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Protein Kinase C Regulates Local Synthesis and Secretion of a Neuropeptide Required for Activity-Dependent Long-Term Synaptic Plasticity

Jiang-Yuan Hu, Yang Chen, and Samuel Schacher

Center for Neurobiology and Behavior, Columbia University College of Physicians and Surgeons, New York State Psychiatric Institute, New York, New York 10032

Long-term facilitation (LTF) of sensory neuron synapses in *Aplysia* is produced by either nonassociative or associative stimuli. Nonassociative LTF can be produced by five spaced applications of serotonin (5-HT) and requires a phosphoinositide 3-kinase (PI3K)-dependent and rapamycin-sensitive increase in the local synthesis of the sensory neuron neuropeptide sensorin and a protein kinase A (PKA)-dependent increase in the secretion of the newly synthesized sensorin. We report here that associative LTF produced by a single pairing of a brief tetanus with one application of 5-HT requires a rapid protein kinase C (PKC)-dependent and rapamycin-sensitive increase in local sensorin synthesis. This rapid increase in sensorin synthesis does not require PI3K activity or the presence of the sensory neuron cell body but does require the presence of the motor neuron. The secretion of newly synthesized sensorin by 2 h after stimulation requires both PKA and PKC activities to produce associative LTF because incubation with exogenous anti-sensorin antibody or the kinase inhibitors after tetanus plus 5-HT blocked LTF. The secreted sensorin leads to phosphorylation and translocation of p42/44 mitogen-activated protein kinase (MAPK) into the nuclei of the sensory neurons. Thus, different stimuli activating different signaling pathways converge by regulating the synthesis and release of a neuropeptide to produce long-term synaptic plasticity.

Key words: long-term facilitation; activity dependence; sensorin; local protein synthesis; protein kinase A; classical conditioning

Introduction

Long-term synaptic plasticity is an important cellular mechanism underlying learning and memory. Nonassociative and associative modifications of withdrawal reflexes in *Aplysia* are accompanied by changes in the properties of sensory neuron synapses (Kandel, 2001). Sensitization training or repeated applications of serotonin (5-HT) [nonassociative long-term facilitation (LTF)] produce increases in the following: (1) sensory neuron branches and varicosities, (2) sensory neuron varicosities with active zones for transmitter release, (3) transmitter release from existing presynaptic varicosities, (4) the size of the active zones, and (5) postsynaptic receptor responses (Bailey and Chen, 1983, 1988, 1989; Glanzman et al., 1990; Schacher et al., 1990; Bailey et al., 1992; Zhu et al., 1997; Kim et al., 2003; Roberts and Glanzman, 2003). Classical conditioning of withdrawal reflexes produced by pairing tactile stimulation with a sensitizing stimulus or temporal pairing of activity in sensory neurons with an application of 5-HT evoke parallel cellular changes in both presynaptic and postsyn-

aptic neurons to produce associative LTF (Buonomano and Byrne, 1990: Sun and Schacher, 1998; Roberts and Glanzman, 2003). Do different stimuli activate the same or different signaling pathways to produce LTF?

5-HT can produce nonassociative LTF by activating several signaling pathways. LTF accompanied by structural plasticity requires new gene and protein expression (Bailey et al., 1992) mediated by the timely activation of phosphoinositide 3-kinase (PI3K), protein kinase A (PKA), and mitogen-activated protein kinase (MAPK) in sensory neurons (Greenberg et al., 1987; Sweatt and Kandel, 1989; Nazif et al., 1991; Schacher et al., 1993; Martin et al., 1997; Michael et al., 1998; Muller and Carew, 1998; Purcell et al., 2003; Udo et al., 2005; Hu et al., 2006). The kinases phosphorylate both local cytoplasmic substrates (Schuster et al., 1985; Bailey et al., 1997; Martin et al., 1997; Michael et al., 1998; Angers et al., 2002; Liu et al., 2004) and transcription factors in the sensory neurons (Dash et al., 1990; Bacskai et al., 1993; Alberini et al., 1994; Martin et al., 1997; Bartsch et al., 1998; Yamamoto et al., 1999; Purcell et al., 2003). Protein kinase C (PKC) activity is enhanced by 5-HT (Sacktor and Schwartz, 1990; Sossin and Schwartz, 1992) and participates in both short- and intermediate-term facilitation (ITF) (Sugita et al., 1992, 1997; Wu et al., 1995; Sutton and Carew, 2000; Manseau et al., 2001; Sutton et al., 2004), but its role in nonassociative LTF is not clear (Sossin et al., 1994; Wu et al., 1995). Activation of MAPK in sensory neurons is mediated by 5-HT-induced increases in the synthesis, secretion, and autocrine action of sensorin, the sensory

neuron neuropeptide (Hu et al., 2004a, 2006) and other secreted factors that activate receptor tyrosine kinase (Purcell et al., 2003; Ormond et al., 2004; Sharma et al., 2006). PI3K- and rapamycin-sensitive translation is required for the rapid increase in sensorin expression, whereas PKA activity is required for the subsequent release of the newly synthesized sensorin and for regulating sensorin signaling (Hu et al., 2006).

Does the same sequence of signal pathway activations and sensorin secretion contribute to associative LTF? The pairing of action potentials in sensory neurons with 5-HT leads to the entry of calcium that acts synergistically with 5-HT through calcium/calmodulin-sensitive adenylyl cyclase to increase PKA activity sufficiently to produce LTF with a single pairing (Occor et al., 1985; Abrams et al., 1991, 1998; Sun and Schacher, 1998). Activity in the postsynaptic neuron and activation of PKC may also contribute to associative LTF (Lin and Glanzman, 1994; Murphy and Glanzman, 1997; Roberts and Glanzman, 2003; Sutton et al., 2004). We report here that sensorin synthesis and secretion are also required for associative LTF, but other signaling pathways than those required for nonassociative LTF are activated by the paired stimuli to regulate sensorin synthesis and secretion.

Materials and Methods

Cell culture and electrophysiology. Sensory neurons were isolated from pleural ganglia dissected from adult animals (60–80 g), and motor neuron L7s were isolated from juvenile abdominal ganglia (2 g) and maintained in coculture for 5 d (Montarolo et al., 1986; Rayport and Schacher, 1986). The coculture contained one sensory neuron and one L7. Some dishes also contained sensory neurons plated alone. Standard electrophysiological techniques were used to record EPSP amplitudes evoked in L7 (Schacher et al., 1993; Hu et al., 2006). L7s were held at −85 mV. EPSP amplitudes were recorded before and either 1 or 24 h after various treatments. Each sensory neuron was stimulated with a brief (0.3–0.5 ms) depolarizing pulse to evoke an action potential using an extracellular electrode placed near the cell body or axon stump of the sensory neuron. Cell bodies of some sensory neurons were removed as described previously (Hu et al., 2006). After 3 h of recovery, the cultures were treated as described below. Changes in EPSP amplitudes were measured by dividing the posttreatment EPSP amplitude by the pretreatment EPSP amplitude times 100%. Some sensory neurons received a tetanus (20 Hz for 2 s) via the extracellular electrode plus a 5 min application of 5-HT (50 μl of 50 μM) applied via micropipette placed near the SN–L7 pair (Eliot et al., 1994; Schacher et al., 1997; Sun and Schacher, 1998). Other sensory neurons received either a 5 min application of 5-HT or tetanus alone. During the paired stimuli (tetanus plus 5-HT), the tetanus alone, or 5-HT alone, the motor neuron was held at its resting potential (−50 to −55 mV). Some cultures were exposed to five applications of 5-HT (5 min each at final concentration of 5 μM) applied at 20 min intervals. Cultures were rinsed after each application with 10 ml of 1:1 mixture of L-15 medium and seawater. The protocol for nonassociative LTF lasted 90 min.

Drug treatments. Cultures were incubated for 1–2 h with anti-sensorin antibody (Ab) or with protein A-purified preimmune serum (400 ng/ml) (Hu et al., 2004a) starting immediately after the washout of the application of 5-HT. The protein synthesis inhibitor rapamycin (50 nM; Sigma, St. Louis, MO), the PI3K inhibitor LY294002 [2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one] (10 μM; Calbiochem, La Jolla, CA), the PKA inhibitor KT5720 [(9*S*,10*S*,12*R*)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid hexyl ester; 10 μM; Calbiochem], or the PKC inhibitor chelyrethrine (10 μM; Calbiochem) was added to some cultures 15 min before stimuli until 30 min after the stimuli. In other cultures LY294002, KT5720, or chelyrethrine was added for 90 min beginning at 30 min after the stimuli. The permeable peptide inhibitor S-Ht31 (10 μM; Promega, Madison, WI) of type II PKA interaction with A-kinase anchoring proteins (AKAPs) or the mutated control peptide S-Ht31P (10 μM; Promega) was added for 90 min

beginning at 30 min after tetanus plus 5-HT (Liu et al., 2004; Hu et al., 2006). Cocultures or sensory neurons plated alone were exposed for 5 min to 100 nM of active phorbol [phorbol dibutyrate (PDBu) dissolved in DMSO; Sigma] or control inactive phorbol (4α-PDBu dissolved in DMSO; Sigma).

Immunocytochemistry. Immunocytochemistry was used to monitor the expression and distribution of sensorin throughout the sensory neurons and total p42/44 MAPK or phosphorylated p42/44 MAPK in the cell bodies of sensory neurons (Hu et al., 2004a, 2006). Cultures at various times after stimuli or after the application of control solutions were rinsed briefly in artificial seawater, fixed in 4% paraformaldehyde, and processed as described previously (Liu et al., 2003; Hu et al., 2004a). Cultures were exposed to rabbit polyclonal antibody specific for sensorin (1:1000) or total p42/44 MAPK and phospho-p42/44 MAPK (1:200; Cell Signaling Technology, Beverly, MA) diluted in 2% normal goat serum in 0.01 M PBS with 0.3% Triton X-100 at 4°C for 24 h. The incubated cultures were washed in 0.01 M PBS and incubated in FITC-conjugated goat anti-rabbit IgG (1:200; Sigma) at 4°C for 4 h. After washing in 0.01 M PBS, cultures were imaged directly with the appropriate filter set for detecting the fluorescent signal. The cultures were viewed with a Nikon (Tokyo, Japan) Diaphot microscope attached to a silicon-intensified target (SIT Dage 68; Dage-MTI, Michigan City, IN) video camera, the images were processed by a Dell computer (Dell Computer Company, Round Rock, TX), and images were captured and processed by the microcomputer-controlled imaging device (MCID) software package (Imaging Research, St. Catharines, Ontario, Canada). Illumination for detecting fluorescent signals was maintained at a constant setting for all experiments. Control experiments were performed to test the specificity of the primary antibody, including the substitution of normal rabbit serum for the primary antibody and omission of the primary antibody. All controls showed little immunocytochemical reaction.

