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Glucocorticoids Prolong Ca^{2+} Transients in Hippocampal-Derived H19-7 Neurons by Repressing the Plasma Membrane Ca^{2+}-ATPase-1

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Calcium ions (Ca^{2+}) play an important role in mediating an array of structural and functional responses in cells. In hippocampal neurons, elevated glucocorticoid (GC) levels, as seen during stress, perturb calcium homeostasis and result in altered neuronal excitability and viability. Ligand- and voltage-gated calcium channels have been the presumed targets of hormonal regulation; however, circumstantial evidence has suggested the possibility that calcium extrusion might be an important target of GC regulation. Here we demonstrate that GC-induced repression of the plasma membrane Ca^{2+}-ATPase-1 (PMCA1) is an essential determinant of intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) in cultured hippocampal H19-7 cells. In particular, GC treatment caused a prolongation of agonist-evoked elevation of $[Ca^{2+}]_i$ that was prevented by the expression of exogenous PMCA1. Furthermore, selective inhibition of PMCA1 using the RNA interference technique caused prolongation of Ca^{2+} transients in the absence of GC treatment. Taken together, these observations suggest that GC-mediated repression of PMCA1 is both necessary and sufficient to increase agonist-evoked Ca^{2+} transients by down-regulating Ca^{2+} extrusion mechanisms in the absence of effects on calcium channels. Prolonged exposure to GCs, resulting in concomitant accumulation of $[Ca^{2+}]_i$, is likely to compromise neuronal function and viability. (*Molecular Endocrinology* 16: 1629–1637, 2002)

THE HIPPOCAMPUS, a brain region critical to learning and memory acquisition, is highly sensitive to the effects of glucocorticoids (GC). GC effects in hippocampal neurons are mediated by two nuclear receptors, a high affinity type I or mineralocorticoid receptor (MR) and a lower affinity type II or glucocorticoid receptor (GR) (1), which exert coordinated effects on gene transcription (2). Although acute rises in GCs are neuroprotective, long-term elevations, as seen in chronic stress, result in hippocampal atrophy and neurodegeneration (3). Hippocampal neurons are also vulnerable to stressful conditions such as seizures, ischemia, and hypoglycemia (4, 5) and at the same time exhibit remarkable plasticity and show dendritic remodeling (6), synaptic turnover, and neurogenesis (7). Although alterations in the intracellular Ca^{2+} concentration are known to play a crucial role in mediating GC effects on the plasticity, excitability, and viability of hippocampal neurons (8, 9), the mechanism underlying these effects remains unclear.

The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is determined by the balance among entry through Ca^{2+} channels (10), release from intracellular stores (11), and buffering and efflux processes. GC regulation of evoked Ca^{2+} transients has focused primarily on increased entry through ligand- and voltage-gated calcium channels (12, 13); however, several lines of circumstantial evidence suggest that GCs might reduce calcium extrusion as well (14–16).

In rat, four isoforms of plasma membrane Ca^{2+}-adenosine triphosphatase-1 (PMCA) exist (17), which are expressed in a developmentally regulated manner (18). PMCA1 and -4 are ubiquitously expressed whereas PMCA2 and -3 exhibit tissue-specific expression, with prominent expression in the brain, heart, skeletal muscle, and kidney. Each isoform has been shown to have four to six alternative splice variants, with variants a and b exhibiting developmental switch and differential calcium-calmodulin affinities (19, 20). The PMCAs are energy-dependent Ca^{2+} pumps that extrude calcium (21, 22) and contribute to maintenance of the low resting level of cytosolic Ca^{2+}.

Recently, we identified PMCA1 as a GC-repressed gene in rat hippocampus (14). In adrenalectomized animals, PMCA1 was repressed by injections of high, but not low, doses of the GC, corticosterone (B), suggesting a GR-mediated effect. Moreover, in response to stress there was an inverse correlation between B

Abbreviations: ATPase, Adenosine triphosphatase; B, corticosterone; $[Ca^{2+}]_i$, intracellular Ca^{2+}; dsRNA, double-stranded RNA; E17, embryonic d 17; GC, glucocorticoid; GFP, green fluorescent protein; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; PMCA1, plasma membrane Ca^{2+}-adenosine triphosphatase-1; RNAi, RNA interference technique; SERCA, sarco/endoplasmic Ca^{2+}-ATPase.

level and PMCA1 mRNA in adrenal-intact animals. Repression of PMCA1 was also observed in cultured conditionally differentiated H19-7 hippocampal neurons, but only when the cells were in the differentiated state. With these observations in mind, we were interested in directly examining whether modulation of PMCA1 was one mechanism by which glucocorticoids regulate $[Ca]_i$. We therefore used H19-7 cells as a model system in which PMCA1 levels could be manipulated in the presence and absence of GCs, whereas calcium transients were monitored.

