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Regulation of excitatory transmission at hippocampal synapses by calbindin D_{28k}

(calcium/adenovirus/synaptic plasticity)

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ABSTRACT **Distinct subpopulations of neurons in the brain contain one or more of the Ca^{2+}-binding proteins calbindin D_{28k}, calretinin, and parvalbumin. Although it has been shown that these high-affinity Ca^{2+}-binding proteins can increase neuronal Ca^{2+} buffering capacity, it is not clear which aspects of neuronal physiology they normally regulate. To investigate this problem, we used a recently developed method for expressing calbindin D_{28k} in the somatic and synaptic regions of cultured hippocampal pyramidal neurons. Ninety-six hours after infection with a replication-defective adenovirus containing the calbindin D_{28k} gene, essentially all cultured hippocampal pyramidal neurons robustly expressed calbindin D_{28k}. Our results demonstrate that while calbindin D_{28k} does not alter evoked neurotransmitter release at excitatory pyramidal cell synapses, this protein has a profound effect on synaptic plasticity. In particular, we show that calbindin D_{28k} expression suppresses posttetanic potentiation.**

Calbindin D_{28k} is a high-affinity Ca^{2+}-binding protein (CABP) found to be widely distributed in the nervous system where it has been shown to modulate Ca^{2+} signaling (1). Light and electron microscopy have clearly demonstrated the existence of calbindin D_{28k} in presynaptic nerve terminals, where it exists in up to millimolar concentrations (2–4). It is therefore tempting to speculate that calbindin D_{28k} also plays a role in modulating synaptic physiology. However, no direct experimental evidence is presently available to validate such a contention.

Several techniques have been developed for the overexpression of proteins in cells. Unfortunately, many of these suffer from very low efficiency or require the exogenous DNA to integrate into the genome of a replicating target cell. Recently, several groups have used adenoviruses as expression vectors because these vectors do not require host cell replication and give rise to highly efficient gene expression in host cells (5, 6). Several other advantages of using adenoviruses include the capacity to propagate the virus to high titers and the large amount of exogenous DNA that may be inserted into the viral vector (≈7 kb if the E1 and E3 regions of the viral genome are deleted).

In vivo injection of a recombinant adenovirus expressing β-galactosidase (*LacZ* gene) into the rat hippocampus and substantia nigra resulted in widespread expression of *LacZ* in neuronal and nonneuronal cells (5). In addition, infecting cultured neurons with a recombinant adenovirus containing the β-galactosidase gene resulted in 80–100% of the cells expressing the protein. Moreover, β-galactosidase expression appeared to be sustained for at least 1 month after infection without any indication of morphological damage (7).

In the present study, we have constructed a replication-defective adenovirus containing the calbindin D_{28k} cDNA under the control of the elongation factor 1α (EF-1α) promoter and the 4F2HC enhancer (8). We have used this vector to express calbindin D_{28k} in cultured hippocampal pyramidal neurons and have studied the protein's effects on synaptic communication.

MATERIALS AND METHODS

Construction of Replication-Defective Calbindin D_{28k}-Expressing Adenovirus. The full-length avian calbindin D_{28k} cDNA was obtained as a generous gift from D. Dowd (St. Louis University Medical Center, St. Louis). A blunt-ended *Ban* II/*Spe* I fragment of calbindin D_{28k} cDNA was inserted into the *Eco*RV site of transfer vector pAdKN—downstream from EF-1α promoter and upstream from the cellular heavy-chain enhancer (4F2) and bovine growth hormone polyadenylation site (pA^+). The pAdKN plasmid also contained 0–1 and 9–16 map units of DNA sequence of adenovirus 5.