Quantification and data analysis. All data are expressed as mean change (percentage) ± SEM produced by the indicated treatments. The intensity of sensorin immunostaining was tested by measuring average fluorescent intensity in the sensory neuron cell body, in the entire main axon, and in varicosities contacting the major processes of L7 with the MCID (7.0) software package from Imaging Systems. Staining intensities for sensorin for the various experimental treatments were normalized to the intensities measured in each cellular compartment after control treatments (100%). The overall staining intensity of total MAPK or phosphorylated MAPK immunofluorescence was determined by averaging intensity for the cell body (cytoplasm plus nucleus). Staining in the nucleus or cytoplasm was determined by measuring average intensity over that area. ANOVA and Scheffé's *F* test were used to gauge significant differences between treatments.

Results

Tetanus plus 5-HT induces a rapid increase in sensorin expression and secretion to produce associative LTF

Nonassociative LTF produced by five applications of 5-HT requires the rapid increase in sensorin synthesis and the release of the newly synthesized sensorin (Hu et al., 2004a, 2006). We therefore tested whether a single pairing of tetanus plus 5-HT, known to produce associative LTF, evokes rapid changes in sensorin expression in the cell body, axon, and varicosities of sensory neurons.

Tetanus or tetanus plus 5-HT, but not 5-HT alone, produced a significant increase in the expression of sensorin, especially in the axon and distal varicosities of the sensory neurons (Fig. 1*A*–*C*). The increase in staining for sensorin was concentrated in punctate granules along the sensory neuron axon and in the distal sensory neuron varicosities contacting L7 (Fig. 1*A*,*B*). Compared with controls (n = 15 cultures; normalized to 100%), staining for sensorin in the axon at 0.5 h increased by 2.5-fold (251 ± 25%) after tetanus (n = 6) or by nearly threefold (293 ± 19%) after tetanus plus 5-HT (n = 11). Sensorin staining in the varicosities at 0.5 h increased by nearly 2.5-fold after tetanus (230 ± 13%) or
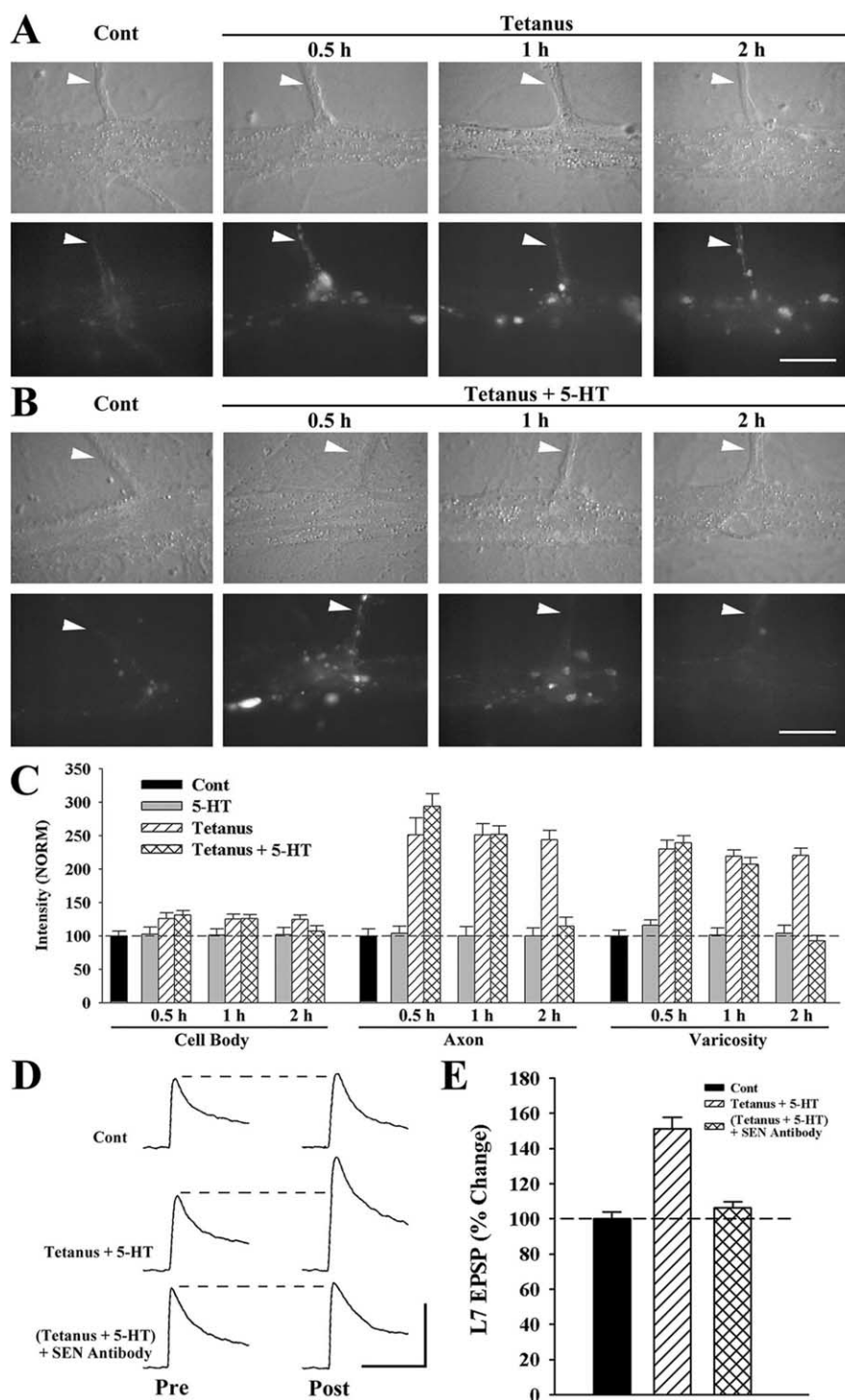
Figure 1. Tetanus plus 5-HT increased both sensorin expression and release required for associative LTF. *A*, *B*, Nomarski contrast (top) and sensorin immunofluorescent images (bottom) of cocultures 0.5 h after control (Cont) (mock application) or at 0.5, 1, and 2 h after tetanus (2 s at 20 Hz; *A*) or after tetanus plus 5-HT (2 s at 20 Hz plus 5 min application; *B*). Staining for sensorin, confined primarily to granules in the sensory neuron axons (arrowheads), regenerated processes or varicosities contacting L7, increased at 0.5 h and 1 h after either stimuli, but decreased to control levels at 2 h only after tetanus plus 5-HT. The axons and processes of L7 are unstained for sensorin. Scale bar, 30 μm. *C*, Summary of sensorin immunostaining in cell body, axon, and varicosities of sensory neurons after control (Cont) and at various times after 5-HT, tetanus, or tetanus plus 5-HT. Staining in each compartment was normalized to the average staining for that compartment after control (dashed line at 100%). ANOVA indicated a significant effect of treatment (df = 18, 162; $F = 48.522$; $p < 0.001$). 5-HT alone produced no significant change in sensorin staining, but tetanus significantly increased staining in axons and varicosities at 0.5 h ($F = 5.34$ and $F = 8.027$; $p < 0.01$), at 1 h ($F = 7.004$ and $F = 8.851$; $p < 0.01$) and at 2 h ($F = 6.321$ and $F = 9.067$; $p < 0.01$). Tetanus plus 5-HT significantly increased staining in axons and varicosities at 0.5 h ($F = 12.976$ and $F = 10.72$; $p < 0.01$) and at 1 h ($F = 8.39$ and $F = 7.185$; $p < 0.01$). Sensorin staining was no longer significantly different from control or 5-HT at 2 h after tetanus plus 5-HT. Sensorin staining in the sensory neuron cell body increased modestly after tetanus or tetanus plus 5-HT but was not significantly different from control

after tetanus plus 5-HT (239 ± 11%). Staining for sensorin in the cell body increased modestly (~30%) but was not significantly different from controls or treatment with 5-HT alone. At 1 h after tetanus ($n = 9$) or tetanus plus 5-HT ($n = 12$), sensorin staining in the axons and varicosities remained 2- to 2.5-fold higher than control or 5-HT alone in both the axons and distal varicosities. At 2 h after tetanus alone ($n = 9$), sensorin staining remained nearly 2.5-fold greater than controls for both axons and varicosities (244 ± 14 and 220 ± 11%). In contrast, by 2 h after tetanus plus 5-HT ($n = 10$) staining for sensorin decreased to control levels both in the axons (115 ± 13%) and varicosities (92 ± 8%). The decline after tetanus plus 5-HT parallel results observed after five applications of 5-HT that produce nonassociative LTF (Hu et al., 2006, their Fig. 1). The results suggest that, although tetanus or tetanus plus 5-HT increased the expression of sensorin, only tetanus plus 5-HT resulted in the rapid disappearance of the newly synthesized sensorin. Because tetanus plus 5-HT produces LTF whereas tetanus alone does not (Schacher et al., 1997; Sun and Schacher, 1998), the secretion of the newly expressed sensorin may account for the reduction in staining and is critical for LTF.