RESULTS

Expression Pattern of PMCAs and Glutamate Receptor Subunits in Cultured Neuronal Cells

We first examined the expression pattern of PMCA isoforms in H19-7 cells. H19-7 cells are conditionally differentiated neuronal cells derived from rat embryonic d 17 (E17) hippocampus (23). In the rat, four PMCA isoforms exist, which are expressed in the hippocampus in a developmentally regulated manner (18). As shown in Fig. 1A, RT-PCR demonstrated that isoforms 1 and 4 were expressed, whereas isoforms 2 and 3 were undetectable in H19-7 cells. This is consistent with the *in vivo* expression pattern of PMCA isoforms; PMCA1 and -4 are expressed throughout development, whereas PMCA2 and -3 are expressed after E18 (24).

Recently it has been shown that *in vivo* both B and stress repress PMCA1 mRNA expression in rat hippocampus (14). Moreover, B treatment of differentiated, but not undifferentiated, H19-7 hippocampal cells resulted in the repression of PMCA1 message, as assessed by Northern blot analysis. We therefore examined the effect of B on PMCA1 and PMCA4 protein levels. As shown in Fig. 1B, 3 h of B treatment resulted in a 1.8- to 2.0-fold (or ~46%) reduction of PMCA1 protein level ($P = 0.029$, by *t* test) and a 2.5-fold or approximately 57% ($P = 0.002$) reduction of PMCA4 protein (Fig. 1B), consistent with previously identified effects on gene expression (14).

We further characterized the H19-7 cells with respect to expression pattern of some of the key subunits of glutamate receptors by RT-PCR. These data are summarized in Table 1. The expression patterns of these subunits are in agreement with those seen *in vivo* in the brain (25).

Measurements of $[Ca^{2+}]_i$ in Single Neuronal Cells

To determine whether B-induced repression of PMCA1 and -4 was associated with a physiologically significant effect on $[Ca^{2+}]_i$, the effect of B on agonist-induced $[Ca^{2+}]_i$ was determined. We first tried the more traditional neurotransmitters such as bradykinin, *N*-methyl-D-aspartate, glutamate, and the synthetic glutamate analog kainic acid to evoke Ca^{2+} transients in H19-7 cells in view of the above results. Surprisingly, these traditional neurotransmitters failed to evoke Ca^{2+} transients in 95–98% of H19-7 cells. KCl was able to evoke Ca^{2+} transients in H19-7 cells; however, the response was not robust. It has been demonstrated that even though the glutamate receptor subunits are expressed as early as stage E14, mere

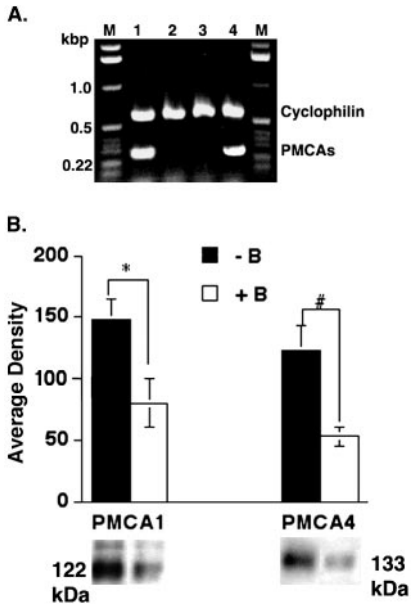

Fig. 1. PMCA1 and PMCA4 Are Expressed in H19-7 Cells and Are GC Regulated

A, RT-PCR was used to amplify PMCA isoforms 1–4 from H19-7 cell RNA. Isoforms 1 and 4 (lanes 1 and 4) were present, whereas 2 and 3 (lanes 2 and 3) were undetectable. B, Effect of B treatment on PMCA1 and -4 immunoreactive protein in H19-7 cells. Cell lysates from control and 3-h B-treated cells were prepared. Two micrograms of total protein were separated by SDS-PAGE, and proteins were transferred to nylon membranes. Blots were probed with PMCA1 (NR1-1) antibody, then stripped and reprobed with PMCA4 antibody (JA9), as described in *Materials and Methods*. Densitometry data were analyzed using NIH Image. *Error bars* represent ± SEM (n = 3–5). A *t* test was used to determine significance. *, $P = 0.029$; #, $P = 0.002$.