To construct the recombinant adenovirus AdCABP-1, the plasmid pAdKN-CABP-1 was first linearized by digesting it with *Nhe* I, while *Xba* I and *Cla* I were used to digest adenovirus 5 (sub 360) DNA. The linear DNA was then cotransfected into human embryonic kidney cells (HEK 293 cells)—a trans-complementing cell line for E1 function (9)—by the calcium phosphate precipitation method. The recombinant AdCABP-1 was plaque purified three times to isolate a homogeneous population of the virus. The virus was further purified by CsCl isopycnic ultracentrifugation and then dialyzed against Hepes-buffered saline to produce a high-titer virus stock.

Hippocampal Culture and Electrophysiology. E17 hippocampal neurons were isolated according to previously described methods (10). To monitor evoked transmitter release, potential presynaptic neurons were stimulated every 10–30 s via an extracellular electrode using a 100-μs (0.1–0.7 mA) field stimulus. A data sweep (100 ms long) was initiated 10 ms before field stimulation. The postsynaptic cell was held at holding potential $V_h = -80$ mV in the whole-cell recording mode, and transmitter release was detected as excitatory postsynaptic currents (EPSCs), which appeared with a typical latency of 2–8 ms and a duration of ≈10 ms. Posttetanic potentiation (PTP) was induced by delivering a 1-s, 30- to 50-Hz tetanus to the presynaptic neuron between the routine stimuli.

Infection Protocol. The multiplicity of infection (moi) was calculated by assuming 1×10^4 neurons per coverslip. Addition of 1×10^7 plaque-forming units of virus (established using HEK 293 cells) would therefore give a moi of 10^3.

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Abbreviations: EPSC, excitatory postsynaptic current; IPSC, inhibitory postsynaptic current; PTP, posttetanic potentiation; moi, multiplicity of infection; BAPTA, bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate.

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Cultured hippocampal neurons were typically infected between 8 and 21 days *in vitro* (DIV)—although most typically at 10 DIV. The coverslips were removed from the glial feeder layer, placed face-up in a 60-mm tissue culture dish, and covered with a "glia-conditioned" medium (2.5 ml). An aliquot of high-titer virus (volume dependent on the level of infection required, although typically 0.1–5 μl) was then added to the medium. The dish was gently agitated and placed in an incubator at 37°C in 5% CO_2/95% air for 2 hr. The coverslips were then removed from the medium and placed face down in their original culture dishes (still containing the original medium). Cells were generally used 48–110 hr postinfection.

Functional $^{45}Ca^{2+}$-Binding Assay. The $^{45}Ca^{2+}$-binding assay adhered to the protocol of Muryama *et al.* (4).

RESULTS

Immunohistochemical studies showed that only a small percentage (13% ± 3%; n = 36 separate experiments) of cultured hippocampal pyramidal neurons normally contained low levels of calbindin D_{28k} (Figs. 1*B* and 2*C*). In contrast, 96 hr after infecting with AdCABP-1 (Fig. 1*A*), the weak endogenous staining observed in control cells was replaced by intense calbindin D_{28k} immunoreactivity in up to 100% of the cells (Fig. 1 *D* and *E*). We obtained similar results when we examined the infectivity of a β-galactosidase-containing replication-defective recombinant adenovirus, Ad*LacZ* (Fig. 1*C*). Ad*LacZ* has been previously shown to stably express β-galactosidase in infected tissue (12). We routinely used Ad*LacZ* infection as a control for the effects of AdCABP-1 (see below). Interestingly, we found that 96 hr after infecting the cells with either Ad*LacZ* or AdCABP-1, immunoreactivity for β-galactosidase and calbindin D_{28k}, respectively, was not restricted to the cell soma but was also widely distributed throughout the fine processes and putative synaptic regions [Fig. 1*C* and Figs. 1 *D* and *E*, representative of n = 32 (Ad*LacZ*) and n = 36 (AdCABP-1) separate experiments, respectively].

