Role for calcium/calmodulin-dependent protein kinase II in the p75-mediated regulation of sympathetic cholinergic transmission

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Neurotrophins regulate sympathetic neuron cotransmission by modulating the activity-dependent release of norepinephrine and acetylcholine. Nerve growth factor promotes excitatory noradrenergic transmission, whereas brain-derived neurotrophic factor (BDNF), acting through the p75 receptor, increases inhibitory cholinergic transmission. This regulation of corelease by target-derived factors leads to the functional modulation of myocyte beat rate in neuron–myocyte cocultures. Calcium/calmodulin-dependent protein kinase II (CaMKII) has been implicated in the control of both pre- and postsynaptic mechanisms of synaptic plasticity. We demonstrate that CaMKII acts in conjunction with p75 signaling to regulate cholinergic transmission between sympathetic neurons and heart cells. Inhibition of presynaptic CaMKII prevents the BDNF-dependent shift to inhibitory neurotransmission, whereas presynaptic expression of a constitutively active CaMKII results in inhibitory neurotransmission in the absence of added BDNF, suggesting that activation of presynaptic CaMKII is both necessary and sufficient for a shift from excitatory to inhibitory transmission. Several isozymes of CaMKII are expressed in sympathetic neurons, with the δ-CaMKII being activated by BDNF and nerve growth factor. Activated CaMKII is less effective at promoting cholinergic transmission in the absence of p75 signaling, demonstrating that p75 and CaMKII act to coordinate neurotransmitter selection in sympathetic neurons.

brain-derived neurotrophic factor  | p75  | sympathetic neurons

Developing and cultured sympathetic neurons can express both noradrenergic and cholinergic properties and are capable of coreleasing norepinephrine (NE) and acetylcholine (ACh) (1–6). Ultrastructural studies of these dual noradrenergic/cholinergic neurons have identified morphologically distinct populations of vesicles (7–9), and localization of the vesicular monoamine transporters and vesicular acetylcholine transporter suggest that NE and ACh are stored in distinct vesicle pools, even within the same synaptic varicosity (10–15). ACh and NE are released from sympathetic neurons in an activity-dependent manner, and this release can be modulated by target-derived factors (16, 17).

Nerve growth factor (NGF) and brain-derived neurotrophic factors (BDNF) are two members of the neurotrophin family of neurotrophic factors that have distinct effects on sympathetic cholinergic and noradrenergic transmission. Neurotransmission between sympathetic neurons and cardiac myocytes can be measured in culture by monitoring the beat rate of spontaneously contracting cardiac myocytes during stimulation of a connected neuron (18). NGF promotes the activity-dependent release of NE from neonatal sympathetic neurons onto myocytes, resulting in an increase in the beat rate of the myocytes (16). In contrast, BDNF potentiates the activity-dependent release of ACh, resulting in the inhibition of myocyte beat rate (17). BDNF-induced cholinergic transmission takes place through a presynaptic mechanism and, because BDNF does not regulate the expression of cholinergic genes or the localization of cholinergic markers (17, 19), is likely to reflect the release of a preexisting cholinergic vesicle pool. Cotransmission of NE is not affected by BDNF (17), suggesting that, in addition to the general effect of activity on the release of both NE and ACh, specific mechanisms exist that regulate the ratio of the release of the transmitters.

Whereas NGF modulates excitatory transmission via the TrkA receptor tyrosine kinase (16), BDNF-dependent modulation of inhibitory transmission is mediated by the p75 receptor (17). These findings contribute to a recent body of work demonstrating that Trk and p75 signaling mediate opposing pathways in nervous system development and function (20). Neurotrophin activation of Trk receptors promotes neuronal survival (21), dendritic growth (22, 23), and synaptic potentiation (24). In contrast, activation of p75 has been linked to neuronal cell death (25), inhibition of dendritic growth (26), and synaptic depression (27, 28). In the sympathetic system, increased p75 signaling rapidly promotes the selective release of ACh, raising the possibility that modifications of synaptic proteins downstream of p75 alter the release properties of some synaptic vesicles.