To examine whether the release of the newly expressed sensorin in required for associative LTF, we incubated cultures with anti-sensorin Ab or control Ab purified from preimmune serum for 90 min starting at 0.5 h after tetanus plus 5-HT. Incubation with anti-sensorin Ab blocked associative LTF (Fig. 1*D,E*). Whereas tetanus plus 5-HT ($n = 11$ cultures incubated with control Ab) produced a significant increase in the EPSP amplitude (151 ± 7%; $p < 0.01$) 24 h after treatment, incubation with anti-sensorin Ab ($n = 13$)

←

or 5-HT. *D*, *E*, Sensorin released after tetanus plus 5-HT is required for associative LTF. EPSPs were recorded before (Pre) and 24 h after (Post) control, tetanus plus 5-HT with control Ab applied between 0.5 h and 2 h, or tetanus plus 5-HT with anti-sensorin Ab applied between 0.5 and 2 h (*D*). The height of the dashed line is the amplitude of each EPSP before treatment. Calibration: 20 mV, 25 ms. Summary of the changes in EPSPs 24 h after treatment (*E*). ANOVA indicated a significant effect of treatment (df = 2, 30; $F = 32.955$; $p < 0.001$). Tetanus plus 5-HT with control Ab produced a significant increase in EPSP amplitude compared with control and with tetanus plus 5-HT with anti-sensorin Ab ($F = 25.503$ and $F = 23.689$; $p < 0.01$). Tetanus plus 5-HT plus anti-sensorin Ab did not significantly affect EPSP amplitude compared with control. The dashed line represents the height of the bar when there was no change in EPSP amplitude.
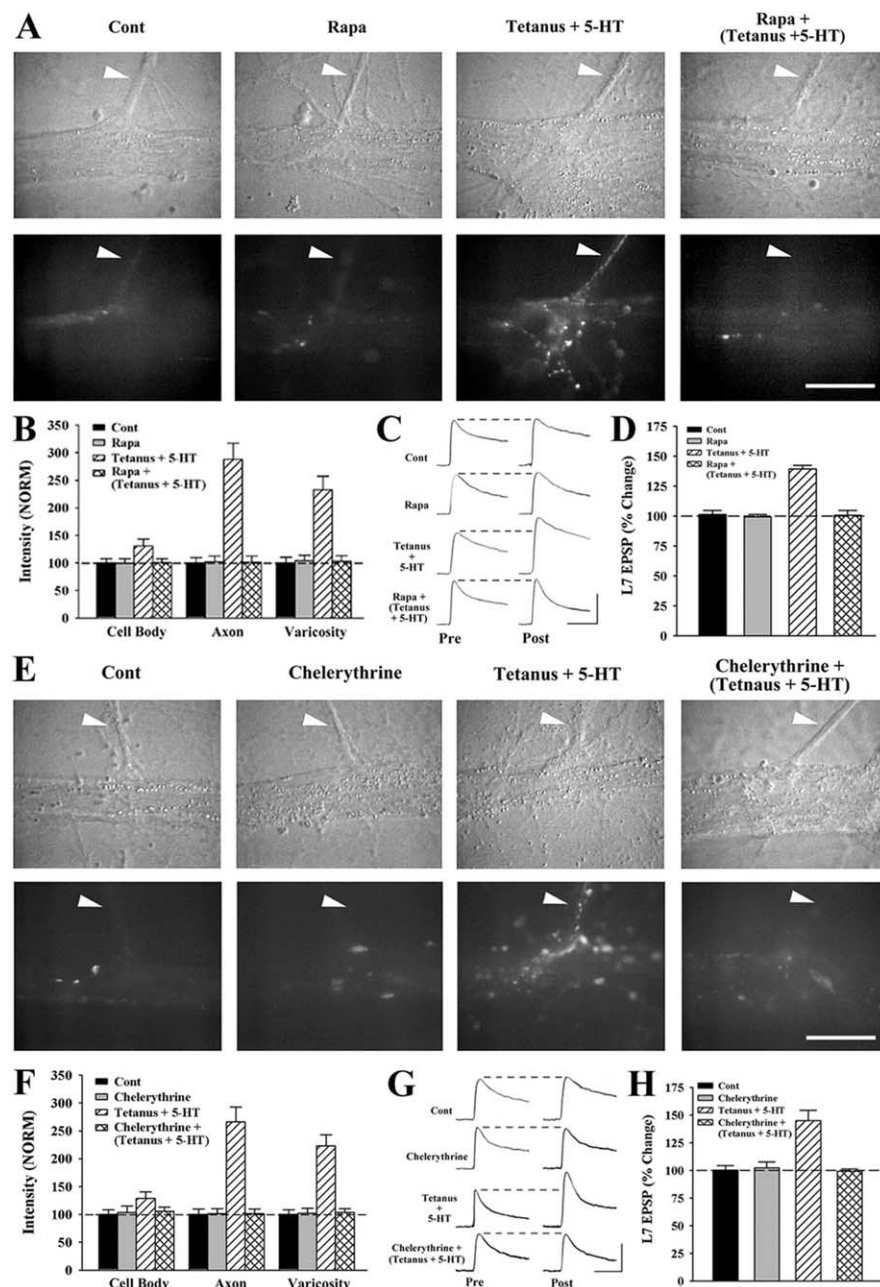
Figure 2. Rapamycin and PKC inhibitor blocked the rapid increase in sensorin and associative LTF produced by tetanus plus 5-HT. **A**, Nomarski contrast (top) and immunofluorescent images of sensorin staining (bottom) after control (Cont), after 45 min incubation with rapamycin (Rapa), 0.5 h after tetanus plus 5-HT, and 0.5 h after tetanus plus 5-HT with rapamycin. Rapamycin blocked the increase in sensorin staining in both axons (arrowheads) and varicosities produced by tetanus plus 5-HT. Scale bar, 30 μm. **B**, Summary of sensorin immunostaining after treatments. Staining in each compartment was normalized to the average staining after control (dashed line at 100%). ANOVA indicated a significant effect of treatment (df = 6, 44; F = 10.095; p < 0.001). Compared with control, tetanus plus 5-HT increased significantly sensorin staining in axons and varicosities (F = 22.131 and F = 13.874; p < 0.01). In the presence of rapamycin, tetanus plus 5-HT failed to significantly change sensorin staining compared with controls, and change in staining levels were significantly lower than the change in sensorin staining produced by tetanus plus 5-HT in the absence of drug in the axons and varicosities (F = 21.73 and F = 13.311; p < 0.01). Sensorin staining in the cell body increased modestly after tetanus plus 5-HT but was not significantly different from control or the other treatments. **C**, **D**, Rapamycin blocked associative LTF. EPSPs were recorded before (Pre) and 24 h after (Post) treatments (**C**). Calibration: 20 mV, 25 ms. Summary of the changes in EPSP amplitudes 24 h after treatment (**D**). ANOVA indicated a significant effect of treatment (df = 3, 20; F = 35.813; p < 0.001). Tetanus plus 5-HT evoked a significant increase in EPSP compared with control or tetanus plus 5-HT with rapamycin (F = 22.682 and F = 25.448; p < 0.01). Tetanus plus 5-HT with rapamycin did not significantly affect EPSP amplitudes compared with control or rapamycin alone. **E**, Nomarski contrast (top) and immunofluorescent images of sensorin staining (bottom) immediately after control (Cont), after 45 min incubation with chelerythrine, 0.5 h after tetanus plus 5-HT, and 0.5 h after tetanus plus 5-HT with chelerythrine. Chelerythrine blocked the increase in sensorin staining in both axons (arrowheads) and varicosities produced by tetanus plus 5-HT. Scale bar, 30 μm. **F**, Summary of sensorin immunostaining after treatments. Staining in each compartment was normalized to the average staining after control (dashed line at 100%).

blocked the increase in EPSP amplitude ($106 \pm 3\%$) at 24 h. The change in amplitude was not significantly different from control treatment ($n = 9$; $100 \pm 4\%$). Incubation with the antibody for 1 h after tetanus plus 5-HT did not block associative ITF measured at 1 h after tetanus plus 5-HT (Sutton and Carew, 2000; Zhao et al., 2006). One hour after tetanus plus 5-HT, EPSP amplitude increased by $191 \pm 12\%$ ($n = 10$). Incubation with anti-sensorin Ab during the 1 h period after stimulation failed to affect the increase in EPSP amplitude; the EPSP amplitude increased by $194 \pm 15\%$ ($n = 14$). Thus, the secretion of newly expressed sensorin during the period when sensorin level in the axons and varicosities of sensory neurons declines precipitously (between 0.5 to 2 h after tetanus plus 5-HT) is required for associative LTF but not associative ITF.

Rapid increase in sensorin expression produced by tetanus plus 5-HT is rapamycin sensitive and PKC dependent

Rapamycin blocks increases in the expression of sensorin after five applications of 5-HT (Hu et al., 2006). Treatment with rapamycin for 45 min beginning 15 min before tetanus plus 5-HT also blocked the increase in sensorin staining at 0.5 h (Fig. 2A,B). In the presence of rapamycin, tetanus plus 5-HT ($n = 7$) failed to increase sensorin staining compared with controls ($n = 7$; normalized to 100% for each compartment) in both axons ($102 \pm 11\%$) and varicosities ($103 \pm 10\%$). Without rapamycin ($n = 6$), tetanus plus 5-HT increased sensorin staining by nearly three-fold in the sensory neuron axons ($288 \pm 29\%$) and by nearly 2.5-fold ($233 \pm 25\%$) in the varicosities (Fig. 2A,B). The 45 min