Western blot analysis confirmed that the immunocytochemical staining was specific for calbindin D_{28k} (Fig. 2*C*). Compared to endogenous levels, calbindin D_{28k} expression was dramatically enhanced in the infected cells. Moreover, the level of protein expression increased with increasing amounts of virus (representative of n = 3 separate experiments). The exact percentage of cells immunoreactive for calbindin D_{28k} or β-galactosidase was dependent on the number of virus particles per neuron (moi) used during the infection and increased linearly with logarithmic increases in moi (Fig. 2*A*). At high moi, ≈100% of the cells expressed calbindin D_{28k}. However, these high concentrations of virus also resulted in toxic side effects. The number of viable neurons per coverslip was reduced to 20–80% of the original culture density—with the exact percentage depending on the age and condition of the culture at the time of infection. When neurons were infected with AdCABP-1 at more moderate concentrations (<700 moi), no toxic side effects were observed and neurons showed normal viability. Virus titers between 500 and 600 moi, which produced intense calbindin D_{28k} expression in ≈75% of the neurons, were used for the remaining experiments outlined in this study.

The time course of calbindin D_{28k} expression in neurons infected at 500 moi is shown in Fig. 2*B*. Compared to control cells, no significant increase in calbindin D_{28k} levels was observed up to 24 hr postinfection. The onset of protein expression occurred between 24 and 48 hr and thereafter remained stable for at least 11 days postinfection, the longest time point examined.

It was necessary to determine whether this expressed immunoreactive protein acted functionally and bound Ca^{2+}. To address this question, lysates were prepared from AdCABP-1-infected HEK 293 cells 45 hr postinfection. Proteins contained in the lysates were separated electrophoretically, transferred onto nitrocellulose, and examined for their $^{45}Ca^{2+}$-binding capacity. Autoradiograms revealed that a protein abundantly expressed in the infected cells but absent from both mock-infected and Ad*LacZ*-infected cells bound $^{45}Ca^{2+}$ ions (Fig. 2*D*). The mobility of this protein was identical to that of purified calbindin D_{28k} and, thus, was concluded to be calbindin D_{28k} (n = 2 separate experiments).

