into various nondividing cells. Nevertheless, these effects of Vpr on nuclear lamin and NE architecture merit further evaluation in terms of their potential to facilitate HIV infection of terminally differentiated macrophages.

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47. We thank D. Morgan, T. Hunter, H. Piwnica-Worms, and H. Worman for the expression plasmids. We also thank R. Givens for administrative assistance and J. Carroll and S. Gonzalez for graphics assistance. This work was supported in part by NIH grant R01 AI145234 (to W.C.G.) and by core facilities provided by the UCSF–Gladstone Institute of Virology and Immunology, Center for AIDS Research, P30 MH59037. M.P.S. was supported by NIH/NIAID grant KO8 AI01866.

2 July 2001; accepted 31 August 2001

Interaction of the Response Regulator ARR4 with Phytochrome B in Modulating Red Light Signaling

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The *Arabidopsis thaliana* response regulator 4, expressed in response to phytochrome B action, specifically interacts with the extreme amino-terminus of the photoreceptor. The response regulator 4 stabilizes the active Pfr form of phytochrome B in yeast and in planta, thus elevates the level of the active photoreceptor in vivo. Accordingly, transgenic *Arabidopsis* plants overexpressing the response regulator 4 display hypersensitivity to red light but not to light of other wavelengths. We propose that the response regulator 4 acts as an output element of a two-component system that modulates red light signaling on the level of the phytochrome B photoreceptor.

Phytochromes (phy) are red/far-red reversible photoreceptors that regulate many aspects of plant development (*1*). They are synthesized in the dark in the inactive Pr form and converted by red light into their active far-red light–absorbing Pfr form. In *Arabidopsis* the phy family is encoded by five genes (*PHYA* to *PHYE*), whose products forward the light signal to downstream transduction components by separate signaling mechanisms (*1*, *2*). Light signal transduction is mediated by proteins that interact with phytochromes in the cytoplasm and the nucleus (*1*, *3*). The cyanobacterial phytochrome, Cph1, is a light-regulated histidine kinase (*4*), therefore it appeared conceivable that eukaryotic phytochromes may also function as sensor kinases (*5*), yet no eukaryotic phytochromes displaying histidine kinase activity have been described (*6*).

The *Arabidopsis* EST and genome databases contain several genes for plant response regulators with similarity to the *Escherichia coli* response regulator CheY (*7*, *8*). One of the identified clones was identical with the recently published *Arabidopsis* response regulator 4 (ARR4, Fig. 1A) (*9*, *10*). We generated an ARR4-specific antiserum to analyze the expression of this protein in planta. This antiserum detected the ARR4 protein in stems, leaves, and flowers but not in roots (Fig. 1B). Thus, the expression pattern of ARR4 overlaps in great part with that of phyB. The amount of ARR4 remained below detection level in seedlings grown in darkness, whereas in light, the accumulation of the protein was detectable 1 day after germination (Fig. 1C). Accumulation of ARR4 was induced by white or red light in dark-grown seedlings, whereas far-red light was ineffective (Fig. 1D). Hourly pulses of 5 min of red light given over a period of 24 hours also induced accumulation of ARR4 protein (Fig. 1D). The inductive effect of red light was reversed by a subsequent far-red light pulse (Fig. 1D). Accumulation of ARR4 was also analyzed in *Arabidopsis phyA* and *phyB* mutants. It was reduced in red light–irradiated *phyB*-mutant seedlings, but was unaffected in *phyA*-mutant (Fig. 1E). These data suggest that phyB dominates the photoregulation of ARR4 expression, although participation of other photoreceptors, especially in white light, should not be excluded.

To investigate a physical interaction of ARR4 with phyB, copurification experiments were performed. Wild-type phyB, a mutated phyB resulting in a reduced red light–sensitive phenotype [phyB-101 (*11*)], and phyA were expressed in yeast (*12*). Functional photoreceptors were obtained by self-assembly with chromophore in crude extracts and far-