ANOVA indicated a significant effect of treatment (df = 6, 44; F = 31.174; p < 0.001). Compared with control, tetanus plus 5-HT produced significant changes in sensorin staining in axons and varicosities (F = 19.454 and F = 17.267; p < 0.01). In the presence of chelerythrine, tetanus plus 5-HT failed to change significantly sensorin staining, and staining levels were significantly lower than the changes in sensorin staining produced by tetanus plus 5-HT without drug both in the axons and varicosities (F = 21.999 and F = 19.013; p < 0.01). **G**, **H**, Chelerythrine blocked associative LTF. EPSPs were recorded before (Pre) and 24 h after (Post) treatments (**G**). Calibration: 20 mV, 25 ms. Summary of the changes in EPSP amplitudes 24 h after treatments (**H**). ANOVA indicated a significant effect of treatment (df = 3, 23; F = 15.387; p < 0.001). Tetanus plus 5-HT evoked a significant increase in EPSP amplitude compared with control and with tetanus plus 5-HT with chelerythrine (F = 9.62 and F = 12.166; p < 0.01). Tetanus plus 5-HT with chelerythrine did not significantly affect EPSP amplitudes compared with control or chelerythrine alone.
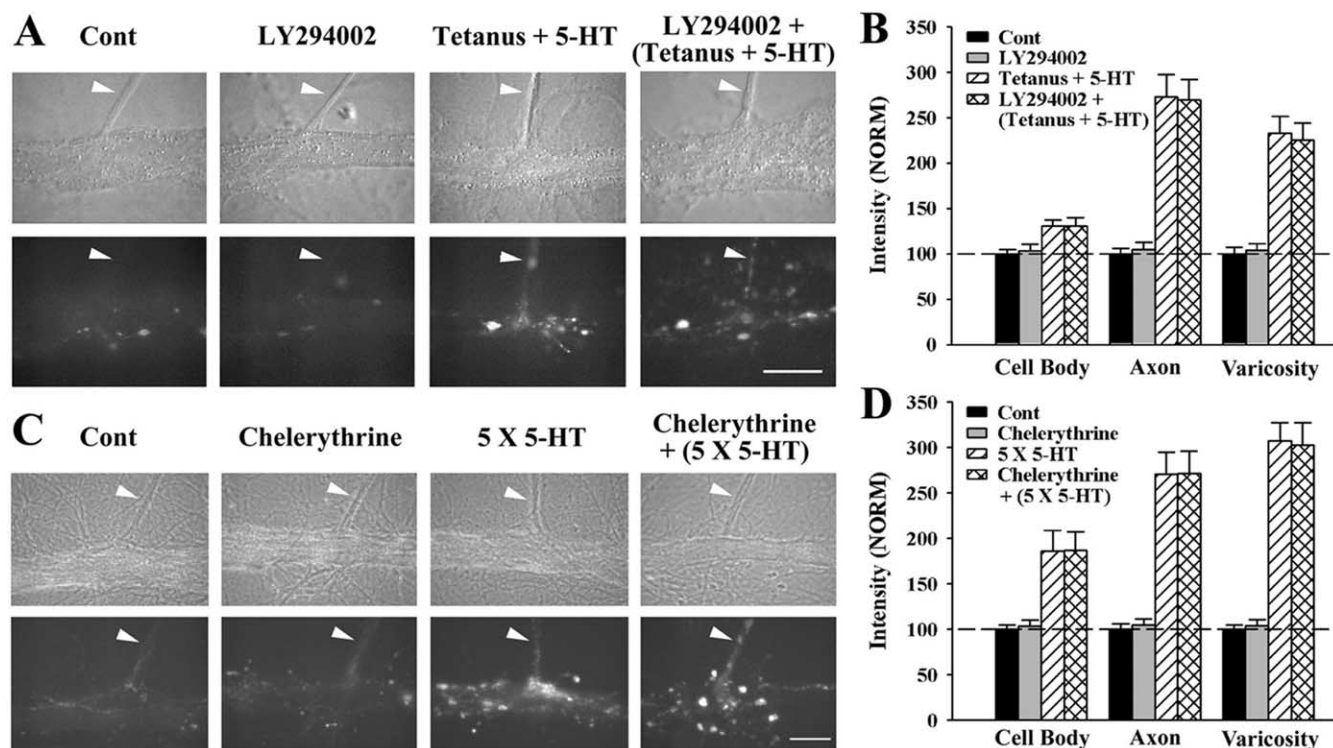
Figure 3. Signaling pathway required for regulating rapid sensorin synthesis differs for associative LTF and nonassociative LTF. *A*, *B*, PI3K activity is not required for rapid sensorin synthesis after tetanus plus 5-HT. Nomarski contrast (top) and immunofluorescent images of sensorin staining (bottom) after control (Cont), after 45 min incubation with PI3K inhibitor (LY294002), 0.5 h after tetanus plus 5-HT, and 0.5 h after tetanus plus 5-HT with LY294002. The inhibitor failed to block the increase in sensorin staining in both axons (arrowheads) and varicosities produced by tetanus plus 5-HT. Scale bar, 30 μm. *B*, Summary of sensorin immunostaining after treatments. Staining in each compartment was normalized to the average staining after control (dashed line at 100%). ANOVA indicated a significant effect of treatment (df = 6, 44; $F = 28.486$; $p < 0.001$). Compared with control, tetanus plus 5-HT significantly increased sensorin staining in axons and varicosities ($F = 14.78$ and $F = 13.332$; $p < 0.01$). In the presence of LY294002, tetanus plus 5-HT also significantly increased sensorin staining in the axons and varicosities ($F = 15.329$ and $F = 12.833$; $p < 0.01$). Staining after tetanus plus 5-HT with drug was not significantly different from the staining detected after tetanus plus 5-HT without drug. Sensorin staining in the cell body increased modestly after tetanus plus 5-HT but was not significantly different from control or the other treatments. *C, D*, PKC activity is not required for the increase in sensorin after five applications of 5-HT. Nomarski contrast (top) and immunofluorescent images of sensorin staining (bottom) after control (Cont), after 2 h incubation with chelerythrine, immediately after five applications of 5-HT, and immediately after five applications of 5-HT plus chelerythrine. The inhibitor failed to block the increase in sensorin staining in both axons (arrowheads) and varicosities produced by five applications of 5-HT. Scale bar, 30 μm. *B*, Summary of sensorin immunostaining after treatments. Staining in each compartment was normalized to the average staining after control (dashed line at 100%). ANOVA indicated a significant effect of treatment (df = 6, 44; $F = 41.679$; $p < 0.001$). Compared with control, five applications of 5-HT significantly increased sensorin staining in cell bodies ($F = 4.272$; $p < 0.05$) and axons and varicosities ($F = 13.421$ and $F = 23.232$; $p < 0.01$). In the presence of chelerythrine, tetanus plus 5-HT also significantly increased sensorin staining in the cell bodies ($F = 4.343$; $p < 0.05$) and axons and varicosities ($F = 13.602$ and $F = 22.281$; $p < 0.01$). Staining after five applications of 5-HT with drug was not significantly different from the staining after five applications of 5-HT without drug.

treatment with rapamycin alone ($n = 6$) did not significantly affect sensorin staining compared with controls ($102 \pm 11\%$ in the axons and $104 \pm 10\%$ in the varicosities). Rapamycin also blocked the increase in sensorin staining in axons and varicosities produced by tetanus alone (data not shown). Thus, the rapid increase in sensorin in both axons and varicosities depend on rapamycin-sensitive protein synthesis.

The same treatment with rapamycin also blocked associative LTF produced by tetanus plus 5-HT (Fig. 2C,D). Whereas tetanus plus 5-HT ($n = 6$) produced a significant increase in the EPSP amplitude ($140 \pm 3\%$) 24 h after stimulation compared with controls ($n = 6$; $102 \pm 3\%$), rapamycin ($n = 7$) blocked the increase in EPSP amplitude ($101 \pm 4\%$) at 24 h after tetanus plus 5-HT. The change in amplitude was not significantly different from the change with rapamycin treatment alone ($n = 5$; $99 \pm 2\%$). Thus, associative LTF that is accompanied by structural plasticity (Sun and Schacher, 1998) and is produced by a cell-wide application of 5-HT requires rapamycin-sensitive protein synthesis that includes the synthesis of sensorin (for protein synthesis-independent form of associative LTF produced by tetanus plus application of 5-HT only at the terminal region of the sensory neuron and the motor neuron, see Bailey et al., 2000).

Protein kinase C inhibitor chelerythrine, which fails to block nonassociative LTF when applied during the five applications of 5-HT (Hu et al., 2003, 2004a), blocked the rapid increase in sensorin synthesis produced by tetanus plus 5-HT (Fig. 2E,F). Treatment with chelerythrine for 45 min beginning 15 min before tetanus plus 5-HT ($n = 8$) reduced significantly the increase in sensorin staining in both the axons ($101 \pm 8\%$) and the varicosities ($103 \pm 7\%$). In contrast, tetanus plus 5-HT without the inhibitor ($n = 6$) produced significant increases in sensorin staining in both the axons ($266 \pm 27\%$) and varicosities ($224 \pm 20\%$). Compared with controls ($n = 6$; normalized to 100%), chelerythrine alone ($n = 6$) did not affect sensorin expression in axons ($101 \pm 9\%$) or varicosities ($103 \pm 9\%$). Chelerythrine also blocked the increases in sensorin staining in the axons and varicosities produced by tetanus alone (data not shown). Thus, the synthesis of sensorin is regulated by two signaling pathways: PI3K activity with five applications of 5-HT and PKC activity with tetanus plus 5-HT (see below).

The brief incubation with chelerythrine also blocked associative LTF produced by tetanus plus 5-HT (Fig. 2G,H). Whereas tetanus plus 5-HT ($n = 7$) produced a significant increase in the EPSP amplitude ($145 \pm 9\%$) 24 h after stimulation compared

with controls ($n = 6$; $100 \pm 4\%$), chelerythrine ($n = 9$) blocked the increase in EPSP amplitude ($99 \pm 2\%$) at 24 h after tetanus plus 5-HT. The change in amplitude was not significantly different from the change with chelerythrine alone ($n = 5$; $102 \pm 5\%$). Thus, associative LTF produced by tetanus plus 5-HT requires PKC activity 15 min before, during, and 30 min after stimuli. In contrast, PKC activity immediately before, during, and immediately after five applications of 5-HT is not required for nonassociative LTF.

The rapid increase in sensorin synthesis with five applications of 5-HT requires PI3K activity (Hu et al., 2006). Does PI3K activity contribute to the increase in sensorin expression after tetanus plus 5-HT? PI3K inhibitor had no effect on the rapid increase in sensorin staining after tetanus plus 5-HT (Fig. 3 *A*,*B*). We incubated cultures with the PI3K inhibitor LY294002 for 45 min beginning 15 min before pairing tetanus plus 5-HT. In the presence of LY294002 ($n = 7$), tetanus plus 5-HT increased sensorin staining by >2.5-fold in axons ($269 \pm 23\%$) and nearly 2.5-fold in varicosities ($225 \pm 19\%$) compared with controls ($n = 6$; normalized to 100%). These increases were comparable with the increase in sensorin staining in axons ($273 \pm 25\%$) and varicosities ($233 \pm 18\%$) after tetanus plus 5-HT without the inhibitor ($n = 6$). Incubation with the PI3K inhibitor alone ($n = 7$) did not significantly affect sensorin staining in axons ($104 \pm 8\%$) or in varicosities ($103 \pm 7\%$). As is the case with five applications of 5-HT (Hu et al., 2006), incubation with the PKA inhibitor KT5720 for 45 min beginning 15 min before tetanus plus 5-HT also failed to block the rapid increase in sensorin expression (data not shown). Thus, PI3K activity, which plays a crucial role in regulating the rapid synthesis of sensorin with nonassociative LTF produced by five applications of 5-HT (Hu et al., 2006), is not essential for regulating the rapid increase in sensorin after tetanus plus 5-HT.

Although blocking PKC activity during five applications of 5-HT applications fails to block nonassociative LTF, does it reduce the rapid increase in sensorin synthesis that is observed immediately after five applications of 5-HT? Treatment with chelerythrine beginning 15 min before and during five applications of 5-HT (total treatment of ~2 h) failed to block the increase in sensorin staining detected immediately after treatment (Fig. 3*C*,*D*). In the presence of the PKC inhibitor ($n = 7$), five applications of 5-HT increased sensorin staining significantly over control ($n = 6$; normalized to 100%) in each compartment: nearly twofold in the sensory neuron cell body ($188 \pm 21\%$) and nearly threefold both in the axons ($272 \pm 24\%$) and in the varicosities ($303 \pm 24\%$). These changes were comparable with the increases in sensorin staining after five applications of 5-HT without the PKC inhibitor ($n = 7$; $186 \pm 23\%$ in the cell body, $270 \pm 24\%$ in the axons, and $308 \pm 20\%$ in the varicosities). Incubation with the inhibitor alone for 2 h ($n = 6$) did not affect sensorin staining in all compartments compared with controls (103 ± 7 in the cell body, 105 ± 6 in the axons, and 104 ± 7 in the varicosities). Thus, blocking PKC activity, which blocks the rapid increase in sensorin with associative LTF (tetanus plus 5-HT), does not interfere with the rapid increase in sensorin with nonassociative LTF (five applications of 5-HT).