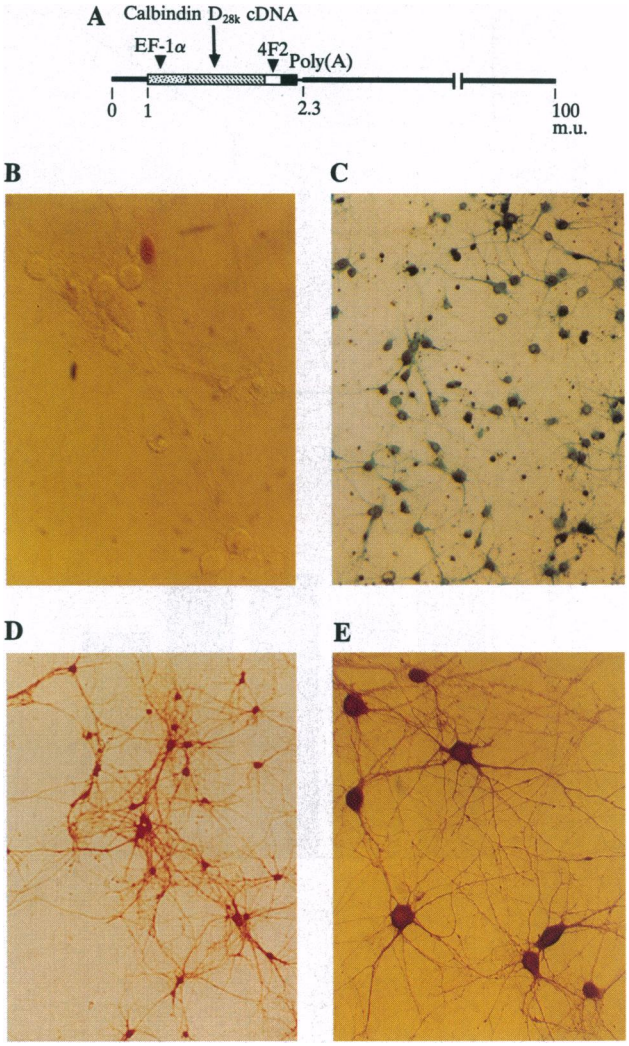

FIG. 1. Expression of calbindin D_{28k} in cultured rat hippocampal neurons infected with AdCABP-1 virus. (*A*) Schematic representation of recombinant adenovirus AdCABP-1 including calbindin D_{28k} cDNA, EF-1α promoter (11), cellular 4F2 heavy-chain enhancer (4F2) (8), bovine growth hormone polyadenylation signal [Poly(A)], and adenovirus map units (m.u.). (*C–E*) Approximately 100% of the hippocampal neurons showed expression of calbindin D_{28k} and β-galactosidase 96 hr after infection with AdCABP-1 or Ad*LacZ* virus. (*B*) Very low levels of calbindin D_{28k} staining in control cultures are shown. This can be compared with *E* and *D*, which illustrate intense staining 96 hr after treatment with AdCABP-1. (*C*) Labeling 96 hr after treatment with Ad*LacZ* is shown. (*B* and *E*, ×400; *C* and *D*, ×60.)

We then examined the consequences of expressing calbindin D_{28k} in hippocampal neurons on aspects of synaptic transmission and PTP. Both evoked neurotransmitter release and PTP are critically dependent on the action potential-mediated influx of Ca^{2+} ions into the synaptic terminal. The consequences of Ca^{2+} influx into the presynaptic terminal can be detected electrophysiologically in the postsynaptic neuron as an EPSC or an inhibitory postsynaptic current (IPSC), depending on the type of neurotransmitter released into the synaptic cleft. Stimulation of presynaptic neurons in these cultures resulted in