Table 1. Expression Pattern of Glutamate Receptor Subunits in H19-7 Cells

Receptor	Subunits	Undifferentiated H19-7 Cells	Differentiated H19-7 Cells
Kainate	KA1	–	–
	KA2	+	+
NMDA	NMDAR1	+	+
	NR2B	+	+
AMPA	GluR1	+	+
	GluR2	+	+
	GluR2 Q/R	+	+
	GluR3	+	+
	GluR4 Flip	–	–
	GluR4 Flop	–	–

expression of these subunits does not result in a functional receptor. It is only toward the end of embryonic development that neurons begin to respond to *N*-methyl-D-aspartate or potassium depolarization (26). Thus, it appears that although H19-7 cells express the glutamate receptor subunits, the resulting receptors are probably not functional. They appear largely to reflect the dynamics of the developing embryonic brain, and although they can be conditionally differentiated and express neuronal markers, they are functionally immature.

Thrombin, a potent inducer of Ca^{2+} transients in most cell types, was recently shown to induce intracellular Ca^{2+} spikes in primary hippocampal neurons (27) and *in vitro* in a neuroblastoma cell line (28). Receptors for thrombin are present throughout the central nervous system, including the hippocampus, and endogenous thrombin appears to protect hippocampal CA1 neurons against ischemic insults and mobilizes Ca^{2+} in Fura-2-loaded CA1 neurons (27). Other studies indicate that thrombin is implicated in a wide variety of processes that include changes in gene expression in the central nervous system, response to injury, long-term potentiation, neuronal plasticity, and dendritic remodeling (29, 30). Thus, thrombin appears to be a physiological regulator of calcium in hippocampal neurons. Importantly for the present purposes, both undifferentiated and differentiated H19-7 cells respond to thrombin with a rapid increase in $[Ca^{2+}]_i$ (Fig. 2). However, in undifferentiated, vehicle-treated cells, the agonist-evoked Ca^{2+} transient returned rapidly to baseline (Fig. 2A). Consistent with the lack of repression of PMCA1, treatment with B had no effect on the duration of evoked Ca^{2+} transients (Fig. 2B). In differentiated vehicle-treated cells, stimulated transients returned rapidly to baseline (Fig. 2C). In contrast, B treatment of differentiated cells markedly prolonged the duration of agonist-evoked Ca^{2+} transients, which remained elevated for at least 5–10 min (compare Fig. 2, C and D). In addition, the amplitude of the peak was consistently reduced by B treatment in differentiated cells. Thus, B treatment both repressed PMCA1 expression and had a profound effect on evoked calcium transients in differentiated, but not undifferentiated, H19-7 cells. Treatment with B for periods shorter than 2 h (15, 30, or 60 min) did not result in repression of PMCA1 or prolongation of agonist-evoked Ca^{2+} transients (data not shown), consistent with the idea that transcriptional repression of PMCA1 is required for this effect.

Exogenous Expression of PMCA1 Prevents B-Mediated Prolongation of Ca^{2+} Transients

It was reasoned that if repression of PMCA1 were necessary for the B-induced prolongation in Ca^{2+} transients, then heterologous expression of PMCA1 from a non-B-regulated promoter would prevent the B-induced prolongation in Ca^{2+} transients. To address this issue directly, a PMCA1 expression vector driven by the cytomegalovirus promoter was transiently transfected into H19-7 cells along with a green fluorescent protein (GFP) expression vector to identify transfected cells. Control cells transfected with the cytomegalovirus-driven empty vector and GFP continued to show B-mediated prolongation in agonist evoked Ca^{2+} transients (Fig. 3, A and B). However, in striking contrast, neuronal cells overexpressing exog-

