 mV, 25 ms. ***B***, Summary data for cultures expressing dominant-negative Apl II (DN) and uninjected controls. Facilitation by 5-HT is not different between the two sets of cultures ($p > 0.05$; two-way ANOVA).

and that this activation can be detected in several cellular compartments, including presynaptic terminals (Fig. 4). Consistent with the physiological experiments of Manseau et al. (2001), Apl II translocates from cytoplasm to membrane in sensory neurons but not in motor neurons (Fig. 4*C*).

ITF induced by pairing 5-HT with sensory neuron firing requires PKC activity for its induction and depends on persistent PKC activity for its maintenance (Sutton and Carew, 2000; Sutton et al., 2004). Our finding that translocation of calcium-dependent isoform Apl I to the membrane also requires conjoint 5-HT and firing (Fig. 6), suggested that Apl I could be the PKC that is responsible for this form of facilitation. This hypothesis is supported by the total block of ITF by dominant-negative Apl I (Fig. 8).

Translocation of Apl II by 5-HT alone

We find that 5-HT alone translocates only Apl II in sensory neurons, using both immunocytochemical detection of endogenous Apl II (Fig. 3) and imaging of eGFP-Apl II (Fig. 4). The fluorescent tag does not affect the requirements of Apl II for activation *in vitro* (Manseau et al., 2001). Also, the level of overexpression is not extremely high, because expressing cells show less than two-fold the immunoreactivity of noninjected cells (data not shown). Although contributions from background staining and/or nonlinearities in immunofluorescence are not known, we are likely looking at translocation under close to physiological conditions. Moreover, these results are consistent with the finding that dominant-negative Apl II, but not Apl I, blocked reversal of synaptic depression by 5-HT (Manseau et al., 2001).

In Sf9 cells, low concentrations of DOG translocated Apl II, but not Apl I (Fig. 1). Thus, a likely explanation for the selective translocation of Apl II in sensory neurons is that 5-HT activates a G-protein coupled receptor (GPCR), which leads to activation of phospholipase C, producing levels of DAG sufficient to translocate Apl II. However, phospholipase C activity should also produce IP-3 that releases calcium from internal stores, and 5-HT does not increase resting calcium levels in sensory neurons, whereas injected IP_3 does (Blumenfeld et al., 1990). Alternatively, it is possible that 5-HT activates phospholipase D, producing phosphatidic acid, which is then converted to DAG (Becker and Hannun, 2005); in this case, IP_3 is not produced. The failure of Apl II to translocate in motor neurons is most simply explained by the lack of a 5-HT GPCR coupled to DAG production.

Our results are inconsistent with previous biochemical experiments that failed to detect Apl II activation after short applications of 5-HT (Sossin and Schwartz, 1992; Sossin et al., 1994). The most likely explanation for this is the limited sensitivity of these assays using whole ganglia to detect translocation in a small subset of neurons.

Differential translocation of Apl I and Apl II

The better translocation of Apl II by DOG alone compared with Apl I could be attributable to a higher affinity of the C1 domain of Apl II for DOG, as has been shown for Ca^{2+}-independent PKCs in vertebrates (Giorgione et al., 2006). Although the C1 domains of Apl I and Apl II bind phorbol esters to a similar extent (Pepio et al., 1998), this does not necessarily predict affinities for DOG (Slater et al., 1996). Alternatively, because the availability of the C1 domain to DAG is restricted by the C2 domain (Pepio and Sossin, 1998; Medkova and Cho, 1999; Slater et al., 2002), calcium may be required for access of DAG to the C1 domain in Apl I, whereas cofactors that allow access are either not required for Apl II, or are already present in cells.

Discrepancy with previous biochemical findings

Our findings contradict models previously generated using assays of purified *Aplysia* PKCs (Pepio et al., 1998) or PKCs from *Aplysia* nervous system extracts (Sossin and Schwartz, 1992; Sossin et al., 1993). In these assays, Apl I did not require calcium for activation and indeed, its kinase activity was increased at similar or lower amounts of DOG than for Apl II, even in the absence of calcium (Pepio et al., 1998). This discrepancy could reflect nonphysiological aspects of the kinase assay, where the lipids are presented to the kinase in a way quite distinct from the way lipids are presented in the cell (Sando, 2003). Thus, it is possible that *in vitro* Apl I is activated by lipids without translocation, whereas *in vivo* translocation is required for activity. In support of this, the physiological results using dominant-negative kinases match the *in vivo* translocation results, not the *in vitro* kinase assays.

Multiple signals in induction of synaptic plasticity

Many forms of synaptic plasticity require a combination of stimuli for their induction. Long-term potentiation and long-term depression induced by concurrent depolarization and binding of glutamate to NMDA receptors at CA3-to-CA1 synapses in the hippocampus are among the best-known examples. The requirement for coincidence of signals limits the induction of the plasticity to a subset of neurons or synapses, thus providing for response specificity. In most cases, induction requires an influx of calcium, either from the outside through plasma membrane channels, or by the release of calcium from intracellular sources

(Zucker, 1999). Increased calcium then activates enzymatic processes that bring about changes in synaptic transmission.

The form of ITF that we examine here requires that the presence of the modulatory transmitter 5-HT coincide with firing in the sensory neuron. This facilitation depends on the generation of a constitutively active protein kinase, termed PKM, that is derived from proteolysis of PKC by the calcium-activated protease calpain (Sutton et al., 2004). Firing in the sensory neuron provides the increase in calcium that activates calpain.

We now show that increased calcium is needed for an additional step in induction: activation of calcium-dependent Apl I. Firing or 5-HT alone does not translocate Apl I to the membrane; both are required. The complete block of ITF by dominant-negative Apl I (Fig. 8) demonstrates that Apl I is necessary for induction. Calcium thus plays a dual role: it is necessary for the initial activation of Apl I, and it is also needed for the generation of the persistently active PKM by calpain. The coincidence requirement is probably a result of the synergy between DAG and calcium required for translocation of Apl I, as seen in Sf9 cells (Fig. 2). It should be noted, however, that coupling of 5-HT and activity leads to higher levels of calcium than activity alone (Boyle et al., 1984; Blumenfeld et al., 1990; Eliot et al., 1993), and higher levels of calcium may also be required for activation of Apl I.

Our experiments indicate that Apl I is apparently the PKC isoform that is cleaved by calpain to yield the persistently active PKM. Sutton et al. (2004) showed that both Apl I and Apl II are cleaved by calpain *in vitro* but were unable to detect proteolytic products of PKC in neurons, most likely because of limitations in the sensitivity of their assay. The failure of dominant-negative Apl II to reduce ITF (Fig. 9) rules out the possibility that Apl II plays a critical role. The fact that functional Apl II and Apl I are required for STF and ITF, respectively, at the same synapses strikingly illustrates how the nervous system exploits the distinct properties of different enzyme species to generate distinct forms of synaptic plasticity and learning.

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