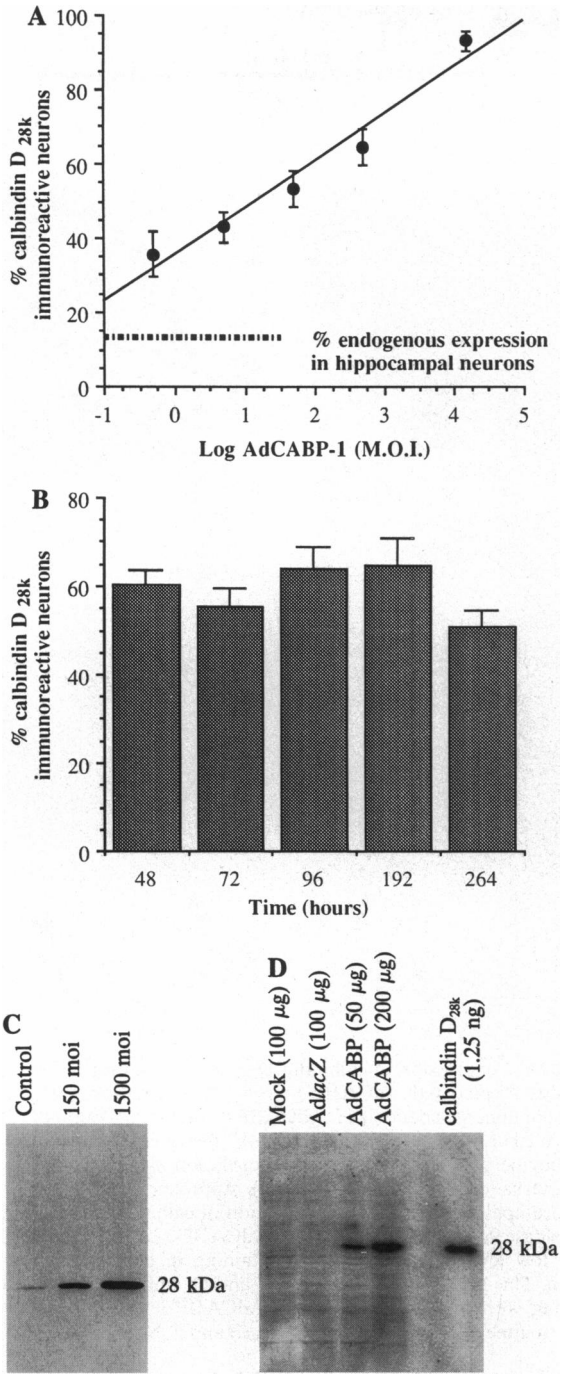

FIG. 2. (*A*) Effect of increasing the concentration of virus on percentage of cells that express calbindin D_{28k}. Endogenous expression of calbindin D_{28k} in cultured hippocampal neurons was ≈13%. Line fit: $y = 35.4 + 12.7x$; $R^2 = 0.96$. Vertical error bars represent means ± SEM ($n = 8$). (*B*) Histogram demonstrates time course and stability of calbindin D_{28k} expression in cultured hippocampal neurons after infection with AdCABP-1 at 500 moi. Vertical error bars represent means ± SEM ($n = 6–8$). (*C*) Endogenous levels of 28-kDa protein expression (control) are compared with expression in cultured hippocampal neurons 96 hr after they were infected with AdCABP-1 at 150 or 1500 moi. Data are representative of $n = 3$ separate experiments. (*D*) Autoradiogram showing $^{45}Ca^{2+}$ binding to pure calbindin D_{28k} (rightmost lane) and to a 28-kDa protein expressed in HEK 293 cells infected with AdCABP-1 (50 or 200 μg of total protein). This Ca^{2+}-binding capacity is absent from mock- or Ad*lacZ*-infected HEK 293 cells. Data are representative of $n = 2$ separate experiments.

a 6-cyano-7-nitroquinoxaline-2,3-dione-sensitive, glutamate-mediated EPSCs, consistent with the presence of glutamate-utilizing synapses between hippocampal pyramidal neurons (10). In addition, potential IPSCs were eliminated from the experiments by the addition of 20 μM bicuculline to the perfusion solution. Fig. 3 *A* and *B* shows typical EPSCs recorded from Ad*LacZ* and AdCABP-1-infected hippocampal cultures. EPSCs recorded from β-galactosidase-expressing neurons had on-rates and peak amplitudes similar to EPSCs recorded from calbindin D_{28k}-expressing neurons. In fact, no significant differences could be discerned between the EPSC characteristics of either neuronal population (Table 1).

Ad*LacZ*-infected cells could be routinely induced to respond to an individual stimulus with a potentiated EPSC by inserting a tetanic stimulus (1 s, 50 Hz) between two normal stimuli (Fig. 3 *A* and *C*). This potentiated response lasted from seconds to minutes and was characteristic of PTP (13). Fig. 3*B* demonstrates that the same tetanic stimulation protocol failed to induce PTP in a hippocampal neuron from an AdCABP-1-infected culture. Fig. 3*C* plots average EPSCs—normalized to the pretetanus EPSC—as a function of time, revealing that the average EPSC amplitude of Ad*LacZ*-infected neurons approximately doubles immediately after tetanization and then gradually decays back to baseline value within 2 min. In contrast, tetanization generally failed to induce PTP in hippocampal neurons from AdCABP-1-infected cultures. However, in other respects the basic properties of neurons from Ad*LacZ*- and AdCABP-1-infected cultures appeared the same (Table 1).

DISCUSSION

We have examined the effect of calbindin D_{28k} expression on synaptic physiology in intact neurons. The use of recombinant adenovirus as a means of delivering calbindin D_{28k} into hippocampal pyramidal neurons provided a useful method for investigating the role of this protein in modulating synaptic physiology. We found that infecting neurons with AdCABP-1 reproducibly resulted in marked expression of calbindin D_{28k} in all regions of the cell. The protein was expressed for the life-span of the host cell and acted functionally to bind Ca^{2+}. Furthermore, our results have indicated that calbindin D_{28k} expression in cultured hippocampal pyramidal neurons can result in the selective suppression of PTP.