Because blocking PKC activity blocks the increase in sensorin after tetanus or tetanus plus 5-HT, we next examined whether activating PKC produces an increase in sensorin. Brief incubation (5 min) with PDBu produced a significant increase in sensorin at 0.5 h (Fig. 4). Compared with control untreated cultures ($n = 6$; normalized to 100% for each compartment), brief exposure to PDBu ($n = 8$) increased sensorin staining in the sensory neuron
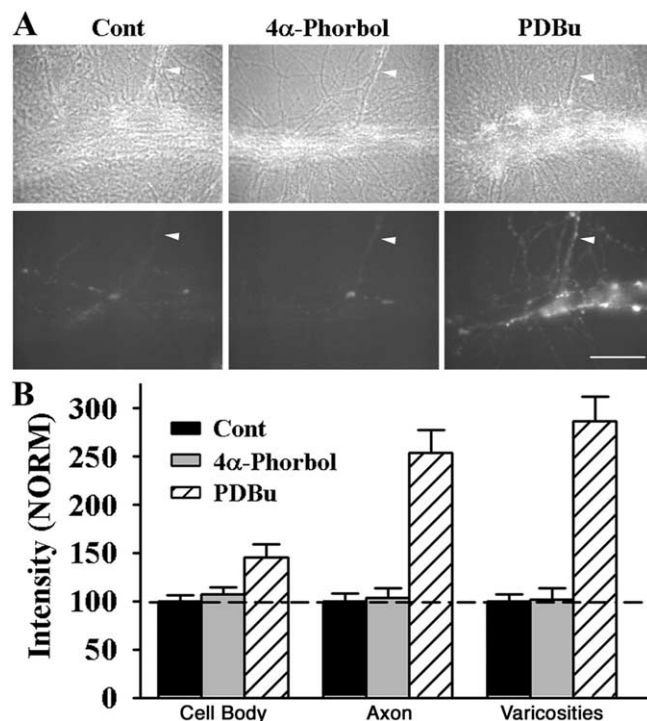

Figure 4. PDBu rapidly increased sensorin in sensory neurons. *A*, Phase contrast (top) and immunofluorescent images of sensorin staining (bottom) in sensory neuron axons (arrowheads) and varicosities immediately after control treatment (Cont), 0.5 h after 5 min application of inactive phorbol (4α-phorbol), and 0.5 h after 5 min application of PDBu. Sensorin staining increased in the axon and varicosities only after treatment with PDBu. Scale bar, 50 μm. *B*, Summary of sensorin immunostaining after treatments. Staining in each compartment was normalized to the average staining after control (dashed line at 100%). ANOVA indicated a significant effect of treatment (df = 4, 34; $F = 51.524$; $p < 0.001$). PDBu significantly increased sensorin staining compared with control or 4α-phorbol in the sensory neuron cell bodies ($F = 5.76$, $p < 0.03$; or $F = 4.195$, $p < 0.05$), axons ($F = 23.379$ or $F = 22.376$; $p < 0.01$), and varicosities ($F = 26.766$ or $F = 25.337$; $p < 0.01$). Treatment with 4α-phorbol did not significantly affect sensorin staining compared with control.

cell body by nearly 50% ($146 \pm 12\%$), by >2.5-fold in the axons ($254 \pm 21\%$), and by nearly threefold in the varicosities ($286 \pm 24\%$). Brief exposure to the inactive 4α-phorbol ($n = 6$) failed to significantly affect sensorin staining ($107 \pm 7\%$ in the cell body, $103 \pm 10\%$ in the axon, and $102 \pm 10\%$ in the varicosities). Thus, activating PKC produces a rapid increase in sensorin synthesis throughout the sensory neuron.

Sensory neuron cell body is not required for the rapid synthesis of sensorin in axon and varicosities

The rapid and significant increase in sensorin at distal varicosities by 0.5 h after stimulation suggested that local translation of sensorin mRNA, which accumulates at varicosities after being transported down sensory neuron axons (Schacher et al., 1999; Hu et al., 2002, 2006; Lyles et al., 2006), contributes to local changes in sensorin levels. Local synthesis of sensorin is detected after removing the sensory neuron cell body; five applications of 5-HT produced a rapid rapamycin-sensitive and PI3K-dependent increase in sensorin synthesis in both the axons and varicosities (Hu et al., 2006). We therefore tested whether, after removing the sensory neuron cell bodies, tetanus plus 5-HT produces a rapid rapamycin-sensitive and PKC-dependent change in the expression of sensorin in the axons and distal varicosities.

Tetanus plus 5-HT produced a significant increase in sensorin in both the axons and the distal varicosities after removal of the
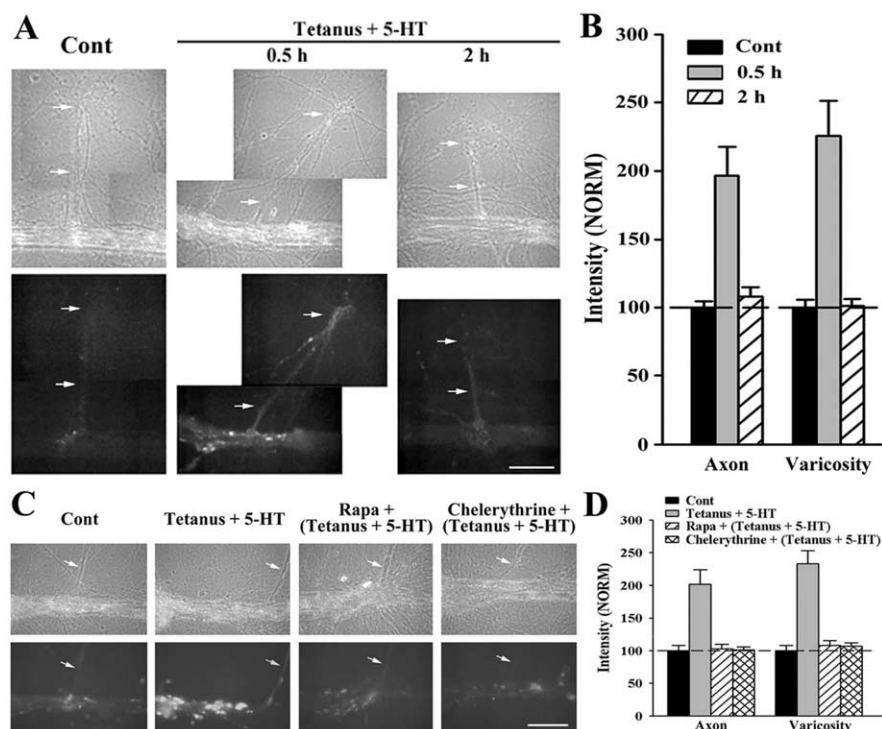
Figure 5. Rapamycin and PKC inhibitor blocked the increase in sensorin produced by tetanus plus 5-HT in the absence of the sensory neuron cell body. **A**, **B**, Without sensory neuron cell bodies, tetanus plus 5-HT rapidly increased sensorin at 0.5 h that recovered to control levels by 2 h. Phase contrast (**A**, top) and immunofluorescent images of sensorin staining (**A**, bottom) in sensory neuron axons (bottom arrows in each image) and varicosities after removal of each sensory neuron cell body (former position indicated by the top arrow in each image). After recovery, cultures were treated either with tetanus plus 5-HT or control (Cont). Sensorin staining increased at 0.5 h after tetanus plus 5-HT and returned to control levels by 2 h after stimuli. Scale bar, 50 μm. Summary of sensorin staining in axons and varicosities of sensory neurons without cell bodies after control and 0.5 and 2 h after tetanus plus 5-HT (**B**). Staining in each compartment was normalized to the average staining after control (dashed line at 100%). ANOVA indicated a significant effect of treatment (df = 2, 17; F = 6.83; p < 0.007). Tetanus plus 5-HT significantly increased sensorin staining at 0.5 h compared with control in both the axons and varicosities (F = 11.86 and F = 14.599; p < 0.01). Sensorin staining in both axons and varicosities 2 h after tetanus plus 5-HT was not significantly different from control and was significantly lower than the staining at 0.5 h after tetanus plus 5-HT in both the axons and varicosities (F = 10.767 and F = 15.53; p < 0.01). **C**, **D**, Rapamycin (Rapa) and PKC inhibitor blocked the increase in sensorin staining at 0.5 h after tetanus plus 5-HT. Phase contrast (**C**, top) and immunofluorescent images of sensorin staining (**C**, bottom) of cocultures without sensory neuron cell bodies after control application (cont), 0.5 h after tetanus plus 5-HT, 0.5 h after tetanus plus 5-HT with rapamycin, or 0.5 h after tetanus plus 5-HT with chelerythrine. Sensorin staining in axons and varicosities increased only after tetanus plus 5-HT without drug. Scale bar, 50 μm. Summary of sensorin staining in axons and varicosities of sensory neurons without cell bodies after control and 0.5 h after tetanus plus 5-HT with or without drug (**D**). Staining in each compartment was normalized to the average staining after control (dashed line at 100%). ANOVA indicated a significant effect of treatment (df = 3, 22; F = 5.794; p < 0.005). Tetanus plus 5-HT significantly increased sensorin staining at 0.5 h compared with control in both the axons and varicosities (F = 9.803 and F = 19.429; p < 0.01). Tetanus plus 5-HT with either drug did not significantly affect sensorin staining in the axons or varicosities. Tetanus plus 5-HT without drug increase sensorin staining compared with tetanus plus 5-HT with rapamycin in both axons and varicosities (F = 9.296 and F = 17.253; p < 0.01) and compared with tetanus plus 5-HT with chelerythrine in both axons and varicosities (F = 10.324 and F = 19.019; p < 0.01).

sensory neuron cell bodies (Fig. 5*A*,*B*). Compared with control (*n* = 6; 100%), tetanus (applied to axon stump) plus 5-HT (*n* = 7) significantly increased sensorin staining at 0.5 h by nearly two-fold in axons (196 ± 21%) and by nearly 2.5-fold in varicosities (226 ± 26%). As was observed for intact sensory neurons (Fig. 1), sensorin staining returned to control levels by 2 h after tetanus plus 5-HT (*n* = 7) in both the axons (108 ± 6%) and varicosities (101 ± 5%). This indicates that local translation of sensorin and the local release of the newly synthesized sensorin may contribute to associative LTF.