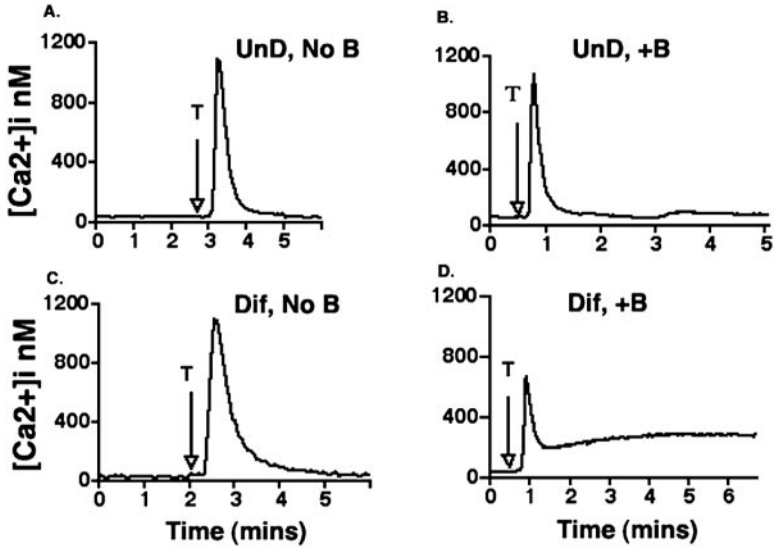

Fig. 2. The Effect of B Treatment on Thrombin-Evoked $[Ca^{2+}]_i$ in H19-7 Cells

Cells were cultured and loaded with Fura-2/AM as described in *Materials and Methods*. A, Undifferentiated (UnD), vehicle-treated H19-7 cells; B, undifferentiated, B-treated cells; C, vehicle-treated, differentiated (Dif) H19-7 cells; D, 10^{-7} M B-treated, differentiated (Dif) H19-7 cells. Measurements were made from at least five to eight independent cells per condition. One representative tracing from each condition is shown.

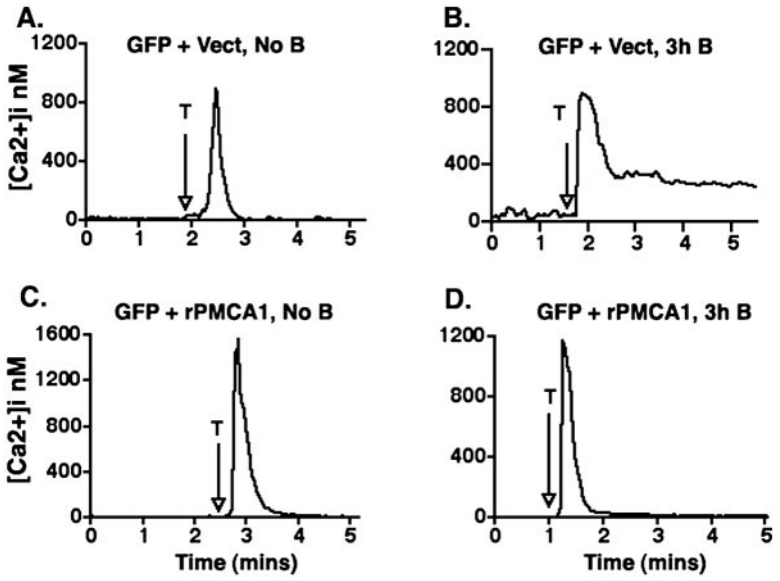

Fig. 3. Exogenous Expression of PMCA1 Rescues B-Mediated Prolongation of $[Ca^{2+}]_i$ in H19-7 Cells

A, GFP- plus vector-transfected, vehicle-treated control cells; B, GFP- plus vector-transfected, 10^{-7} M B-treated cells; C, GFP- plus PMCA1-transfected, vehicle-treated cells; D, GFP- plus PMCA1-transfected, 10^{-7} M B-treated cells. Measurements were made from at least 8–10 independent cells/condition.

enous PMCA1 were resistant to the B-induced prolongation of Ca^{2+} transients (Fig. 3, C and D). Heterologous overexpression of PMCA1 did not have any effect on basal cytosolic levels of calcium, consistent with previous observations in other cell types (31, 32). It was also determined by immunocytochemistry that the heterologously overexpressed PMCA1 was targeted appropriately to the plasma membrane and was not retained in the endoplasmic reticulum, whereas the endogenously expressed PMCA1 was undetectable in control cells [data not shown and Brini *et al.* (32)]. These data demonstrate that PMCA1 overexpression prevents the B-induced prolongation in Ca^{2+} transients, consistent with the idea that repression of PMCA1 is necessary for this effect.

