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Plasma Membrane Calcium Pump Isoform 1 Gene Expression Is Repressed by Corticosterone and Stress in Rat Hippocampus

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Glucocorticoids (GCs) are critical to learning and memory, in large part because of their actions in the hippocampus. Chronic high levels of GCs have profound effects on hippocampal structure and function and can even result in irreversible neurodegeneration. Hippocampal GC actions are mediated by intracellular receptors that modulate the transcription of specific target genes. In a screen for genes repressed by GCs in rat hippocampus, we identified plasma membrane calcium pump isoform 1 (PMCA1), a plasma membrane calcium ATPase. In Northern blots, PMCA1 was repressed ~33% after a high, but not a low dose of the GC, corticosterone (B), suggesting glucocorticoid (but not mineralocorticoid) receptor-mediated repression. Furthermore, *in situ* hybridization demonstrated that B significantly downregulated PMCA1 mRNA in all brain regions examined. Repression of PMCA1 was also observed in cultured hippocampal neurons, but only when the cells were in the differentiated state. Stress also repressed PMCA1 expression in hippocampus of adrenal-intact animals, and a clear inverse correlation between B level and PMCA1 mRNA could be discerned. However, other non-B-dependent factors appeared to be involved in the response of PMCA1 to stress because, unlike exogenous B, cold stress did not repress PMCA1 in brain regions other than hippocampus. Moreover, in the presence of constant B (B-replaced, adrenalectomized animals), cold stress led to increased hippocampal PMCA1 expression. These observations suggest that repression of PMCA1 represents one molecular mechanism by which corticosteroids regulate Ca^{2+} homeostasis and hence influence neuronal activity. Moreover, other stress-related neurohumoral factors appear to counter the repressive effects of B. Defects in the balance between GC-mediated and non-GC-mediated effects on PMCA1 expression may have adverse effects on neuronal function and ultimately result in irreversible neuronal damage.

Key words: corticosteroids; hippocampus; target genes; gene repression; plasma membrane calcium pump; cell-death

Through their actions in a variety of brain regions, corticosteroids have potent effects on mood (Dinan 1994; Barden et al., 1995), feeding (Dallman et al., 1993), memory, and cognition (McEwen and Sapolsky, 1995). Whereas nonstress levels of corticosteroids influence vegetative circadian functions such as feeding and sleep (Dallman et al., 1995), stress levels are essential to the autonomic and higher order cortical processes involved in developing and implementing strategies for coping with stressors in the environment (McEwen and Sapolsky, 1995; Gray and Bingaman, 1996). In the hippocampus, corticosteroids have been shown to modulate several key processes including development (Gould et al., 1992), neurotransmitter synthesis and release (McEwen et al., 1990; de Kloet, 1991), neuronal excitability (Joëls and de Kloet, 1989; Kerr et al., 1989), and Ca^{2+} homeostasis (Choi, 1988).

Although the high levels of corticosteroids secreted in response to stressful stimuli have a transient protective effect (Munck et al., 1984), prolonged elevations can cause irreversible damage and permanent cognitive deficits (Joëls and de Kloet, 1994). Whereas there is considerable interindividual variability, chronically elevated GC levels or chronic stress have been shown to cause neurodegenerative changes in the pyramidal cell layer of the hippocampus, similar to those seen during aging (Sapolsky et al., 1986; Kerr et al., 1991). The mechanistic basis of this effect remains unknown; however, it has been suggested that perturbations of Ca^{2+} homeostasis may play an important role (Siesjo, 1988; Elliot and Sapolsky 1993). Sustained elevations of GC, as seen during stress, have been associated with an increase in free intracellular levels of Ca^{2+} (Elliot and Sapolsky 1993), and increased intracellular Ca^{2+} levels caused by loss of regulatory mechanisms controlling influx and efflux of Ca^{2+} ion have been associated with events such as brain trauma, stroke, and epilepsy (DeCoster, 1995).

Two intracellular receptors, the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) mediate most, or perhaps all GC effects in the brain. We were interested in identifying novel target genes for these receptors, particularly those that might be involved in controlling hippocampal neuronal activity and Ca^{2+} homeostasis. We used suppression subtractive hybridization (Diatchenko et al., 1996) to identify genes repressed by high levels of GC in rat hippocampus. From this screen, we identified the plasma membrane calcium pump isoform 1 (Shull and Greeb, 1988).