Modulation of components of synaptic vesicle exocytosis can be achieved by kinases that phosphorylate a wide variety of synaptic proteins (29–32). Several kinases, including mitogen-activated protein kinases, PKA, and calcium/calmodulin-dependent protein kinase II (CaMKII) have been implicated in modulating presynaptic activity (31, 33, 34). The relationship between any of these kinases and the p75-dependent modulation of cholinergic transmission is not known. The p75 receptor has no intrinsic catalytic activity but can initiate downstream signaling cascades that involve activation of kinases, including interleukin-1 receptor-associated kinase (35) and extracellular signal-regulated kinases (36). Here, we show that activation of CaMKII is necessary for p75-mediated, activity-dependent ACh release. In the context of concurrent p75 signaling, CaMKII promotes activity-dependent cholinergic transmission, suggesting that multiple parallel pathways affecting vesicle-specific synaptic release are required for ACh release from predominantly noradrenergic sympathetic neurons.

Results

Cultured neonatal sympathetic neurons form excitatory, predominantly noradrenergic connections to cardiac myocytes that can be measured as an increase in myocyte beat rate during neuronal stimulation (2, 16, 18). Growth of neuron–myocyte cocultures in BDNF results in inhibition of beat rate during neuronal stimulation (17). This inhibition results from an increase in cholinergic transmission, acting through a p75-dependent mechanism. We investigated the role of CaMKII in

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Abbreviations: ACh, acetylcholine; BDNF, brain-derived neurotrophic factor; CaMKII, calcium/calmodulin-dependent protein kinase II; NE, norepinephrine; NGF, nerve growth factor.*To whom correspondence should be addressed. E-mail: birren@brandeis.edu.

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this regulation by expressing a CaMKII inhibitory peptide (ala) in sympathetic neurons and examining the effect of BDNF on myocyte beat rate during neuronal stimulation. Neurons grown in BDNF (100 ng/ml) or the control condition were transfected with either yellow fluorescent protein (YFP) or with YFP plus the ala peptide (37). After 3 days, whole-cell recordings were obtained on individual, YFP-expressing, sympathetic neurons in close proximity to beating myocytes. The myocyte response to a series of current injections sufficient to elicit single action potentials at 2.5 Hz was recorded (16). Consistent with previous results in untransfected cells (17), we observed excitatory transmission in YFP-transfected control cultures, seen as an increase in myocyte beat rate during neuronal stimulation (Fig. 1A). Stimulation resulted in a decrease in myocyte beat rate in YFP-transfected cultures treated with BDNF, demonstrating an increase in inhibitory transmission. This shift to inhibitory transmission was blocked when the CaMKII inhibitory ala peptide was coexpressed with YFP in BDNF-treated cultures. These data demonstrate that CaMKII activation is required for the BDNF-dependent release of ACh during sympathetic neuron activity.

We investigated whether CaMKII activation was sufficient to promote cholinergic transmission by transfecting cultures with the constitutively active CaMKII(T286D) or with the kinase-dead CaMKII(K42M, T286D). Stimulation of CaMKII(T286D)-expressing neurons resulted in a decrease in the beat rate of myocytes for a majority of connected neuron–myocyte pairs. This decrease in beat rate indicates that expression of the constitutively active kinase increased inhibitory transmission, as opposed to simply blocking excitatory effects of stimulation (17). The shift to more inhibitory transmission was seen in cultures expressing CaMKII(T286D) but not in cultures transfected with the kinase-dead construct (Fig. 1B). Thus, activation of CaMKII is sufficient for a shift to inhibitory transmission in neuron–myocyte cultures. This shift to inhibitory transmission encompasses variability in the responses of individual connected neurons (see the scatter plot in Fig. 1B). This variability is also seen in the BDNF-treated cultures, suggesting that it reflects intrinsic properties of the neurons, as opposed to variability in plasmid expression levels.

Recent evidence suggests that specific CaMKII isozymes have distinct cellular localizations and function (38). We identified mRNAs for specific CaMKII isozymes present in the coculture system using RT-PCR (Fig. 2A). The α- and γ-CaMKII isozyme mRNAs were detectable in both myocytes and neurons. There was no detectable expression of β-CaMKII mRNA in neurons, although it was present in myocytes. The δ-CaMKII isozyme mRNA was seen as a strong band in both neurons and myocytes. Interestingly, δ-CaMKII is associated with neuronal development and axonal localization in P19 cells (39), whereas the α-CaMKII, but not β-, δ-, or γ has been reported to colocalize with synapsin 1 in the rat striatum (40). Thus, sympathetic neurons express CaMKII isozymes associated with presynaptic processes.