RNA Interference Technique (RNAi) against PMCA1 Results in Prolongation of Intracellular Calcium Transients

To determine whether down-regulation of PMCA1 by itself caused prolongation of agonist-induced Ca^{2+} transients, RNAi was used to selectively decrease PMCA1 levels. RNAi is a highly specific and effective method for generating phenotypic knockouts in a variety of animal and cell culture models, including *Caenorhabditis elegans* (33, 34), *Drosophila* (35), and mouse (36) tissues and cultured cells (37, 38), although it has not been effective in all cell types. To our knowledge this is the first report showing that RNAi works in cultured mammalian cells to produce a specific downstream effect of physiological significance. We first established that transfection of PMCA1-specific RNAi resulted in a reduction of PMCA1 protein expression relative to that in cells transfected with control RNAi (Fig. 4A). Expression of PMCA4 remained unaffected by either RNAi treatment (Fig. 4A).

Next, the effect of RNAi on agonist-stimulated $[Ca^{2+}]_i$ was determined. As shown in Fig. 4B, H19-7 cells transfected with RNAi against PMCA1 consistently exhibited prolonged evoked Ca^{2+} transients, although not to the same extent as with B treatment. Importantly, transfection of several nonspecific species of RNAi had no effect on Ca^{2+} transients (Fig. 4B and data not shown). Hence, we conclude that selective repression of PMCA1 protein levels is sufficient to prolong Ca^{2+} transients in the absence of GCs.

DISCUSSION

These results provide direct support for the idea that regulation of PMCA1 levels is an important mechanism by which GCs modulate evoked Ca^{2+} transients in neurons and possibly other cell types. The B-induced prolongation in Ca^{2+} transients was completely suppressed by heterologous expression of PMCA1, on the one hand, and was partially mimicked by specific inhibition of PMCA1 with RNAi, on the other. In support of the relevance of our findings to the intact adult animal, Ca^{2+} transients in neurons from hippocampal explants treated with high doses of B (sufficient to activate GR) are prolonged, whereas those treated with low doses of B (sufficient to activate the higher affinity MR, but not GR) are foreshortened (39).

Heterologous overexpression of PMCA1 in differentiated cultured cells was able to overcome the repres-