The PMCAs play an essential role in the regulation of intracellular Ca^{2+} levels in most cells, by coupling ATP hydrolysis with Ca^{2+} extrusion from cells (Jencks, 1989; Carafoli, 1992). In

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the rat, four PMCA isoforms are expressed with isoforms 1 and 4 showing a ubiquitous tissue expression pattern (Greeb and Shull, 1989; Stahl et al., 1992). Four different splice variants for plasma membrane calcium pump isoform 1 (PMCA1) exist (Stauffer et al., 1995; Filoteo et al., 1997). The physiological function of these variants is not clear, although some variants appear to be cell-specific (Brandt et al., 1992; Keeton et al., 1993; Stauffer et al., 1995).

We found that PMCA1 was repressed by B in hippocampus and other brain regions. In adrenal-intact animals, repression of PMCA1 mRNA exhibited an inverse correlation with the B levels. In contrast, under relatively constant levels of B, cold stress increased PMCA1 expression. Although different stressors appear to elicit different neuroendocrine responses, B secretion appears to be an important component of a stress response in determining the level of PMCA1 expression.

MATERIALS AND METHODS

Animal care and in vivo *experiments*

Male Sprague Dawley rats (Bantin-Kingman, Freemont, CA), weighing between 200 and 225 gm at arrival, were used for all experiments. Animals were housed individually, maintained on a 12 hr light/dark schedule (lights on 6:30 A.M.) with ad libitum access to food and water, and were allowed at least 2 d to acclimatize to the housing conditions before any experiments were conducted. All efforts were made to minimize animal suffering and to reduce the number of animals used. All procedures were approved by the University of California, San Francisco Committee on Animal Research. After decapitation, the hippocampus was dissected and snap frozen in liquid nitrogen for RNA isolation and subtraction hybridization or the brains were immersed in OCT (Tissue Tek, Sakura, CA) and frozen in a dry ice-ethanol bath for sectioning. Trunk blood from all the animals was collected in tubes containing 0.3 M EDTA, centrifuged, and the plasma was stored at −20°C until radioimmunoassay for corticosterone and ACTH.

Experiment 1. Rats were bilaterally adrenalectomized (adx) and provided with 0.5% saline to drink for the next 5 d. On day 5 they were injected with vehicle (DMSO), 50 μg/kg or 10 mg/kg B, and killed 4 hr later to determine genes in hippocampus that were repressed by B.

Experiment 2. Rats treated similarly to those in experiment 1 with the addition of a sham-adx group injected with vehicle were killed 4 hr after injections for measurement of PMCA1 by *in situ* hybridization.

Experiment 3. To determine whether acute or repeated stress alters PMCA1 mRNA expression, rats were subjected to 2 hr restraint stress once, or repeated daily for 5 d. The animals were killed 4 hr after the onset of a single or final restraint stress together with a control, unstressed group.

Experiments 4a and b. To determine whether sustained chronic stress alters PMCA1 mRNA through effects on corticosterone, rats were exposed to either room temperature or 4°C cold for 5 d and killed in the morning under initial conditions (4a). To determine the role of chronic cold stimulus in the absence of adrenal B secretory responses to cold, rats were bilaterally adx, replaced with constant release pellet of B implanted subcutaneously (one 100 mg pellet designed to mimic mean plasma B levels achieved over a diurnal cycle in adrenal-intact animals), and either placed in the cold at 4°C for 5 d or left at room temperature (4b). The animals were killed in the morning of day 5.

Experiment 5. To test the acute interaction between exposure to the stimulus of restraint for 30 min and the B response, we measured PMCA1 30 min after the onset of restraint in rats that had been maintained at room temperature or in the cold for preceding 5 d.

Subtractive hybridization (PCR select)