Neurotrophins could influence CaMKII signaling by regulating the production of CaMKII protein and/or by changing the level of CaMKII activation. Western blot analysis using antibodies specific for α-, δ-, and autophosphorylated (pT286) CaMKII suggests that both types of regulation take place (Fig. 2B and C). BDNF (100 ng/ml) and NGF (50 ng/ml) increased the level of activated δ-CaMKII but not α-CaMKII. We examined total CaMKII protein levels and found that NGF induced an increase in α-CaMKII protein. Smaller, but nonsignificant increases were seen for α-CaMKII protein levels in BDNF and for δ-CaMKII after either NGF or BDNF treatment. Thus, while α-CaMKII protein levels were higher in NGF-treated cultures, the fraction of enzyme in the activated state actually decreased. In contrast, there were only modest increases in δ-CaMKII protein levels in both NGF and BDNF but significant increases in the amount of δ-CaMKII in the activated state. These results suggest that δ-CaMKII is the major CaMKII isozyme that undergoes activation in response to neurotrophins in sympathetic neurons.

NGF responses in sympathetic neurons are mediated through TrkA and p75 signaling. Sympathetic neurons lack TrkB expression, however, and p75 acts as the BDNF receptor in these cells (17, 41). CaMKII activation could act downstream of the p75 receptor to promote cholinergic transmission. Alternatively, activation of p75 by BDNF or from low levels of NGF in our cultures could be required in addition to activated CaMKII. We expressed constitutively active CaMKII (T286D) in cultures prepared from wild-type and p75−/− mice (exon III, 42) to further define the relationship between p75 and CaMKII. We previously showed that mouse sympathetic neurons form excitatory connections to cardiac myocytes and that treatment with BDNF caused a shift to inhibitory transmission (17). Similar to the result in rat cultures, the majority of wild-type mouse neurons expressing the constitutively active CaMKII (T286D) displayed inhibitory transmission in the absence of BDNF (Fig. 3). In contrast, most connected p75−/− neurons showed excitatory transmission, even in the presence of constitutively active CaMKII, suggesting that, even in the presence of constitutively...
active CaMKII, concurrent activation of p75 contributes to the shift to inhibitory transmission.

Discussion
Activation of p75 by BDNF promotes inhibitory cholinergic transmission between predominantly noradrenergic sympathetic neurons and cardiac myocytes (17). We have defined a role for presynaptic CaMKII in this shift, demonstrating that expression of constitutively active CaMKII in sympathetic neurons is sufficient to drive inhibitory neurotransmission. Inhibition of endogenous CaMKII resulted in loss of BDNF-induced cholinergic transmission, defining a pathway linking p75 signaling and CaMKII with the modulation of neurotransmitter release.

Activation of CaMKII could be downstream of p75, or p75 and CaMKII may act in parallel pathways, both required for ACh release. The activation of δ-CaMKII by BDNF supports a role for CaMKII downstream of p75. However, the finding that activated CaMKII promotes inhibitory transmission more strongly in wild-type than in p75−/− neurons suggests that p75 activates additional pathways that converge to regulate sympathetic corelease properties (Fig. 4). A number of diverse signaling pathways have been linked to the p75 receptor. In addition to modulating signaling from the Trk receptors, p75 activates independent pathways that involve kinases such as c-Jun N-terminal kinase and Akt (43). Activation of p75 also promotes the release of ceramide from membrane lipids (44). Ceramide is a second messenger that has been linked to neurotrophin-mediated events such as cell death and process outgrowth (45, 46). Increased ceramide levels also promote sympathetic cholinergic transmission in the absence of BDNF (17), suggesting ceramide signaling as a candidate second pathway for the BDNF-dependent shift to cholinergic transmission.