The reduction in PTP produced by calbindin D_{28k} coupled with its lack of effect on evoked transmitter release can be explained in terms of the time course of these events and the Ca^{2+}-binding kinetics of calbindin D_{28k}. Different molecular mechanisms seem to be responsible for evoked neurotransmitter release and PTP. The former is a consequence of the nonlinear relationship between intraterminal Ca^{2+} and evoked release—a function of external Ca^{2+} concentration $([Ca^{2+}]_o)^4$—whereas the latter is linearly dependent on the intraterminal $[Ca^{2+}]$ $([Ca^{2+}]_i)$ (14). During evoked transmitter release, action potential-induced opening of voltage-sensitive Ca^{2+} channels results in the establishment of Ca^{2+} microdomains (>100 μM $[Ca^{2+}]_i$), which arise within microseconds directly beneath clusters of Ca^{2+} channels in the terminal membrane. These transient Ca^{2+} microdomains are responsible for initiating neurotransmitter release with a latency of <500 μs (15). PTP, on the other hand, is thought to be a consequence of the accumulation of Ca^{2+} during tetanization in the presynaptic terminal and the decay of this summated Ca^{2+} signal appears to parallel the decay of potentiated transmitter release (16, 17). Thus, the simplest explanation for the differential actions of calbindin D_{28k} in the nerve terminal may be speed. Calbindin D_{28k}'s Ca^{2+}-binding kinetics are too slow to prevent the establishment of Ca^{2+} microdomains but are sufficiently fast to reduce the global $[Ca^{2+}]_i$ increases responsible for mediating potentiation of neurotransmitter release on a second to minute time scale.