The suppression subtractive hybridization technique (Diatchenko et al., 1996) (PCR select; Clontech, Cambridge, UK), was used to construct a cDNA library from hippocampus of adx rats. Rats were adx and 5 d later were treated with a high dose of B (10 mg/kg) or vehicle (DMSO). Four hours later, rats were decapitated, and RNA isolated from their hippocampus was used in the PCR-based method to identify steroid-regulated messages according to the manufacturer's protocol and as described earlier (Chen et al., 1999). In brief, 2 μg of poly(A^+) RNA was used to synthesize cDNA. First and second strand synthesis was performed on both groups of RNA, and the cDNA was subjected to digestion with *Rsa*I. To look for repressed genes, cDNA from hippocampus of adx animals (no hormone) were used as "tester" group. The tester cDNA was split into two groups, and each was ligated to different adapters. A first set of hybridization was performed using excess of cDNA from B-treated group (driver) against its corresponding tester cDNA. A second hybridization step was then used, in which the two tester cDNA sets were mixed with driver cDNA (forward subtraction). Next, two rounds of PCR amplification were performed using a primer sequences present in the adapter. The final PCR product was subsequently cloned into a TA cloning vector pCR2.1 (Invitrogen, San Diego, CA), transformed into *Escherichia coli*, plated, and individual colonies were picked and analyzed further. A secondary screen was used to eliminate false positives. Dot blots from individual colonies were prepared and probed with forward and reverse subtracted probes. Clones that were detected by the "forward-subtracted" probe and were absent from the "reverse-subtracted probe" were thought to be true positive clones (Fig. 1).

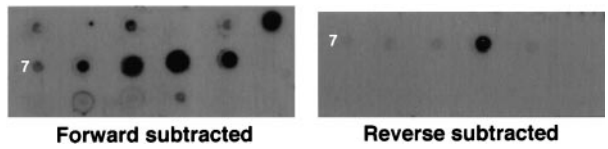


Figure 1. Subtracted cDNA library screened with forward- and reverse-subtracted probe. cDNA inserts from individual clones were amplified using nested primers, as described in Materials and Methods, and two identical dot blots were prepared. Forward- and reverse-subtracted cDNAs were digested with appropriate restriction enzymes to remove adaptor sequences, fractionated by electrophoresis, and used as probes to screen for putative positive clones. Blots were hybridized and exposed to film for 3 d to visualize weakly expressed clones. Clones that gave signals with the forward subtracted probe and either weak or no signal with the reverse-subtracted probe were treated as putative B-repressed clones. *Dot* corresponding to PMCA1 (VH7) is labeled.

RNA isolation and Northern blot analysis

Rats were treated as described above and were killed by decapitation. Tissues were dissected and snap frozen in liquid nitrogen. RNA was isolated using STAT-60 method (Tel-Test). Poly(A^+) RNA was prepared using oligo-dT column (Invitrogen). For Northern analysis, 10 μg of total RNAs were fractionated on a 1.2% agarose/formaldehyde denaturing gel and transferred onto nylon membrane (Hybond N; Amersham, Arlington Heights, IL). A multiple tissue poly(A^+) RNA blot from Clontech was used to determine tissue distribution of PMCA1. Radiolabeled probes were prepared by the random primer method. The 477 bp VH7/PMCA1 insert was obtained by restriction digestion of the plasmid with *Eco*RI, and the insert was purified, labeled, and used as a probe in this study. Signal intensity was quantitated on a PhosphorImager. Actin, a widely expressed housekeeping gene, was used as control mRNA for tissue distribution experiments (see Fig. 3). However, actin shows a complex response to glucocorticoids in the brain (Beaman-Hall et al., 1996), and hence was not used as a normalizing factor in the analysis of glucocorticoid effects on PMCA1. Cyclophilin is unaffected by B (Beaman-Hall et al., 1996), and in subsequent Northern blots this gene was used for normalization instead of actin.

Sequence analysis

Clones were sequenced by the ABI Prism dye terminator PCR cycle sequencing method from Perkin-Elmer (Emeryville, CA). Sequence was visualized and edited with Editview. Both strands were sequenced, and the sequences were analyzed by Blast search.

In situ *hybridization*

For detailed analysis of PMCA1 regulation in brain, rats from different groups were killed by decapitation, and their brains were frozen in a powdered dry ice and ethanol bath. Tissue sections (15 μm) were cut on a cryostat, thaw-mounted on Superfrost slides (three sections per slide), and fixed as described (Chen et al., 1999). Riboprobes using ^{33}P-UTP label were generated from 1 μg of linearized pCRII plasmid containing the 477 bp long VH7 insert with T7 (antisense; linearized with *Spe*I) and SP6 (sense; linearized with *Eco*RV) RNA polymerase using a standard *in vitro* transcription protocol (Promega, Madison, WI). Probe was dena-

tured at 65°C for 10 min, and 2×10^6 cpm of probe was applied to each slide. Sections were coverslipped and hybridized overnight at 55°C in a moist chamber. Hybridization and wash conditions used were as described previously (Chen et al., 1999). Sections were finally washed in 0.1× SSC at 65°C for 45 min, passed through an alcohol series, air-dried, and exposed to X-OMAT AR film overnight. The autoradiogram was scanned by Adobe Photoshop, and sections were quantitated using NIH Image program.