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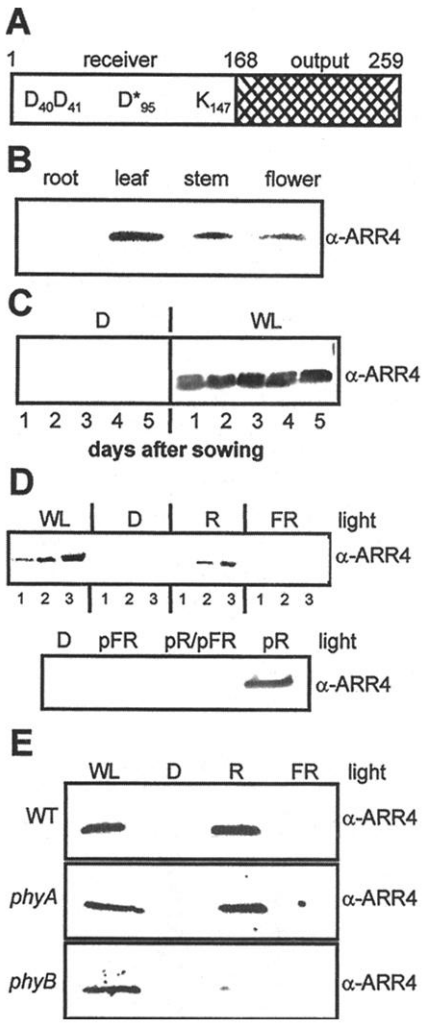

Fig. 1. Expression of ARR4 in *Arabidopsis* seedlings. (**A**) Representation of ARR4. The NH_2-terminal receiver and COOH-terminal output domains and the relative position of amino acids conserved in receiver modules are indicated (*9*, *10*). D^*_{95} is the phospho-accepting aspartate (*21*). (**B**) ARR4 is differentially expressed in *Arabidopsis*. Immunoblot with protein extracts from different tissues of 3-month-old *Arabidopsis* plants. (**C**) ARR4 accumulates in light-grown *Arabidopsis* seedlings. Immunoblot with protein extracts from seedlings grown for 1 to 5 days in darkness (D) or under continuous white light (WL). (**D**) Induction of ARR4 accumulation depends on light quality and phyB. Immunoblots with protein extracts from seedlings grown for 3 days in darkness and then exposed for 12 hours (lanes 1), 18 hours (lanes 2) or 24 hours (lanes 3) to continuous WL, R, or FR light or were exposed for 24 hours to hourly light pulses of 5 min of red (pR), 5 min of red followed by 5 min of far-red (pR/pFR) or 5 min of far-red (pFR). D, darkness. (**E**) Expression of ARR4 is differentially regulated by phytochrome photoreceptors. Immunoblots with protein extracts from wild-type (WT), *phyB* mutant (*phyB*), and *phyA* mutant (*phyA*) *Arabidopsis* seedlings grown for 3 days under continuous WL, R, or FR light or kept in darkness (D). ARR4 was detected with a specific antiserum. All techniques in Figs. 1 to 4 are as described in (*16*).

red or red light was applied to produce the inactive Pr or active Pfr forms, respectively (*12*). Equal amounts of photoreversible phyB, phyB-101, or phyA were then incubated with recombinant, Strep-tagged ARR4. In contrast to phyA, phyB, and phyB-101 copurified with ARR4 (Fig. 2A). Although the Pr form and Pfr form of phytochromes exhibit different protein conformations (*13*), ARR4 binds to both (Fig. 2A). Coimmunoprecipitation experiments with the ARR4 antiserum using protein extracts from ARR4-overexpressing *Arabidopsis* seedlings confirmed the interaction of the response regulator with phyB (Fig. 2B). To map the domains of the phyB protein that mediate this interaction we performed yeast two-hybrid analyses. ARR4 interacted with full-length phyB, the extreme NH_2-terminus of phyB ($phyB^{1-173}$) but not with any other phyB domains or with the NH_2-terminal domain of phyA ($phyA^{1-137}$; Fig. 2C). The NH_2-terminus of phyB did not interact with a different, type-A ARR, namely ARR5, and the B-type response regulator ARR2 (Fig. 2C; *9*, *14*). Thus, it appears that red light–induced accumulation of ARR4 can lead to the formation of a specific phyB:ARR4 complex in vivo because of unique properties of these molecules (*2*, *9*, *10*).

Recently, it was observed that a fraction of cytoplasmic phyB is transported into the nucleus in response to light treatment (*3*, *15*). Intracellular partitioning of an ARR4-GFP fusion protein in parsley protoplasts demonstrated that ARR4-GFP was detectable in the cytoplasm and the nucleus independent of irradiation (Fig. 2D), suggesting that the interaction of ARR4 with phyB is not restricted to any of these cellular compartments.