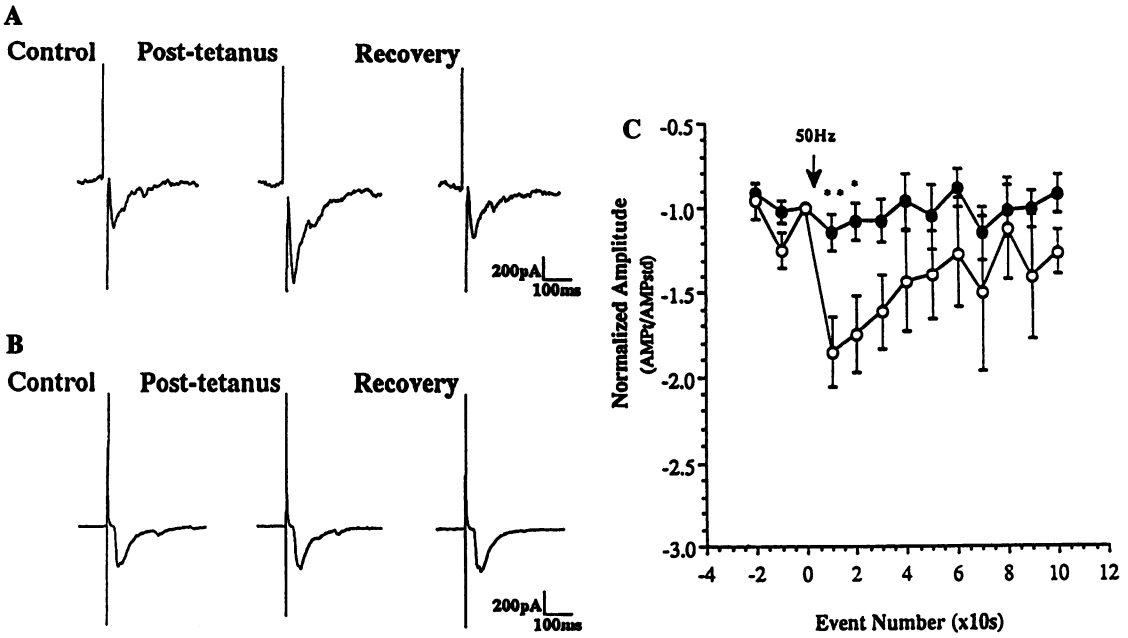

FIG. 3. (*A* and *B*) Shown are typical control, 5 s posttetanus, and recovery sweeps of data (each 100 ms in duration) recorded from two individual neurons in the presence of 20 μM bicuculline. *A* and *B* show EPSCs recorded in an individual postsynaptic neuron from an Ad*LacZ*- and AdCABP-1-infected culture, respectively. Traces are taken (from left to right) immediately prior to, immediately after, and 70 s after tetanus. (*C*) EPSC amplitudes were normalized to the value obtained two sweeps before tetanic stimulation. For each experiment, subsequent responses were expressed as a fraction of this normalized response. Averaged data are plotted for Ad*LacZ*-infected (open circle, up to 12 neurons per point from at least six separate cultures) and AdCABP-1-infected (solid circle, up to 10 neurons per point from at least six separate cultures) neurons. Vertical error bars represent means ± SEM. **, $P < 0.01$; *, $P < 0.05$, Student's unpaired *t* test.

The concept that Ca^{2+} accumulation in the nerve terminal is responsible for the time course of PTP is a central tenet of the "residual Ca^{2+}" hypothesis. According to this hypothesis, the amount of Ca^{2+} that reaches the active zone is proportional to $[Ca^{2+}]_o$. Therefore, the probability of release must also vary with intraterminal $[Ca^{2+}]^n$ (where $n > 1$) (18). It has been predicted that when $n = 4$, an action potential will rapidly raise the intraterminal $[Ca^{2+}]$ to a peak value (m), which will subsequently decay rapidly back to 30% of m (18). The remaining 30% will decay slowly and, in the absence of a suitable buffer, will still be present during the next action potential. As a result, the depolarization-induced Ca^{2+} influx will raise the intraterminal $[Ca^{2+}]$ to $1.3m$. This new $[Ca^{2+}]_i$ will cause a $(1.3)^4$ increase in transmitter release, an ≈3-fold increase over the amount of transmitter released by the first action potential. In light of this model, we might predict that calbindin D_{28k} is too slow to alter the onset of m. However, it should increase the rate of decay of the Ca^{2+} signal such that, prior to the next action potential, $[Ca^{2+}]_i$ will return to basal levels. Consequently, the next action potential-induced increase in $[Ca^{2+}]_i$ will not exceed m and transmitter release will not be potentiated.

The apparent kinetic properties of calbindin D_{28k} raise the possibility that its biological effects are closer to those of EGTA than to those of bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetate (BAPTA). For example, studies in the squid giant synapse have demonstrated that BAPTA attenuates neurotransmitter release, while EGTA has no effect (19, 20). At the same time, however, EGTA is effective in preventing PTP (21). It is thought that EGTA fails to affect transmitter release for the same reason that it is less effective than BAPTA in preventing Ca^{2+}-dependent inactivation of Ca^{2+} channels—i.e., its time constant of Ca^{2+} binding is too slow. The importance of k_{on} resides in the fact that it determines how rapidly a mobile buffer, such as calbindin D_{28k}, will facilitate the diffusion of Ca^{2+} ions away from the Ca^{2+} microdomains and, as a result, collapse the Ca^{2+} gradient (22, 23). The k_{on} values for EGTA and BAPTA (1.5×10^6 $M^{-1}s^{-1}$ and 6.0×10^8 $M^{-1}s^{-1}$, respectively), for example, parallel their effectiveness in reducing transmitter release, while their similar K_d

Table 1. Summary of the consequences of calbindin D_{28k} expression on synaptic physiology

	Ad*LacZ*-infected hippocampal neurons	AdCABP-infected hippocampal neurons
% of cells in which PTP was induced	75; $n = 12$	20; $n = 10$
Average evoked EPSC amplitude, pA	−915 ± 66; $n = 263$ events	−858 ± 48; $n = 174$ events
Average % increase in EPSC amplitude after tetanus (posttetanus amp./pretetanus amp.)	85 ± 22; $n = 12$	13 ± 11; $n = 10$
Average evoked EPSC slope, pA/ms	−498 ± 35; $n = 263$ events	−462 ± 27; $n = 174$ events
Average % increase in EPSC slope after tetanus (posttetanus slope/pretetanus slope)	44 ± 7.5; $n = 12$	11 ± 8; $n = 10$
Average kainate-activated current, pA	−986 ± 169; $n = 13$	−802 ± 253; $n = 8$
Average NMDA-activated current, pA	−731 ± 243; $n = 9$	−819 ± 171; $n = 8$