Quantification of hybridization signal and statistical analysis

Hybridization signal from each brain region was quantified by measuring mean average density using the NIH Image (version 1.61; W. Rasband, National Institutes of Health). Data shown here represent the mean of four to six animals per group and three tissue sections per animal ± SEM. Group means were initially analyzed for overall statistical significance using ANOVA. Fisher's and Scheffe analysis were used to test the significance of *post hoc* effects. A p value of 0.05 was considered significant. Regression analysis with slope comparisons was used to test the effect of B on PMCA1 mRNA levels in different brain regions. StatView (SAS Institute, Carey, NC) was the commercial package used for all analysis.

Cell culture

H19–7 cells of hippocampal origin (Eves et al., 1992; Beaman-Hall et al., 1996) were grown on Petri plates coated with poly-L-lysine, in 1% DMEM containing penicillin/streptomycin (50 U/ml and 50 μg/ml), 200 μg/ml of G418, and 10% FBS and grown at 33°C in 5% CO_2. For differentiation, cells were grown in medium comprised of 1% DMEM, penicillin/streptomycin, G418, 1% FBS, 10^{-10} M T3, 0.1 mM putrescine, 50 ng/ml insulin, 0.1 mg/ml transferrin, and 30 nM sodium selenite, and the process was accelerated by adding 10 ng/ml bFGF and incubating at 39°C in 5% CO_2. One day before hormone treatment, cells were grown in medium containing stripped FCS. Undifferentiated or differentiated cells were then treated with 10^{-7} M B for 0, 2, and 24 hr. RNA was isolated, and Northern blots were prepared as described above.

RESULTS

Isolation of corticosteroid downregulated genes

We generated a subtracted library representing genes repressed by corticosteroids in hippocampus from adx rats (experiment 1) using suppression subtractive hybridization. From this library, we identified 18 putative repressed clones, 12 of which passed a secondary screen (see Materials and Methods for details); the remaining six clones did not hybridize with either probe used in the secondary screen (Fig. 1). Partial sequences of all 12 clones were obtained. One clone, designated VH7, with an insert size of 477 bp was found to be identical to rat brain PMCA1 from nucleotides 1506–1983 (within the ORF) (Shull and Greeb, 1988) as determined by Blast search.

Hormone responsiveness and tissue distribution of PMCA1

Basal corticosteroid levels predominantly occupy MR, whereas high B levels during the circadian peak and stress progressively occupy both MR and GR (Dallman et al., 1992; Joëls and DeKloet, 1994), thus providing an opportunity to study either largely MR-mediated effects or effects mediated by MR and GR together. To confirm that rat PMCA1 was indeed repressed by B, RNA was isolated from hippocampus of sham-operated, adx animals and adx animals treated with either a high (10 mg/kg) or a low (50 μg/kg) dose of B as described in Materials and Methods. Northern blots of total hippocampal RNA from different groups of animals revealed that PMCA1 message was repressed ~33% by a high dose of B when compared with adx animals (Fig. 2), suggesting a GR-mediated (or MR–GR-mediated) effect on PMCA1. Significant repression was not observed in animals treated with a low dose of B, suggesting that MR by itself does not mediate repression of PMCA1. That sufficient levels of B were present to occupy MR in the low B group is demonstrated by plasma ACTH values, which were moderately suppressed by low dose of B and strongly suppressed by high dose of B (mean ACTH values in picograms per milliliter ± SEM: sham, 49 ± 9; adx, 615 ± 91; adx + low B, 332 ± 38; adx + high B, 66 ± 31).