To examine the functional relevance of the observed phyB:ARR4 interaction, we generated transgenic *Arabidopsis* lines overexpressing ARR4 under the control of the constitutive viral 35S promoter (Fig. 3A). These transgenic lines had a shortened hypocotyl compared with the wild type in red light, but no difference was observed in darkness or after irradiation with far-red or blue light (Fig. 3B). A fluence-rate/response analysis of hypocotyl elongation revealed that the photoresponsiveness of a representative ARR4-overexpressing line to red light was enhanced over a broad range of light intensities (Fig. 3C). A similar hypersensitivity was observed when the root length and number of leaves developed at flowering of wild-type and transgenic seedlings were compared (*16*). These data suggest that overexpression of ARR4 specifically interferes with phyB-dependent photomorphogenic responses and that ARR4 acts on the phyB photoreceptor itself.

The photoconversion of the inactive Pr into the active Pfr form and the Pfr-to-Pr dark reversion involves major structural changes in the NH_2-terminus of the phytochrome mol-

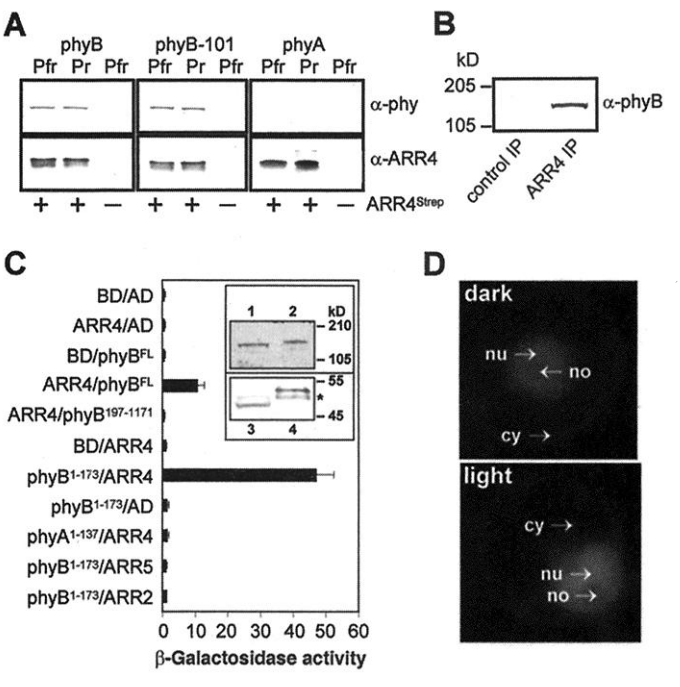

Fig. 2. ARR4 interacts with B-type phytochromes and is localized in the cytoplasmic and nuclear compartment. (**A**) ARR4 copurifies B-type but not A-type phytochromes from yeast extracts containing the corresponding Pr or Pfr forms. Copurified phytochromes were monitored by using phyB- and phyA-detecting antibodies (*22*) and ARR4 with a specific antiserum. (**B**) In planta interaction of ARR4 with phyB. The ARR4-specific antiserum coimmunoprecipitates phyB from protein extracts derived from ARR4-overexpressing seedlings. As a control the corresponding preimmune serum was used. (**C**) Phytochrome:ARR4 interaction analysis in the yeast two-hybrid system. The interaction of the indicated fusion proteins was monitored by β-galactosidase activity. The immunoblot of the inset shows the expression of AD-$phyB^{197-1171}$ (lane 1), AD-$phyB^{FL}$ (lane 2), BD-$phyA^{1-137}$ (lane 3), and BD-$phyB^{1-173}$ (lane 4). Asterisk, nonspecific band. (**D**) Intracellular partitioning of ARR4. Epifluorescence images of transiently transformed parsley protoplasts (*23*) expressing ARR4:GFP that were irradiated for 12 hours with white light (light) or kept in darkness (dark). nu, nucleus; no, nucleolus; cy, cytoplasm.