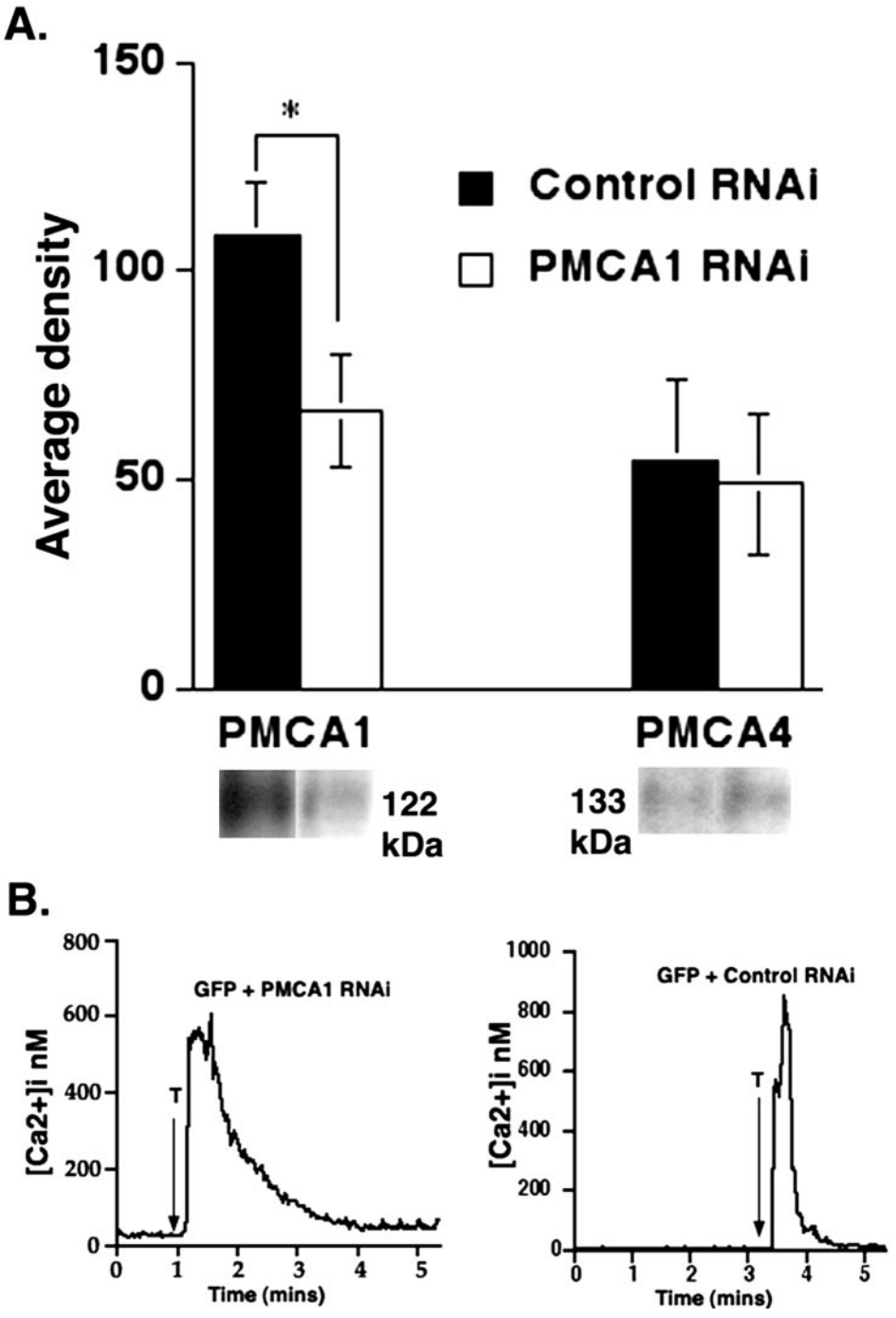

Fig. 4. RNAi Against PMCA1 Results in Selective Degradation of PMCA1 Protein and Prolonged Evoked Ca^{2+} Transients

Cells were transfected with dsRNA as described in *Materials and Methods*. A, PMCA1 protein is selectively reduced in cells that received RNAi against PMCA1 compared with cells treated with control dsRNA. The same blot was stripped and probed with antibody against PMCA4, whose expression remains unchanged with treatment. Densitometry data were obtained from above blots, and the mean average density was calculated using NIH Image. *Error bars* represent ± SEM (n = 3–5). A *t* test was used to calculate *P* values. *, $P = 0.04$. B, Selective removal of PMCA1 by RNAi results in prolongation of $[Ca^{2+}]_i$ in the absence of GCs. B, H19-7 cells were transfected with dsRNA against PMCA1 (*left tracing*) or control dsRNA (*right tracing*). Measurements were made from at least five to eight independent cells per condition, with similar results.

sive effects of B treatment. Similar to the effect of B, heterologous expression of PMCA1 did not affect basal calcium levels, consistent with previous findings in other cell types overexpressing PMCA1 that do not show altered basal $[Ca^{2+}]_i$ (31, 32). Interestingly, differentiated H19-7 cells overexpressing PMCA1 were

resistant to B-induced prolongation of evoked Ca^{2+} transients, suggesting that repression of PMCA1 is necessary to decrease the rate of cytosolic Ca^{2+} extrusion. However, the caveat exists that this overexpression may rescue or compensate for the influence other candidate proteins may exert on recovery, including the endoplasmic Ca^{2+}-adenosine triphosphatases (SERCAs), other PMCA isoforms, or the Na^{+}/Ca^{2+} exchanger when cells are challenged with B. Overexpression of PMCA1 does not seem to dramatically alter the amplitude of the $[Ca^{2+}]_i$ response (after agonist stimulation) and is not qualitatively different from that seen in untreated cells. Notably, this also suggests that B-mediated decreases in efflux of Ca^{2+} are not due to diminished ATP, because isolated overexpression of PMCA1 (whose activity is ATP dependent) rescues the inhibitory effects of B.

The other calcium extrusion candidate, the Na^{+}/Ca^{2+} exchanger, has been shown to be present mainly in the dendrites in neurons and in presynaptic nerve terminals (40). In addition, the Na^{+}/Ca^{2+} exchanger and mitochondria play a minor role during $[Ca^{2+}]_i$ recovery from $[Ca^{2+}]_i$ elevations that do not exceed 50 nM (41, 42). In our study, $[Ca^{2+}]_i$ measurements were made from the cell body and not the dendrites; hence, the role played by the Na^{+}/Ca^{2+} exchanger in the extrusion phase may not be very significant. Nonetheless, its role in $[Ca^{2+}]_i$ extrusion in H19-7 neurons will be of interest to determine.