Although tetanus plus 5-HT increases sensorin in both the axons and varicosities even in the absence of sensory neuron cell bodies, these changes require the presence of the motor neuron L7. Stimulating sensory neurons plated without L7 with tetanus

plus 5-HT failed to significantly alter sensorin expression at 0.5, 1, or 2 h (*n* = 4 for each time point) after stimulation (data not shown). However, incubating sensory neurons for 5 min with PDBu (*n* = 10) compared with incubation with 4α-phorbol (*n* = 8; normalized to 100%) significantly increased sensorin staining at 0.5 h by 144 ± 14% in the cell body, 157 ± 11% in the axons, and 150 ± 13% in the distal neurites and varicosities. Although significant, the increases in sensorin expression in the axon and distal neurites and varicosities were lower than the increases produced by PDBu when sensory neurons contact L7 (Fig. 4). Thus, although tetanus plus 5-HT failed to increase sensorin expression, bypassing the stimulus-induced activation of PKC by direct activation of PKC with phorbol regulated the rapid expression of sensorin in isolated sensory neurons but at a lower level than observed when sensory neurons formed synapses with L7.

Because rapamycin and chelerythrine block the increases in sensorin in the intact sensory neuron after tetanus plus 5-HT, we examined whether the inhibitors interfered with the increases in sensorin staining at 0.5 h when tetanus plus 5-HT was applied after removing the sensory neuron cell bodies. The inhibitors, applied for 45 min beginning 15 min before stimulation, interfered with the local increases in sensorin staining produced by tetanus plus 5-HT (Fig. 5*C*,*D*). Compared with control (*n* = 6; 100%), rapamycin blocked the increase in sensorin staining produced by tetanus plus 5-HT (*n* = 6) in both the axons (103 ± 7%) and varicosities (107 ± 5%). The PKC inhibitor also blocked the increases in sensorin staining produced by tetanus plus 5-HT (*n* = 7) in both the axons (101 ± 4%) and varicosities (107 ± 6%). Without drugs, tetanus plus 5-HT (*n* = 7) produced a significant increase in sensorin staining in both the axons (202 ± 22%) and varicosities (233 ± 20%). Thus, much of the increase in sensorin in axons

and varicosities is produced by the actions of paired stimuli on the rapamycin-sensitive and PKC-dependent pathways that regulate local translation of sensorin mRNA present in axons and varicosities of sensory neurons. In addition, the disappearance of the newly synthesized sensorin by 2 h suggests that the machinery for packaging and releasing the newly synthesized neuropeptide is present at the distal sites.

Both PKA and PKC activities regulate the secretion of newly synthesized sensorin

The rapid release of the newly synthesized sensorin after five applications of 5-HT or tetanus plus 5-HT is required for nonassociative or associative LTF (Hu et al., 2004a, 2006) (Fig. 1). Type II PKA activity is required for the release of the newly synthesized

sensorin after five applications of 5-HT (Hu et al., 2006). We therefore examined whether blocking the signaling pathways affected the release of sensorin after tetanus plus 5-HT. Blocking either PKA activity or PKC activity blocked both the apparent release of sensorin and associative LTF.

Blocking activity of all PKA isoforms with KT5720 blocked the decline of the newly synthesized sensorin in axons and varicosities (Fig. 6*A*,*B*). Applying KT5720 between 0.5 and 2 h after stimulation interfered with the reduction in sensorin staining that normally occurs after tetanus plus 5-HT (Fig. 6*A*,*B*). The nearly 2.5-fold increase in sensorin staining in both the axons (236 ± 21%) and varicosities (265 ± 24%) at 0.5 h after tetanus plus 5-HT ($n = 6$) failed to decline by 2 h after stimulation in the presence of the drug (229 ± 15% in axons and 251 ± 24% in varicosities). In the absence of KT5720 ($n = 6$), sensorin staining in the axons declined to 105 ± 13% and in the varicosities declined to 100 ± 11% by 2 h after tetanus plus 5-HT. A similar result was obtained when cultures were incubated for 90 min starting 0.5 h after stimuli with the membrane-permeable form of a peptide sHt-31 that interferes with the interactions between type II PKA and AKAPs. Sensorin staining that increased by >2.5-fold in both the axons and varicosities 0.5 h after tetanus plus 5-HT failed to decline when cultures were incubated with the active peptide sHt-31 ($n = 7$; 231 ± 17% in axons and 243 ± 22% in varicosities) but returned to control levels ($n = 6$; 100%) when cultures were incubated with the permeable control peptide sHt-31P that has a single amino acid substitution ($n = 6$; 108 ± 11% in axons and 112 ± 15% in varicosities). Associative LTF was blocked when cultures were incubated with KT5720 between 0.5 and 2 h (Fig. 6*C*,*D*). In the presence of the drug ($n = 8$), tetanus plus 5-HT failed to increase significantly EPSP amplitudes 24 h later (109 ± 3% compared with 165 ± 7% produced by tetanus plus 5-HT without KT5720; $n = 7$). Associative LTF was also blocked by incubation with the permeable peptide sHt-31 that interferes with type II PKA–AKAP interaction. At 24 h after stimulation, EPSP amplitudes changed by only 105 ± 5% ($n = 6$). Associative LTF was produced by tetanus plus 5-HT when cultures were incubated with the control peptide sHt-31P ($n = 6$; 149 ± 6%). Thus, type II PKA activity is critical for the release of the newly synthesized sensorin and is required for associative LTF.
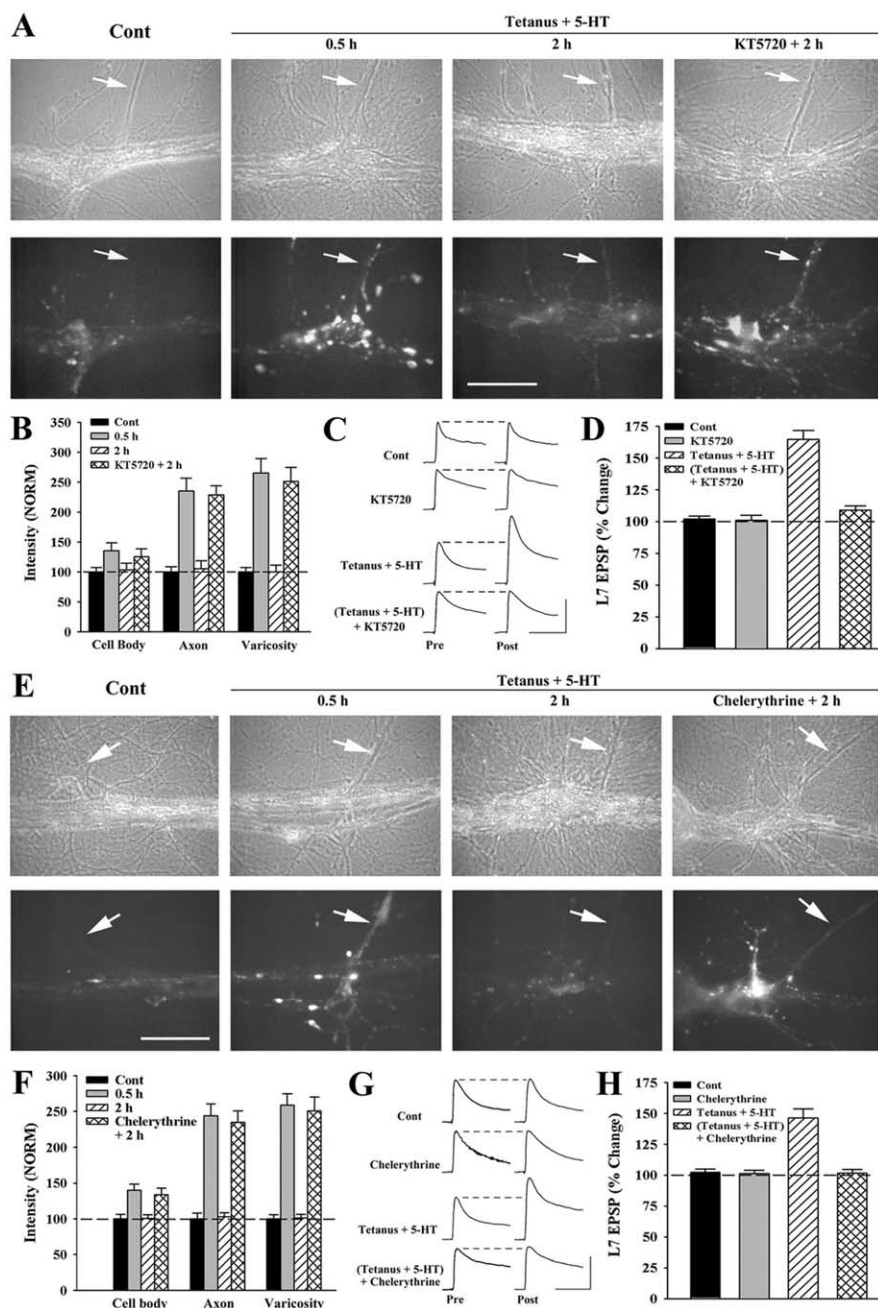