ecule (*13*). The interaction of phyB with ARR4 may interfere with these changes and could be the reason for the hypersensitive phenotype of the ARR4-overexpressing lines in red light. This interference can be determined by measuring the degree and kinetics of the Pfr-to-Pr dark reversion in yeast cells (*17*). Therefore, yeast cells containing functional phyB were irradiated with a red light pulse and then transferred to darkness. The levels of total phyB and Pfr only were spectroscopically determined (*17*). About 20 and 45% of the initial pool of Pfr reverted back into the Pr form within 20 min after irradiation for phyB (Fig. 4A) and phyB-101 (Fig. 4B), respectively. This indicates that the mutant phenotype of the *Arabidopsis phy-B101* allele, which results in an amino acid exchange in the COOH-terminus of the phyB molecule, is indeed caused by a higher Pfr-to-Pr conversion rate (*11*). Dark reversion of the Pfr form was reduced to about 15%, when ARR4 was coexpressed with phyB or phyB-101 (Fig. 4, A and B). This reduction was specific for ARR4, because ARR2 did not effect dark reversion (Fig. 4C). These data show that ARR4 stabilizes phyB in its active Pfr form by inhibiting the Pfr-to-Pr dark reversion in yeast. Moreover, the inhibition of the phyB-101 dark reversion suggests that NH_2-terminus–bound ARR4 modulates the interdomain crosstalk within the phy molecule.

To investigate this aspect in plants we generated ARR4-overexpressing *Arabidopsis* lines in the ABO/A$^-$ background (Fig. 4D). Absence of phyA (A$^-$) and overexpression of phyB (ABO) allows in planta spectroscopic measurements of the photochemical properties of total phyB and Pfr (*18*). The ARR4-overexpressing seedlings again exhibited an enhanced reduction of hypocotyl length in red light compared with ABO/A$^-$ (Fig. 4E) indicating that the observed hypersensitivity response is not dependent on phyA. Analysis of dark reversion showed that about 60% of the initial pool of Pfr reverted back into the Pr form within 120 min after irradiation (Fig. 4F). If ARR4 was overexpressed, the amount of dark-reverted Pfr was strongly reduced (Fig. 4F). Thus, our data demonstrate that ARR4 stabilizes the active Pfr form of phyB in yeast and in plants—an effect that is manifested in the increased hypersensitivity of ARR4-overexpressing plants to red light.

Stabilization of the active Pfr form by regulating dark reversion of Pfr appears to be a process used by plants to regulate

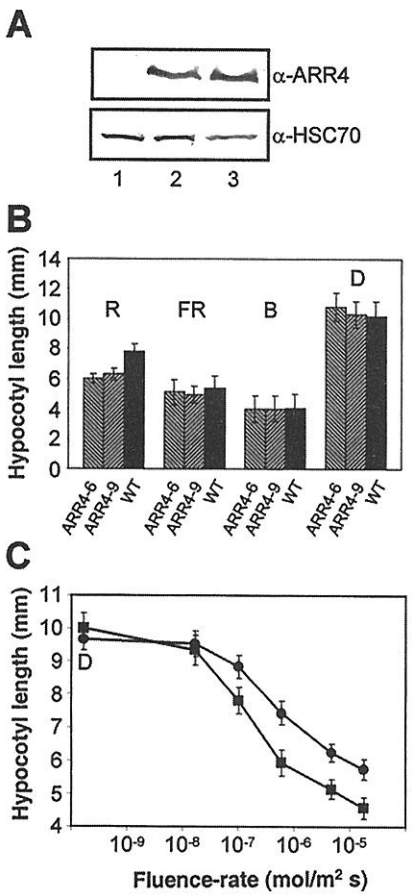

Fig. 3. Overexpression of ARR4 modulates the responsiveness of *Arabidopsis* plants to red light. (**A**) Immunoblot of ARR4 protein levels in 4-day-old, dark-grown wild-type (lane 1) seedlings and two independent ARR4-overexpressing *Arabidopsis* lines [ARR4-6 (lane 2), ARR4-9 (lane 3)] by using an ARR4-specific antiserum. HSC70, control immunoblot. (**B**) Hypocotyl elongation response of ARR4-6 and ARR4-9 in comparison with wild type (WT). Seedlings were grown for 72 hours under continuous red light (R), far-red light (FR), or blue light (B) or in darkness (D). (**C**) Fluence-rate dependence of red light inhibition of hypocotyl elongation in wild type (●) and ARR4-6 (■). Seedlings were grown for 72 hours under continuous red light (R) at the indicated light intensities. D, hypocotyl length in darkness.