Compared are mean responses for AdCABP-1- and Ad*LacZ*-infected neurons. An EPSC was generated by a 100-μs, 0.3- to 0.7-mA stimulus. Excitatory amino acid-activated currents were elicited by bath application of either 30 μM kainate (in standard buffer) or 30 μM *N*-methyl-D-aspartate (NMDA) (in Mg^{2+}-free medium supplemented with 50 μM glycine) in the presence of 1 μM tetrodotoxin and 20 μM bicuculline.

values (8.6×10^{-7} M vs. 5.0×10^{-7} M, respectively) bear no relation to either chelator's efficacy in affecting the release process. How rapidly calbindin D_{28k} binds Ca^{2+} ions is a function of both its k_{on} value and concentration at the active site. Roberts (24) calculated the mean time for binding Ca^{2+} ions to be 3.3 μs. In addition, the k_{on} was estimated to be $\approx 4 \times 10^7$ $M^{-1} \cdot s^{-1}$, which does, in fact, place its Ca^{2+}-binding kinetics somewhere between those of EGTA and BAPTA [1.5×10^6 $M^{-1} \cdot s^{-1}$ (EGTA) $< 4 \times 10^7$ $M^{-1} \cdot s^{-1}$ (calbindin D_{28k}) $< 6.0 \times 10^8$ $M^{-1} \cdot s^{-1}$ (BAPTA)]. These calculations support the experimental observation that, while calbindin D_{28k} does not alter routine action potential-induced neurotransmitter release, it can modulate the plasticity of synaptic responses by regulating phenomena such as PTP. One should also note the protein's action in the intestine where its role in the facilitated transcellular diffusion of Ca^{2+} ions has been well characterized (11). In enterocytes, 100 μM calbindin D_{28k} is thought to augment the transcellular flow of Ca^{2+} ions by $\approx$70-fold. Diffusion is facilitated as a consequence of the constant binding and release of Ca^{2+} to calbindin D_{28k} molecules as the two tend toward dynamic equilibrium, so that calbindin D_{28k} molecules contribute to the transport of a Ca^{2+} ion across the cell. The principle of facilitated diffusion, demonstrated for calbindin D_{28k} in the gut, may therefore also explain the protein's actions in the nerve terminal. Assuming that the concentration of calbindin D_{28k} in a presynaptic terminal is $\approx$1 mM (23), then, as Ca^{2+} ions enter the cell and rapidly saturate the local unbound protein, two steep diffusion gradients would be set up. The first would be a 4 mM (four Ca^{2+}-binding sites per protein molecule) to 100 nM Ca^{2+}-bound calbindin D_{28k} gradient extending from the site of influx across the cell. The second diffusion gradient would extend toward the site of Ca^{2+} influx and be composed of 4 mM to 100 nM unbound calbindin D_{28k}. Clearly, in the absence of calbindin D_{28k}, the Ca^{2+} microdomain is more diffuse and penetrates further into the nerve terminal, whereas in the presence of the bound vs. unbound calbindin D_{28k} gradients described above, the microdomain would be more restricted, less penetrative, and shorter-lived (23).

We might speculate that CABP acts by restricting and destabilizing evoked nerve terminal Ca^{2+} signals and, consequently, preventing them from directly or indirectly enhancing neurotransmitter release. Such a mechanism coupled with the understanding that the protein's kinetic properties are too slow to affect evoked transmitter release would suffice to explain calbindin D_{28k}'s selective suppression of PTP at the presynaptic nerve terminal.

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