Figure 6. Both PKA and PKC activities are required for the release of sensorin after tetanus plus 5-HT and associative LTF. *A*, *B*, PKA inhibitor blocked the decline in sensorin staining 2 h after tetanus plus 5-HT. Phase contrast (*A*, top) and immunofluorescent images of sensorin staining (*A*, bottom) after control (Cont), 0.5 h after tetanus plus 5-HT, 2 h after tetanus plus 5-HT, and 2 h after tetanus plus 5-HT with KT5720 present between 0.5 and 2 h. The rapid increase in sensorin staining in axons (arrows) and varicosities at 0.5 h does not decline at 2 h with the PKA inhibitor. Scale bar, 50 μm. Summary of the changes in sensorin staining after treatments (*B*). Staining in each compartment was normalized to the average staining after control (dashed line at 100%). ANOVA indicated a significant effect of treatment (df = 6, 40; $F = 37.858$; $p < 0.001$). Compared with control, tetanus plus 5-HT at 0.5 h produced a significant increase in sensorin staining in both the axons and varicosities ($F = 13.208$ and $F = 13.86$; $p < 0.01$). Without PKA inhibitor, sensorin staining in all compartments at 2 h after tetanus plus 5-HT was not significantly different from control. Sensorin staining at 2 h after tetanus plus 5-HT with KT5720 was not significantly different from the staining at 0.5 h after tetanus plus 5-HT, remained significantly higher than control in both the axons and varicosities ($F = 11.941$ and $F = 11.61$; $p < 0.01$), and was significantly higher than the staining 2 h after tetanus plus 5-HT without drug both in the axons and varicosities ($F = 11.002$ and $F = 11.16$; $p < 0.01$). *C*, *D*, PKA inhibition between 0.5 and 2 h after tetanus plus 5-HT blocked associative LTF. EPSPs were recorded before (Pre) and 24 h after (post) control, 90 min of KT5720, tetanus plus 5-HT, and tetanus plus 5-HT with KT5720 added between 0.5 and 2 h after paired stimuli (*C*). Calibration: 20 mV, 25 ms. Summary of the changes in EPSP amplitudes 24 h after treatment (*D*). ANOVA indicated a significant effect of treatment (df = 3, 23; $F = 145.627$; $p < 0.001$). Change after tetanus plus 5-HT with KT5720 was not significantly different from control or treatment with KT5720 alone but was significantly lower than the increase produced tetanus plus 5-HT without drug ($F = 26.509$; $p < 0.01$). Tetanus plus 5-HT significantly increased EPSP amplitude compared with control ($F = 29.108$; $p < 0.01$). *E*, *F*, PKC (*Figure legend continues*)
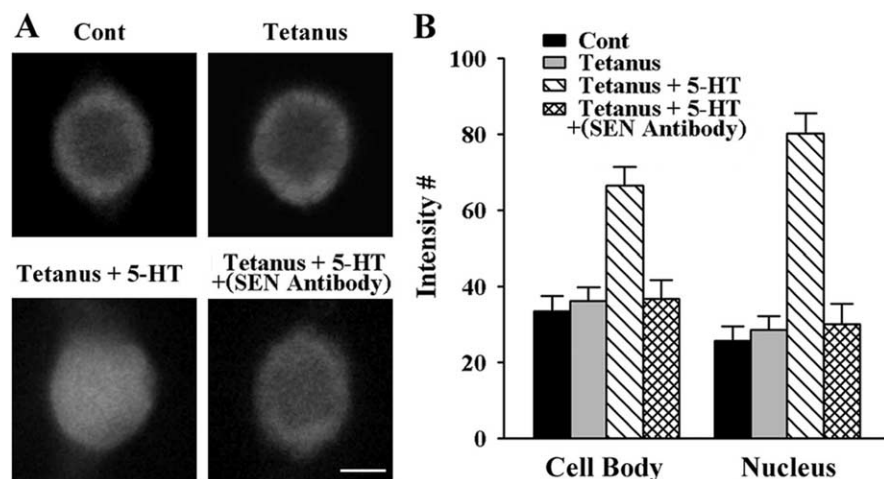
Figure 7. Activation and translocation of p42/44 MAPK in the cell bodies of sensory neurons 1 h after tetanus plus 5-HT is blocked by anti-sensorin Ab. **A**, Immunofluorescent images of phospho MAPK staining in the cell body of sensory neurons 1 h after control (Cont), tetanus, tetanus plus 5-HT, and tetanus plus 5-HT with incubation for 1 h with anti-sensorin (SEN) Ab. Note the increase in overall staining in the cell body and staining in the nucleus only after tetanus plus 5-HT. Scale bar, 25 μm. **B**, Summary of phospho-MAPK immunostaining in the whole cell body and nucleus after treatments. ANOVA indicated a significant effect of treatment (df = 3, 38; F = 35.255; $p < 0.001$). Tetanus plus 5-HT significantly increased staining in both the cell body and nucleus compared with control (F = 8.992 and F = 22.317; $p < 0.01$), tetanus alone (F = 7.234 and F = 18.89; $p < 0.01$), and tetanus plus 5-HT with anti-sensorin Ab (F = 7.748 and F = 19.843; $p < 0.01$). There was no significant difference in staining in the cell body or nucleus after tetanus plus 5-HT with anti-sensorin Ab compared with control or tetanus alone.

Blocking PKC activity also blocked the release of newly synthesized sensorin and associative LTF (Fig. 6 *E*–*H*). Incubation with chelerythrine between 0.5 and 2 h after tetanus plus 5-HT blocked the decline in sensorin staining (Fig. 6 *E,F*) normally detected by 2 h after stimulation. Staining for sensorin that increased by 2.5-fold in both the axons (244 ± 17%) and varicosities (259 ± 16%) at 0.5 h after tetanus plus 5-HT (n = 7) remained at that high level at 2 h in both the axons (235 ± 16%) and varicosities (251 ± 20%) when chelerythrine was present between 0.5 and 2 h (n = 8). In the absence of the inhibitor (n = 7), sensorin staining declined to control levels in both the axons (103 ± 5%) and varicosities (101 ± 6%). Incubation with chelerythrine between 0.5 and 2 h also blocked LTF produced by

←

(*Figure legend continued.*)

inhibitor blocked the decline in sensorin staining 2 h after tetanus plus 5-HT. Phase contrast (**E**, top) and immunofluorescent images of sensorin staining (**E**, bottom) after control, 0.5 h after tetanus plus 5-HT, 2 h after tetanus plus 5-HT, and 2 h after tetanus plus 5-HT with chelerythrine present between 0.5 and 2 h. The rapid increase in sensorin staining in axons (arrows) and varicosities at 0.5 h does not decline at 2 h with the PKC inhibitor. Scale bar, 50 μm. Summary of the changes in sensorin staining after treatments (**F**). Staining in each compartment was normalized to the average staining after control (dashed line at 100%). ANOVA indicated a significant effect of treatment (df = 6, 48; F = 42.206; $p < 0.001$). Compared with control, tetanus plus 5-HT at 0.5 h produced a significant increase in sensorin staining in the sensory neuron cell bodies (F = 3.951; $p < 0.05$) and axons and varicosities (F = 18.05 and F = 18.986; $p < 0.01$). Without PKC inhibitor, sensorin staining in all compartments at 2 h after tetanus plus 5-HT was not significantly different from control. Sensorin staining at 2 h after tetanus plus 5-HT with chelerythrine was not significantly different from the staining at 0.5 h after tetanus plus 5-HT, remained significantly higher than control both in the axons and varicosities (F = 16.071 and F = 18.052; $p < 0.01$), and was significantly higher than the staining 2 h after tetanus plus 5-HT without drug in both the axons and varicosities (F = 17.563 and F = 19.396; $p < 0.01$). **G**, **H**, PKC inhibition between 0.5 and 2 h after tetanus plus 5-HT blocked associative LTF. EPSPs were recorded before (Pre) and 24 h after (Post) control, 90 min of chelerythrine, tetanus plus 5-HT, and tetanus plus 5-HT with chelerythrine added between 0.5 and 2 h after paired stimuli (**G**). Calibration: 20 mV, 25 ms. Summary of the changes in EPSP amplitudes 24 h after treatment (**H**). An ANOVA indicated a significant effect of treatment (df = 3, 22; F = 125.062; $p < 0.001$). Change in EPSP amplitude after tetanus plus 5-HT with chelerythrine was not significantly different from control or treatment with chelerythrine alone but was significantly lower than the increase produced by tetanus plus 5-HT without drug (F = 18.471; $p < 0.01$). Tetanus plus 5-HT significantly increased EPSP amplitude compared with control (F = 15.48; $p < 0.01$).

tetanus plus 5-HT (Fig. 6*G,H*). The increase in the EPSP amplitudes 24 h after tetanus plus 5-HT (146 ± 7%; n = 6) was reduced to control levels (101 ± 3%) when chelerythrine was added between 0.5 and 2 h after tetanus plus 5-HT (n = 8). Thus, PKC activity is also required for the release of the newly synthesized sensorin and associative LTF.

Does PKC activity also contribute to the release of newly synthesized sensorin after five applications of 5-HT? Blocking PKC activity with chelyrethrine during the period of release 0–2 h after five applications of 5-HT also blocked the decline in sensorin staining normally produced in the absence of the drug. In the presence of chelerythrine (n = 8), sensorin staining remained nearly 2.5-fold higher in both the axons and varicosities (242 ± 16 and 246 ± 12%) compared with the decline to control levels in both the axons and varicosities (105 ± 5 and 108 ± 5%) 2 h after five applications of 5-HT in the absence of chelyrethrine (n = 6). The presence of chelerythrine during the 2 h period after five applications of 5-HT (n = 8) also blocked nonassociative LTF. EPSP amplitudes at 24 h changed 105 ± 3% compared with 101 ± 3% for control (n = 6) and 151 ± 4% for five applications of 5-HT in the absence of drug (n = 7). Thus, PKC activity after stimulation appears to have an important role in sensorin release after five applications of 5-HT and nonassociative LTF. PKC activity after five applications of 5-HT, but not during the applications, is critical for nonassociative LTF.

Newly released sensorin after tetanus plus 5-HT activates and translocates MAPK

Phosphorylation (activation) and translocation of p42/44 MAPK into the nuclei of sensory neurons is critical for nonassociative LTF (Martin et al., 1997; Purcell et al., 2003; Hu et al., 2004a, 2006; Sharma et al., 2006). The release of the newly synthesized sensorin after five applications of 5-HT binds to autoreceptors, leading to the activation and translocation of the MAPK (Hu et al., 2004a, 2006). We used immunocytochemistry to measure the expression and distribution of both phosphorylated p42/44 MAPK and total p42/44 MAPK in sensory neurons to determine whether the release of the newly synthesized sensorin after tetanus plus 5-HT leads to p42/44 MAPK phosphorylation and translocation into the nuclei of sensory neurons 1 h after stimulation.