Interestingly, we found that B treatment does not inhibit PMCA1 expression in undifferentiated cells, nor does it alter agonist-stimulated Ca^{2+} transients. This argues that repression of PMCA1 by B is state and context dependent; however, repression of PMCA1 appears to be both necessary and sufficient for prolongation of evoked $[Ca^{2+}]_i$ transients. Differentiated H19-7 cells show inhibition of PMCA1 upon B treatment, and the duration of agonist-induced $[Ca^{2+}]_i$ is also increased. In addition, B treatment reduced the amplitude of the peak in differentiated cells. The reduction in peak height could be due to decreased release from the endoplasmic reticulum or more rapid uptake into the intracellular stores upon agonist treatment. It is conceivable that B treatment alters the expression of other components of the system (such as SERCAs) that influence intracellular levels of calcium. Shorter periods of treatment with B did not repress PMCA1 mRNA, nor did it result in prolongation of agonist-evoked Ca^{2+} transients consistent with a transcriptional mechanism.

In explants from CA3/CA1 regions of the hippocampus, treatment with low doses of B result in small ionic conductance and transmitter responses (43) and decreased Ca^{2+} conductance (44). The evoked Ca^{2+} transients return faster to baseline under conditions of low B administration, whereas neuronal cells treated with high B show a decrease in the clearance rate of $[Ca^{2+}]_i$ (39). Large $[Ca^{2+}]_i$ transients are associated with long-term potentiation modulating synaptic plasticity associated with learning and memory, whereas smaller magnitudes of $[Ca^{2+}]_i$ transients are associated with long-term depression (45, 46). Qualitatively, our observations in conditionally differentiated cultured hippocampal neurons are similar to those seen in dissociated hippocampal cells under conditions of high B treatment. Thus, our findings in H19-7 cells appear to reflect *in vivo* regulation of $[Ca^{2+}]_i$ transients by high levels of GC, with the caveat that H19-7 cells express only low levels of MR, and hence, the effects of low B concentrations mediated by MR could not be studied.

It is striking that repression of PMCA1 is not only necessary but is also sufficient to produce prolongation of stimulated Ca^{2+} transients in differentiated H19-7 cells. However, neither PMCA1 repression nor overexpression had a detectable effect on basal Ca^{2+} levels in H19-7 cells, similar to previous observations in a variety of other neuronal and nonneuronal cell types (31, 32, 39, 47, 48). These observations are consistent with the idea that under resting conditions, pump levels are in excess, and turnover number is substrate limited even when PMCA levels are repressed (47). In contrast, under conditions of elevated $[Ca^{2+}]_i$, as seen during evoked transients, Ca^{2+} extrusion becomes limited when PMCA1 is repressed.

Although the effects of B and RNAi on evoked transients were similar, it is noteworthy that the $[Ca^{2+}]_i$ elevation during the later phase (after 3–5 min) was substantially greater in the B-treated cells. In view of the complete suppression of the B effect by heterologous PMCA1 (Fig. 3) and the lack of increase in peak $[Ca^{2+}]_i$, this difference suggests that B treatment alters the expression of other genes involved in the removal of cytoplasmic Ca^{2+} (for example, PMCA4 or SERCAs); however, late effects on Ca^{2+} channels could also be implicated. In support of a role for PMCA4, this PMCA isoform was also repressed by B treatment in differentiated H19-7 cells, whereas its levels remained unchanged in neurons treated with RNAi against PMCA1. It remains to be established whether selectively inhibiting PMCA4 by RNAi also results in prolongation of Ca^{2+} transients in H19-7 cells.

During fetal/embryonic development, hippocampal neurons are in a dynamic state, establishing new synaptic contacts and undergoing a plethora of active remodeling (49). At this stage of development, the onus of maintaining $[Ca^{2+}]_i$ by actively extruding elevated levels of Ca^{2+} falls upon PMCA1 and -4, whereas tissue-specific PMCA isoforms 2 and 3 do not begin to be expressed until later during embryonic development (24). Thus, any modulations or oscillations in $[Ca^{2+}]_i$ required by the neurons will be achieved by regulating the activity and/or levels of PMCA1 and -4. H19-7 cells derived from E17 hippocampal neurons express both PMCA1 and –4, and both isoforms are regulated by B in differentiated cells. Thus, H19-7 cells provide an attractive model system to study dynamic changes taking place in the developing embryonic hippocampus.