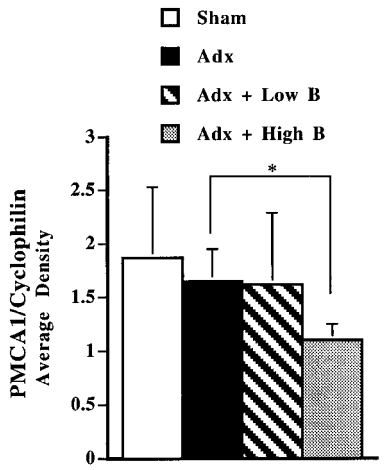


Figure 2. PMCA1 expression is repressed by B in rat hippocampus. PMCA1 expression was quantitated from Northern blots by densitometry and normalized using cyclophilin ($n = 3$; ± SEM). PMCA1 message was found to be repressed ~1.5-fold by high-dose B (10 mg/kg); low B (50 μg/kg) had no effect on PMCA1 expression. Differences between the Adx and Adx + high B groups were significant ($^*p < 0.05$ using Student's unpaired t test); other differences were not statistically significant.

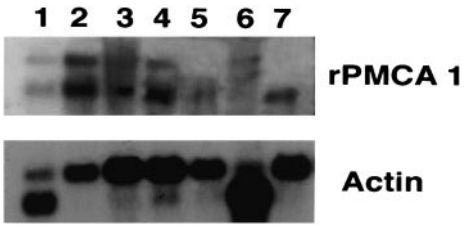


Figure 3. Expression pattern of PMCA1. Multiple tissue Northern blot (Clontech) containing 2 μg of poly(A^+) RNA was probed with the 477 bp PMCA1/VH7 insert. Lanes: *1,* heart; *2,* brain; *3,* lung; *4,* liver; *5,* spleen; *6,* skeletal muscle; *7,* kidney. *Bottom panel* shows the same blot probed with β-actin.

We also determined the tissue distribution of PMCA1 using a rat multiple tissue RNA blot. Two transcripts of approximate sizes 7.6 and 5.5 kb were identified in Northern blot probed with the 477 bp insert of VH7. The sizes and ubiquitous expression pattern of PMCA1 conforms with the previously reported sizes and tissue distribution (Greeb and Shull, 1989): the highest level of signal intensity was observed in brain, followed by lung and liver. Heart and kidney appeared to have moderate amounts of PMCA1, and low levels of signals were seen in spleen and skeletal muscle (Fig. 3).

Localization and regulation of PMCA1 in brain regions by *in situ* hybridization

Having established that PMCA1 was indeed repressed by B in hippocampus, we wanted to examine the distribution of PMCA1 in brain and ascertain whether the extent of its downregulation varied in different subfields of hippocampus and other brain regions (experiment 2). *In situ* hybridization was performed on coronal sections of rat brain from sham-operated, adx, and B-treated animals (Fig. 4*a*). PMCA1 was found to be highly

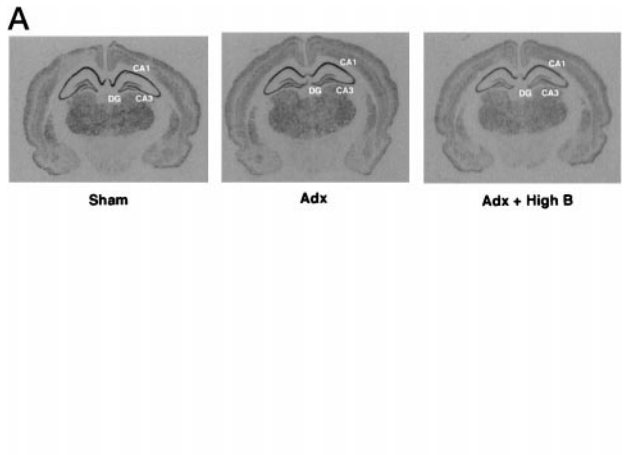


Figure 4. Repression of PMCA1 in rat brain detected by *in situ* hybridization. *A*, Coronal sections were hybridized with PMCA1 riboprobe. *Left*, Sham-operated; *middle*, Adx; *right*, Adx + high B. *DG,* Dentate gyrus. *B*, Signal density was measured using NIH Image. Signals were quantitated from three sections per animal with a total of four animals per group (± SEM). In dentate gyrus and cortex of adx + B-treated animals, PMCA1 levels were down by 33% [$F_{(2,8)} = 36.3$; $p < 0.001$; and cortex: ($F_{(2,8)} = 41.1$; $p < 0.001$)] as compared with Adx and sham-operated animals. PMCA1 levels in CA1 and CA3 subfields of hippocampus were down by 13% ($F_{(2,8)} = 44.3$, $p < 0.001$; $F_{(2,8)} = 40.5$, $p < 0.002$, respectively) in adx + B group. #$p < 0.001$ between sham and adx group; **$p < 0.001$ between sham and adx + B groups; *significant differences between adx and adx + B groups.