Activation of CaMKII by neurotrophins has been described in several systems. BDNF activates CaMKII in hippocampal neurons (47), and NGF and neuronal activity collaborate through CaMKII-dependent mechanisms to regulate glutamate release (48) and dendrite growth (49). The activation of δ-CaMKII in sympathetic neurons by NGF and BDNF suggests that neurotrophin-dependent activation of CaMKII can take place through activation of either Trk or p75 receptors. NGF acts through TrkA to promote noradrenergic transmission in sympathetic neurons (16), whereas p75 and CaMKII interact to promote cholinergic transmission, suggesting that the balance of p75 and Trk signaling provides a dynamic mechanism for the regulation of sympathetic cotransmission. In this model, a p75-dependent shift in the cellular context of CaMKII activity underlies alterations in synaptic vesicle release properties to favor release of the cholinergic vesicle pool (Fig. 4). The importance of balancing p75 and Trk signaling is also seen in the regulation of survival and death (50) and in the activity-dependent regulation of sympathetic dendrite growth (51). In addition to the activation of signaling components such as ceramide, signaling through TrkA may play a dominant role in this balance. Trk activity suppresses apoptotic pathways activated by p75 (50) and inhibits p75-dependent production of ceramide (52). Thus, Trk signaling may act to limit cholinergic transmission by sympathetic neurons while promoting noradrenergic properties (16). Together, these data suggest that activation of CaMKII via multiple pathways enhances synaptic transmission with the specificity of neurotransmitter release further regulated by additional signals downstream of p75.

CaMKII is a key modulator of synaptic plasticity (53). It is present in postsynaptic densities (54), and modulates long-term potentiation (LTP) by a postsynaptic mechanism (55, 56). CaMKII is also important for presynaptic modulation (57, 58), with loss of presynaptic CaMKII at the CA3-CA1 synapse altering the response to high-frequency stimulation (59). Here, we show that CaMKII acts presynaptically to promote cholinergic transmission in sympathetic neurons, mimicking the effect of BDNF. Transmission remains excitatory when CaMKII is
The change in myocyte beat rate during neuronal stimulation was measured. Neuronal cultures were transfected with YFP and the constitutively active T286D CaMKII. The change in myocyte beat rate during neuronal stimulation was measured after a 3- to 4-day culture period. (Upper) Averaged data from all pairs showing a functional connection, mean ± SEM, n = 4 for p75+/+ T286D; n = 6 for p75−/− T286D. P < 0.06, unpaired Student’s t test. (Lower) Scatter plot showing the results of the beat rate assays performed with all YFP-expressing neurons in each condition. Horizontal lines indicate the change in beat rate cutoff for synaptic connectivity.

Fig. 3. p75 promotes the CaMKII-mediated shift to inhibitory transmission. Cocultures from wild-type mice (+/+ or p75 knockout mice (Exon III knockout, 42) were transfected with YFP and the constitutively active T286D CaMKII. The change in myocyte beat rate due to neuronal stimulation was measured 42). The plasmids used were pJPA5-CD8-YFP (gift of Gary Banker, Oregon Health Sciences University, Portland, OR), pSRα-T286D (constitutively active CaMKII) (61), pSRα-K42M/T286D (kinase-dead CaMKII) (62), and pSRα-eala (CaMKII-inhibiting peptide) (37). All CaMKII constructs were coloaded onto the gold particles with the YFP construct. On average, two to four neurons per culture dish were found to express YFP. Inhibited in the presence of BDNF, defining a role for CaMKII in the regulation of cholinergic corelease. Neither CaMKII activation nor BDNF (17) affects noradrenergic cotransmission, suggesting that CaMKII activation is not required for basal synaptic transmission. Thus, CaMKII is likely to act with p75 as part of a pathway to selectively modulate neurotransmitter release from cotransmitting neurons.

Materials and Methods

Cell Culture. Neonatal rat and mouse superior cervical ganglion neurons and cardiac myocytes were prepared as described in ref. 16. Posterior ventricular cardiac myocytes (75,000) and 15,000 neurons were plated on 35-mm collagen-coated dishes and cultured in L15CO2 medium as modified by Hawrot and Patterson (60). Cells to be used for physiology experiments were plated on glass-bottom microwell dishes (MatTek, Ashland, MA). All cultures contained 5 ng/ml NGF, a concentration sufficient to support neuronal survival but not modulate synaptic transmission. Thus, CaMKII is likely to act with p75 as part of a pathway to selectively modulate neurotransmitter release from cotransmitting neurons.