Although the transient high levels of GCs associated with circadian peaks or brief stress promote adaptive behavioral responses, persistently elevated levels in conjunction with chronic stress or Cushing's syndrome contribute to depression, posttraumatic stress disorder, and dementia (4, 50). At the cellular level, acute GC-induced elevations in $[Ca^{2+}]_i$ result in changes in synaptic plasticity and neuronal excitability, whereas chronic elevations lead to remodeling of neurons, enhanced apoptosis (51), and, ultimately, neurodegeneration (3). Delineating the role of GC regulation of PMCA1 in neuronal Ca^{2+} homeostasis provides a mechanistic basis for these GC-induced effects.

MATERIALS AND METHODS

Cell Culture

H19-7 cells of hippocampal origin were grown on poly-L-lysine-coated dishes (or coverslips), in 1% DMEM at 33 C at 5% CO_2 as described previously (14). H19-7 cells are transformed with a temperature-sensitive mutant of simian virus 40 T antigen and are conditionally differentiated (23). For differentiation, cells were incubated at 39 C at 5% CO_2 in DMEM and N2 supplements, and the process was accelerated by adding 10 ng/ml basic fibroblast growth factor as previously described (23). One day before hormone treatment, cells were provided with medium containing charcoal-stripped fetal bovine serum. Differentiated cells were then treated with 10^{-7} M B for 0, 30, 60, and 120 min. RNA or cell extracts were prepared as previously described (14).

Western Blot Analysis

H19-7 cells were differentiated and treated with B as described above. Three hours after B treatment, cells were washed twice with ice-cold PBS, and 30 μl lysis buffer were added to the cells. Proteins were electroblotted onto a nylon membrane and immunoprobed as previously described (52). Blots were incubated in appropriate secondary antibody, washed in PBS-Tween 20, and developed using an ECL Plus kit (Amersham Pharmacia Biotech, Arlington Heights, IL) according to the supplier's specifications.

Preparation of RNAi and Cell Transfection

Double-stranded RNA was prepared by synthesizing sense and antisense RNA *in vitro* using the Riboprobe kit (Promega Corp., Madison, WI) and plasmid pCDNA3.1/rPMCA1 linearized with appropriate restriction enzymes, as described previously (33). For RNAi experiments, at least 15 μg PMCA1 double-stranded RNA (dsRNA) or equivalent pmol amounts of nonspecific dsRNA (pBluescript) were used per well.

For transfection studies, cells were seeded on coverslips at a density of 5×10^4 cells/well in a six-well dish. Sixteen hours later, PMCA1 expression plasmid (or vector) and GFP were transfected using the Lipofectamine method (BRL, Gaithersburg, MD) according to the supplier's specifications. Twenty-four hours after transfection, cells were differentiated using the conditions described above. DNA-transfected cells were then treated with either vehicle (ethanol) or 10^{-7} M B for various times (15 min to 3 h). $[Ca^{2+}]_i$ measurements were performed as described below. After RNAi transfection, cells were differentiated, and Ca^{2+} measurements were performed without B treatment.

Microscopy and $[Ca^{2+}]_i$ Measurements

Cells were incubated in loading buffer containing 5 mM Fura-2/AM for 60 min. Transfected cells were identified by their GFP expression. The coverslips were mounted on a temperature-controlled chamber at 31 C in assay medium (53). Fura-2 fluorescence was measured using a Nikon epifluorescence inverted microscope (Melville, NY) fitted with a rotating holder for excitation filters (340 and 380 nm) as previously described (53). Signals were digitized using a Labmaster interface board (Scientific Solutions, Solon, OH) and were recorded in an IBM style computer using the UMANS software package (Chester Regen, Bio-Rad Laboratories, Inc., Hercules, CA). $[Ca^{2+}]_i$ was calculated as previously described (54). Agonist (thrombin, 1.5 U/ml) was added to evoke intracellular calcium transients. Signal from three to five cells per condition per experiment were collected. Each experimental condition was repeated at least three times on different days.

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