expressed in cerebral cortex, piriform cortex, hippocampus, thalamus, and striatum, a pattern previously reported specific for PMCA1 (Stahl et al., 1992). No detectable signal was seen in the choroid plexus, confirming that no cross-hybridization with PMCA isoform 3 (previously shown to be highly expressed in choroid plexus) was occurring. In hippocampus, dense signal was seen in pyramidal cells of CA1, whereas pyramidal cells of CA3 and the granule cell layer of dentate gyrus were less densely labeled. High levels of signal were also observed in different layers of cerebral cortex. No signal was detected when the sense riboprobe was used (data not shown).

We analyzed expression of PMCA1 in hippocampus and cortex in sham, adx, and adx + high B-treated animals. ANOVA revealed that there were significant effects of brain region and B-treatment ($p < 0.001$ for both). *Post hoc* tests revealed that differences in dentate gyrus ($F_{(2,8)} = 36.3$; $p < 0.001$) and CA1 ($F_{(2,8)} = 44.3$; $p < 0.001$) regions of hippocampus between all three groups. Moreover, PMCA1 expression in CA3 ($F_{(2,8)} = 40.5$; $p < 0.002$) and cortex ($F_{(2,8)} = 41.1$; $p < 0.001$) of B-treated

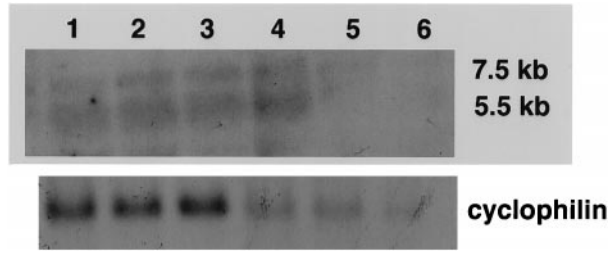


Figure 5. Total RNA was isolated from undifferentiated and differentiated H19–7 cells. Ten micrograms of total RNA were fractionated by agarose/formaldehyde gel electrophoresis, and Northern blots (repeated a total of three times) were hybridized to the 477 bp VH7 insert. A representative blot is shown here. *Lanes 1–3,* RNA from undifferentiated H19–7 cells; *lanes 4–6,* RNA from differentiated cells; *lanes 1 and 4,* no hormone treatment; *lanes 2 and 5,* 2 hr of B treatment; *lanes 3 and 6,* 24 hr B treatment. *Bottom panel,* The above blot was stripped and reprobed with cyclophilin, a housekeeping gene used for normalization and whose expression is unaffected by B treatment.

adx animals was significantly lower than both adx and sham groups, however, the differences in CA3 and cortex regions of sham and adx animals did not reach statistical significance (Fig. 4*b*).

Selective repression of PMCA1 in a neuronal cell line

To identify a cell culture system to study the mechanistic basis of PMCA1 repression, we examined the expression and steroid response of PMCA1 in H19–7 cells of hippocampal origin (Eves et al., 1992). These cells, transformed by stable expression of a temperature-sensitive mutant of SV-40 T-antigen, demonstrate characteristics of a neuronal phenotype after serum retraction and growth at high temperature. The differentiated H19–7 cells attain the morphology of neurons with protruding neurites and express neuronal markers such as neurofilamin protein and nerve growth factor (NGF) (Eves et al., 1992). They also express GR in both differentiated and undifferentiated states, however, they do not express significant levels of MR (Beaman-Hall et al., 1996; O. C. Meijer, unpublished results). H19–7 cells were cultured as described in Materials and Methods. Undifferentiated and differentiated cells were treated for 2 and 24 hr with 10^{-7} M B or vehicle (ethanol), and transcripts were detected by Northern blot probed with PMCA1 (Fig. 5). Both the 7.6 and 5.5 kb transcripts were found to be expressed in undifferentiated and differentiated H19–7 cells, a pattern identical to that seen in hippocampus. However, although undifferentiated H19–7 cells express PMCA1 transcripts, B does not affect their expression level. In contrast, significant downregulation of the message was observed as early as 2 hr after B treatment in differentiated H19–7 cells and was maintained for the duration of B treatment.