Transfection of Cultured Neurons. A Bio-Rad gene gun was used to shoot plasmid-coated gold spheres into each culture dish. Two micrograms of DNA was used per milligram of gold particles. The plasmids used were pJPA5-CD8-YFP (gift of Gary Banker, Oregon Health Sciences University, Portland, OR), pSRα-T286D (constitutively active CaMKII) (61), pSRα-K42M/T286D (kinase-dead CaMKII) (62), and pSRα-eala (CaMKII-inhibiting peptide) (37). All CaMKII constructs were coloaded onto the gold particles with the YFP construct. On average, two to four neurons per culture dish were found to express YFP. Under these conditions, we have confirmed >90% coexpression of two plasmid vectors colocalized on the same gold particles (J.D.S. and S.J.B., unpublished work).

Electrophysiology. Whole-cell current-clamp recordings were obtained from YFP-expressing neurons as described in ref. 16. Whole-cell recordings were obtained by using 3–4 MΩ patch electrodes with 2-μm diameter tips. The intracellular solution contained 130 mM KMeSO4, 10 mM KCl, 10 mM potassium-Hepes (pH 7.4), 2 mM MgSO4, 0.5 mM EGTA (Sigma), and 3 mM ATP (Sigma). Cultures were continuously perfused with artificial cerebrospinal fluid (126 mM NaCl, 3 mM KCl, 2 mM MgSO4, 1 mM NaH2PO4, 25 mM NaHCO3, 11 mM dextrose, and 2 mM CaCl2), bubbled with 5% CO2 and 95% O2. Signals were amplified and current injection controlled through an Axoclamp 2B (Axon Instruments). Evoked myocyte beating was examined by injecting 200-ms pulses of current into a neuron, eliciting single action potentials at 2.5 Hz for 3 min. The number of spontaneous contractions per minute (beat rate) of connected myocytes was counted before and during the stimulation. A neuron–myocyte pair was considered to be connected if it met the criterion described in ref. 16, based on the variability in the baseline myocyte beat rate.

RT-PCR. RNA was prepared from cocultures of neurons and myocytes by using RNeasy mini (Qiagen). Primer sets were designed by using the program PRIMER3 (63): Rat α-CaMKII, 5′-CTC TGA GAG CAC CAA CAC CA-3′; rat β-CaMKII, 5′-TTT TCA GTG GGC AGA CAG AC-3′; rat γ-CaMKII, 5′-GTT TGT CTG GGG CTT GCT GAC TC-3′; rat δ-CaMKII, 5′-CAC CTG AAG CCA AAG ACC TC-3′; rat ε-CaMKII, 5′-GAG CAG CAC CTC TTG GTG CTC TCT GGT GTA-3′; rat δ-CaMKII, 5′-GAA CTT TCT AGT TGG CAG GC-3′; rat γ-CaMKII, 5′-GAG CTA GCT GTG CTC TCT GTG GA-3′. GAPDH primer sequences were obtained from Clontech: 5′-ACC ACA GTC CAT GCC ATC AC-3′, 5′-TCC ACC ACC CTC TGG TAT TA-3′. RT-PCR was carried out as described in ref. 19 by using 35 cycles for CaMKII primers and 25 cycles for GAPDH primers. Each primer set yielded a single band whose identity was confirmed by direct sequencing.

Immunoblot Analysis. Cultures were harvested as described in ref. 16, and protein concentration was determined by using Bio-Rad Protein Assay Reagent. Equal amounts of protein were loaded for SDS/PAGE followed by transfer to Hybond-P membrane (Amersham Pharmacia Biosciences). Equal loading was confirmed by reprobing blots with anti-tubulin in three of four experiments. Primary antibodies included monoclonal anti-α
polyclonal anti-Slonimsky et al. fluorescence detection was performed with ECF substrate (Am-alkaline phosphatase-conjugated secondary antibodies, chemifcia Biosciences) and anti-goat (Jackson ImmunoResearch). For peroxidase (HRP)-conjugated anti-mouse (Amersham Pharmacia Biosciences), anti-goat (Sigma), and horseradish (Amersham Pharmacia Biosciences) antibodies used were alkaline phosphatase-conjugated anti-mouse 11. Liu, Y., Schweitzer, E. S., Nirenberg, M. J., Pickel, V. M., Evans, C. J. & Pickel, V. M. (1995) Proc. Natl. Acad. Sci. USA 92, 7362–7367.


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