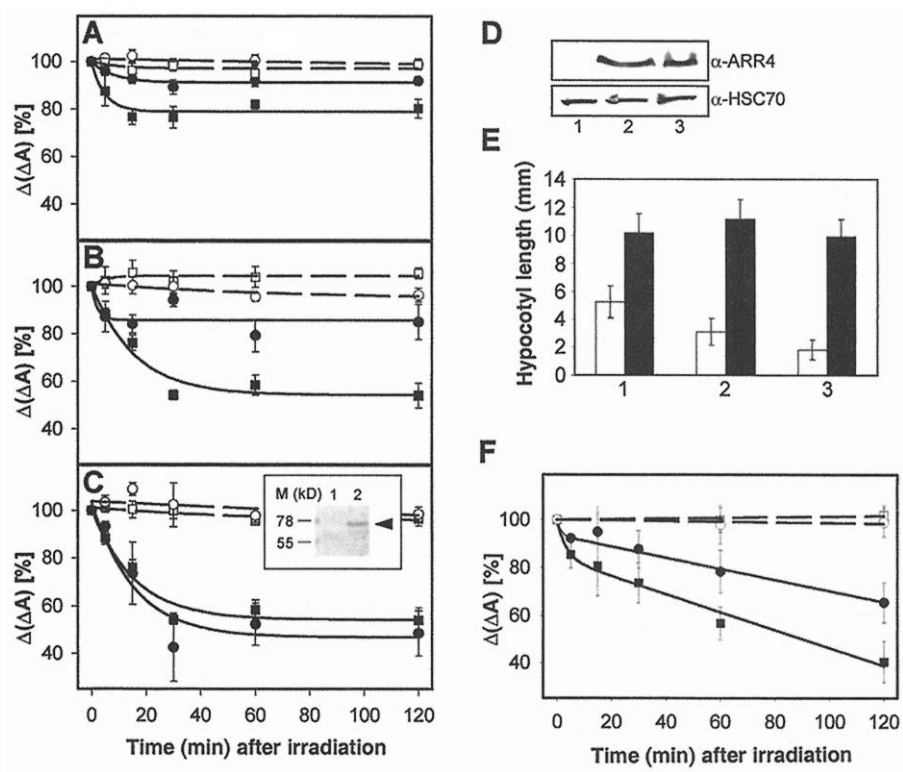

Fig. 4. ARR4 stabilizes the active Pfr form of B-type phytochromes in yeast and in planta. Yeast cells expressing photoreversible phyB (**A**) or phyB-101 (**B** and **C**) together with ARR4 [circles in (**A**) and (**B**)], ARR2 [circles in (**C**)], or no response regulator [squares in (**A** to **C**)] were irradiated with a red light pulse and transferred to darkness. At the indicated time points the amount of Pfr (black symbols) and total phy (white symbols) was spectroscopically measured in vivo (*17*). The inset of (**C**) shows an immunoblot with protein extracts from phyB-101–expressing yeast cells (lane 1) and from cells expressing phyB-101 plus ARR2 (lane 2) by using an ARR2-specific antiserum. (**D**) Immunoblots of ARR4 protein levels in 4-day-old, dark-grown *Arabidopsis* seedlings of the ABO/A$^-$ genotype (lane 1) and of two ARR4-overexpressing ABO/A$^-$ lines [ABO/A$^-$-ARR4-δ (lane 2), ABO/A$^-$-ARR4-ε (lane 3)] by using an ARR4-specific antiserum. HSC70, control immunoblot. (**E**) Hypocotyl elongation response of ABO/A$^-$-ARR4-δ (2) and ABO/A$^-$-ARR4-ε (3) in comparison with ABO/A$^-$ seedlings (1), which were grown for 72 hours either under continuous red light (white bars) or in darkness (black bars). (**F**) ARR4 stabilizes the active Pfr form of phyB in the ABO/A$^-$-ARR4-ε line. Four-day-old, dark-grown ABO/A$^-$ (squares) or ABO/A$^-$-ARR4-ε (circles) seedlings were irradiated with a red light pulse and transferred to darkness. At the indicated time points the amount of Pfr (black symbols) and total phy (white symbols) was measured.