The staining for phospho-p42/44 MAPK that increased significantly in both the cell body and nucleus of sensory neurons 1 h after tetanus plus 5-HT was blocked when the cultures were incubated after stimulation with anti-sensorin Ab (Fig. 7). Compared with controls, staining in the cell body increased nearly twofold and in the nucleus increased by more than threefold after tetanus plus 5-HT. In contrast, the presence of anti-sensorin Ab after tetanus plus 5-HT blocked the increases in

staining in both the cell body and the nucleus. Tetanus alone, which fails to evoke the secretion of the newly synthesized sensorin (Fig. 1), did not affect staining for phospho-p42/44 MAPK either in the cell body or nucleus. As was found after five applications of 5-HT (Hu et al., 2004a, 2006), the increase in the nucleus of phospho-p42/44 MAPK is mediated by translocation. Compared with controls (normalized to 100% in each compartment), staining for total p42/44 MAPK in the nucleus increased to 158 ± 8% at 1 h after tetanus plus 5-HT ($n = 4$). The increase in the nucleus was accompanied by a compensatory decrease to 71 ± 4% in the MAPK staining in the cytoplasm of the sensory neuron cell body. Incubation with anti-sensorin Ab ($n = 4$) blocked the change in the distribution of total p42/44 MAPK produced by tetanus plus 5-HT (normalized to control at 100% in each compartment, 101 ± 6% in the cytoplasm, and 98 ± 4% in the nucleus). Total staining for p42/44 MAPK in the cell body was unaffected by stimulation or incubation with anti-sensorin Ab. Thus, the secretion of the newly synthesized sensorin from the sensory neurons after tetanus plus 5-HT evokes the autocrine activation and translocation of phospho-p42/44 MAPK that is critical for LTF.

Discussion

Our results indicate that different stimuli activating both unique and common signaling pathways converge in regulating the synthesis and secretion of a neuropeptide whose downstream actions are critical for LTF at *Aplysia* sensory neurons synapses. With nonassociative LTF (five applications of 5-HT), PI3K, but not PKC, activity is required for the rapid, local increase in sensorin synthesis (Hu et al., 2006). In contrast, with associative LTF (tetanus plus 5-HT), PKC, but not PI3K, activity is required for the rapid, local increase in sensorin synthesis. The increase in sensorin synthesis is rapamycin sensitive. The increase in sensorin synthesis is then followed by the same sequence of events for both nonassociative (Hu et al., 2006) and associative forms of LTF: PKA- and PKC-dependent increases in sensorin secretion and sensorin-dependent activation and translocation of p42/44 MAPK into sensory neuron nuclei. Secretion of newly synthesized sensorin is required primarily for LTF and not short-term facilitation (Hu et al., 2004a) or ITF. The timely and sequential activation of the different signaling pathways mediated in part by the secretion of newly synthesized sensorin are required for LTF that is accompanied by structural plasticity (Glanzman et al., 1990; Sun and Schacher, 1998; Hu et al., 2004a).

PKC and rapamycin regulate the local synthesis of sensorin after tetanus plus 5-HT

A single pairing produced a rapid increase in sensorin translation from sensorin mRNA that is located in the cell body, axons, and the distal varicosities (Schacher et al., 1999; Hu et al., 2002; Lyles et al., 2006). Tetanus plus 5-HT, however, modestly increased sensorin in the cell body of the sensory neurons. Overall, the increase in the cell body, when sensorin levels in other compartments were at their maximums, was ~33% compared with increases in sensorin of ~90% in the sensory neuron cell bodies after five applications of 5-HT (Fig. 3C) (Hu et al., 2006). In addition, sensorin in the cell body returned to control levels by 2 h after tetanus plus 5-HT but remained significantly higher than control levels at 2 h after five applications of 5-HT (Hu et al., 2006). The quantitative differences could arise from the number of stimuli (five compared with a single pairing) and the amplitude and time course of regulating sensorin synthesis in the cell body by the two signaling pathways (PI3K compared with PKC).

When PKC is activated by phorbol, sensorin levels also increased modestly (~45%) in the cell body. The quantitative differences in regulating sensorin synthesis in the cell body may lie in the efficacy of the two signaling pathways in regulating protein synthesis.

Because 5-HT activates PKC, it was surprising that PKC did not play any role in regulating sensorin synthesis with five applications of 5-HT. However, *Aplysia* sensory neurons express several PKC isoforms (Kruger et al., 1991; Sossin, 2007). Recent studies demonstrated that 5-HT activates the calcium-independent PKC (Apl-II) in sensory neurons, whereas activity plus 5-HT activates the calcium-dependent PKC (Apl-I) (Manseau et al., 2001; Zhao et al., 2006) that is required to produce associative ITF at this synapse. Thus, differences in the activation of specific isoforms of PKC by the two types of stimulation (Apl-II by five applications of 5-HT compared with Apl-I by tetanus plus 5-HT) might explain why PKC regulates sensorin synthesis only after a single strong tetanus (20 Hz for 2 s), a single strong tetanus plus 5-HT, multiple tetani in the presence of 5-HT (Zhao et al., 2006), or maximum activation by PDBu. PI3K inhibitor failed to reduce synthesis of sensorin after tetanus plus 5-HT is likely the result of the absence of a significant activation of this pathway with a single application of 5-HT or a single pairing of tetanus plus 5-HT. Increased phosphorylation of a PI3K substrate, Akt/protein kinase B, was observed in isolated ganglia after five applications of 5-HT, whereas one application of 5-HT did not significantly increase phosphorylation compared with controls (J. Liu, J.-Y. Hu, J. H. Schwartz, and S. Schacher, unpublished observations). Thus, the activation of a specific isoform of PKC might influence sensorin synthesis after tetanus plus 5-HT, whereas sufficient activation of PI3K by repeated applications of 5-HT (Udo et al., 2005) may regulate sensorin synthesis after five applications of 5-HT. Either signaling pathway could then lead to the regulation of rapamycin-sensitive protein synthesis (Khan et al., 2001; Pepio et al., 2002) required for increasing sensorin levels and other proteins at distal varicosities even without the sensory neuron cell body. Thus, the machinery for local protein synthesis and its various regulatory pathways (PKC or PI3K dependent and rapamycin sensitive) are distributed in the cell body, axons, and distal varicosities.

Although isolated sensory neurons contain autoreceptors capable of responding to exogenous sensorin (Hu et al., 2004a), sensorin synthesis in sensory neurons did not increase after tetanus plus 5-HT when the motor neuron L7 was absent. However, bypassing stimulus-dependent activation of receptors with direct activation of PKC by phorbol led to a smaller but significant increase in sensorin expression throughout the sensory neuron when L7 was absent. This increase parallels that found by Zhao et al. (2006) who found that strong paired stimulation, repeated tetani applied to isolated sensory neurons in the presence of 5-HT, also activated type 1 PKC (Zhao et al., 2006). The absence of a significant change in sensorin expression in isolated sensory neurons with a single pairing or the smaller increase with PDBu compared with cocultures could arise from a reduction in sensorin mRNA levels at distal sites (Hu et al., 2002; Lyles et al., 2006) and a reduction in the levels of the 5-HT receptors at distal sites that lead to the activation of the appropriate PKC isoform after a single tetanus plus 5-HT (Sun and Schacher, 1996, 1998; Hu et al., 2002, 2004b). In addition, activity plus 5-HT might regulate a retrograde signal from the target cell L7 that contributes to the changes in sensorin expression in the sensory neuron. In the absence of the motor cell, this missing signal could prevent the changes in sensorin synthesis produced by tetanus plus 5-HT.

PKA and PKC regulate sensorin secretion that leads to the activation and translocation of p42/44 MAPK and LTF

Both PKA and PKC activities contribute to sensorin secretion after five applications of 5-HT (Hu et al., 2006) or tetanus plus 5-HT. Type II PKA is enriched at synapses of *Aplysia* and is maintained close to the plasma membrane by interaction between the regulatory subunit RII and membrane anchoring proteins (AKAPs) (Liu et al., 2004). This interaction is required for the release of sensorin (Hu et al., 2006). The persistent activation of type II PKA by cAMP by either five applications of 5-HT (Bernier et al., 1982; Greenberg et al., 1987; Muller and Carew, 1998; Liu et al., 2004) or pairing tetanus plus 5-HT (Abrams et al., 1991, 1998) could phosphorylate key proteins that enhance the secretion of the neuropeptide (Goodman et al., 1996; Han et al., 1999; Jovanovic et al., 2000; Angers et al., 2002). Persistent activation of PKC at synaptic terminals of sensory neurons (Sossin et al., 1994; Sutton et al., 2000, 2004; Zhao et al., 2006) may also lead to the phosphorylation of synaptic proteins necessary for secretion of neuropeptides (Nakhost et al., 2003; Houeland et al., 2007; Sieburth et al., 2007) or the phosphorylation of cytoskeletal proteins or other substrates that regulate the recruitment of neuropeptide-containing vesicles to appropriate positions near the plasma membrane for subsequent release (Knox et al., 1992; Nakhost et al., 1998, 2002; White et al., 1998; Smith, 1999; Nagy et al., 2002; Gruenbaum et al., 2003). Inhibition of either kinase immediately after stimulation blocks both release and LTF. Thus, persistent activation of the two kinases are likely to act cooperatively by either phosphorylating different sets of synaptic proteins with complementary and essential functions and/or different sites on the same proteins that regulate neuropeptide release. Because different PKC isoforms appear to be activated by five applications of 5-HT and tetanus plus 5-HT, it is unclear which isoform of PKC is regulating neuropeptide secretion during the period after stimulation. Persistent activation of an atypical PKC isoform recently identified in *Aplysia* (Sossin, 2007) after tetanus plus 5-HT or five applications of 5-HT may function in regulating secretion in a manner analogous to the persistent activation of an atypical PKC important for maintaining long-term potentiation (LTP) in hippocampus (Ling et al., 2002; Serrano et al., 2005). Thus, the persistent activation of both PKA and PKC lead to the release of the newly synthesized sensorin for different forms of LTF. Because sensorin release can occur without the sensory neuron cell body, the distal processes and varicosities must contain the appropriate apparatus for packaging and secreting this locally synthesized neuropeptide to activate other signaling pathways critical for LTF. Our results for sensorin parallel the stimulus-induced regulation of local synthesis and secretion of BDNF that are important for long-lasting forms of LTP in the hippocampus (Hall et al., 2000; Pang et al., 2004).

One such pathway activated by sensorin (and BDNF) is the p42/44 MAPK pathway, whose phosphorylated form is translocated into the nucleus of the sensory neuron. This autocrine action of sensorin is critical for the expression of both nonassociative LTF and associative LTF (Hu et al., 2004a, 2006). The activation of PKA in the cell body by 5-HT also leads to the translocation of PKA into the nucleus (Bacskai et al., 1993) and may facilitate the translocation of MAPK. PKA activation by 5-HT is a prerequisite for the activation and translocation of MAPK by sensorin (Hu et al., 2004a, 2006). The translocated MAPK and PKA phosphorylate transcription factors and other components within the nucleus to regulate gene expression required for LTF. Thus, long-lasting change in behaviorally relevant synapses is orchestrated by the timely and persistent activa- tion of signaling pathways first by the initial stimuli and then followed by the actions of a secreted neuropeptide.

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