photomorphogenesis (*17*). Here, we show that the enhanced stabilization of phyB-Pfr is responsible for the light-hypersensitive phenotypes in ARR4-overexpressing lines. Moreover, ARR4 contributes to this regulatory mechanism, and the ARR4:phyB interaction requires the extreme NH_2-terminus domain of phyB. These results raise the question whether phyB is the cognate sensor kinase of ARR4 or whether it is the target of a two-component signaling system comprising ARR4. Because histidine kinase activity of phyB has not been reported and because *ARR4* gene expression is also induced by cytokinin (*9*), phyB may be the target of a hormone-modulated signaling system rather than the sensor kinase itself. The cytokinin receptor CRE1 is a functional sensor histidine kinase (*19*, *20*), which might regulate the activity of ARR4. *Arabidopsis* seedlings expressing a nonphosphorylable form of ARR4, display a hyposensitive phenotype to red light (*21*). This observation demonstrates the importance of phosphorylation of ARR4 for the modulation of phyB signaling. Therefore we propose that ARR4 may act as a signal module integrating both red light (*via* phyB) and hormone (cytokinin) signaling.

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24. We thank C. Fankhauser for the *PHYB* and *PHYB-101* cDNAs; L. Hennig for the *PHYA* construct; A. Nagatani, M. Furuya, and L. Nover for antibodies; and the *Arabidopsis* Biological Resource Center, Ohio State University, for expressed sequence tag clones. This work was supported by a Sonderforschungsbereich grant to K.H. and E.S., and a Howard Hughes Medical Institute International Scholarship and a Human Frontier Science Program grant to F.N. U.S. and K.E. were recipients of fellowships from the Landesgraduiertenförderung and the Evangelische Studentenwerk, respectively.

2 August 2001; accepted 19 September 2001

Synaptotagmin Modulation of Fusion Pore Kinetics in Regulated Exocytosis of Dense-Core Vesicles

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In the exocytosis of neurotransmitter, fusion pore opening represents the first instant of fluid contact between the vesicle lumen and extracellular space. The existence of the fusion pore has been established by electrical measurements, but its molecular composition is unknown. The possibility that synaptotagmin regulates fusion pores was investigated with amperometry to monitor exocytosis of single dense-core vesicles. Overexpression of synaptotagmin I prolonged the time from fusion pore opening to dilation, whereas synaptotagmin IV shortened this time. Both synaptotagmin isoforms reduced norepinephrine flux through open fusion pores. Thus, synaptotagmin interacts with fusion pores, possibly by associating with a core complex of membrane proteins and/or lipid.

The fusion pore represents a pivotal intermediate in Ca^{2+}-triggered neurotransmitter exocytosis. Contact sites between the vesicle and the plasma membrane presumably contain special components capable of forming a fusion pore after the binding of Ca^{2+}. The fusion pore has been likened to the channels that control ion flux across cell membranes (*1*), and like ion channels, single fusion pore activity can be observed in high-resolution electrical measurements (*2–5*). Whereas plasma membrane ion channels are formed by well-characterized proteins, the structural components of the fusion pore have yet to be identified. A molecule likely to be intimately associated with fusion pores is synaptotagmin, a synaptic vesicle protein that binds Ca^{2+} and phospholipid (*6*). Genetic and biochemical studies have suggested a number of possible functions for synaptotagmin in vesicle trafficking and fusion (*7–10*). One appealing scenario is that soluble *N*-ethylmaleimide–sensitive factor attachment protein receptors (SNAREs) form fusion pores. These proteins catalyze the fusion of lipid vesicles (*11*), and putative fusion pores formed by these proteins could be regulated by synaptotagmin through rapid Ca^{2+}-stimulated binding to the SNARE complex (*12*). Here, we examined the role of synaptotagmin in specific steps of secretion in norepinephrine (NE)–secreting PC12 cells using amperometry to monitor release from individual dense-core vesicles.

When an amperometry electrode was positioned at the surface of a PC12 cell, exocytosis of a single vesicle registered as a spike of current as NE was oxidized at the electrode surface (*3*, *13*) (Fig. 1). These spikes were elicited by depolarization, in this case induced by rapid local application of KCl (Fig. 1) (*14*). Responses to KCl were characterized by a latent period with no spikes, followed by a period of spikes at irregular intervals. Recordings such as these provide information about the kinetics of exocytosis, allowing us to compare control PC12 cells (Fig. 1A) with cells transfected with either synaptotagmin I (Fig. 1B), synaptotagmin IV (Fig. 1C) (*15*), or the α1A (P/Q-type) Ca^{2+} channel (Fig. 1D). Because of the irregular nature of the release process in a single trial, we counted spikes after a given time interval and constructed normalized waiting time distributions (Fig. 1E). These plots provide an overall time course for secretion that is in

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