Effect of stress on PMCA1 expression

Stress invokes a complex array of early and late responses, only one of which is a marked increase in corticosteroids. Elevated corticosteroid levels and chronic stress may interfere with metabolically demanding processes like extrusion of Ca^{2+}, reduce the number of dendritic spines, and become detrimental (McEwen, 1994). Having established that PMCA1 is repressed by high B in hippocampus and other brain regions of adx rats and that similar repression is seen in cultured hippocampal neuronal cells (sug-

Table 1. Summary of *in vivo* manipulations

Treatment	B levels (μg/dl)	PMCA1 levels
Experiment 3. (Stress I measured at 4 hr)		
Control	8.8 ± 10.5[a]	Basal
Singly restraint	9.5 ± 8.5	No change
Repeated restraint (for 5 d)	4.8 ± 4.2	No change
Individual animals (from 3 groups)	Variable	Inverse correlation with B
Experiments 4–5. (Stress II measured at 0 and 30 min)		
Room temperature (controls)		
Intact (control for cold)	≤0.1	Basal
Adx + B	4.4 ± 0.3	Basal
Intact + 30 min restraint	50.5 ± 5.0	Variable - inverse correlation with B
Chronic cold (4°C for 5 d)		
Intact	2.3 ± 0.8	Downregulated
Adx + B	6.9 ± 1.0	Upregulated
Intact + 30 min restraint	62.3 ± 2.8	Variable - inverse correlation with B

[a] ± SEM.

gesting that the B effect is intrinsic to hippocampal neurons), we next wanted to determine the effects of stress itself on PMCA1 message *in vivo*.

In experiments directed at assessing this (experiment 3), neither single nor repeated acute restraint resulted in consistent elevations in B 4 hr after the stress (Table 1). Indeed, at this time (chosen to allow PMCA1 repression to be manifest), there was no significant difference in B level between stressed and nonstressed animals, when the groups were compared as a whole. Similarly, there was no significant difference in PMCA1 mRNA in any brain region (Fig. 6*a,b*). However, the concentrations of B varied widely within groups, prompting us to determine if there was a possible correlation between B level and PMCA1 expression. As shown in Figure 6*c*, there was a strong inverse correlation between B level and PMCA1 mRNA in CA3 and dentate by linear regression analysis ($r^2 = 0.41$; $p < 0.04$), consistent with the idea that stress-induced increases in B repress PMCA1.

Acute restraint stimulates marked but transient elevation in B (Bradbury et al., 1991) and, moreover, there is a marked blunting of the rise in B in response to repeated bouts of acute restraint (Lachuer et al., 1994). Therefore, we next tested the effect of the more sustained stressor of chronic cold (experiment 4), which has been shown to double the integrated urinary B output throughout a 5 d exposure to cold (Akana et al., 1996). Chronically cold-stressed rats had significantly elevated B levels as compared to room temperature controls, (2.3 ± 0.8 vs = 0.1 μg/dl, respectively). In these rats, cold stress resulted in significantly (27–30%) decreased expression of PMCA1 in all regions of hippocampus (Fig. 7*a,b*) but not cortex. In hippocampus, there were significant decreases of PMCA1 mRNA in CA1 (27.5%; $F_{(1,6)} = 7.2$; $p = 0.04$), CA3 (30%; $F_{(1,6)} = 12.8$; $p = 0.01$), and DG (28%; $F_{(1,6)} = 11.3$; $p = 0.02$). Unlike B, stress did not repress PMCA1 in all brain regions examined, suggesting the interesting possibility that stress offsets a variety of effects, and only one of them is enhanced production of B.

To determine the effect of stress on PMCA1 in the presence of constant B, we examined the response of PMCA1 mRNA to cold stress in adx, B-replaced rats. Adx rats were provided with constant B replacement (35% B; 65% cholesterol w/w) and exposed to room temperature or cold for 5 d (experiment 4b). B levels in cold rats were slightly, but significantly higher than those at room temperature (6.9 ± 1.0 vs 4.4 ± 0.3 μg/dl, respectively; $p < 0.05$), probably as a consequence of decreased hepatic B metabolism in the cold. Despite this modest difference in B, the stimulus of cold resulted in increased (26%), not decreased, expression of PMCA1 (Fig. 8*a,b*). ANOVA showed significant main effects of temperature condition ($p < 0.001$) and brain region ($p < 0.001$). *Post hoc* (Scheffe) analysis revealed significant cold-associated increases in CA1 ($F_{(1,10)} = 5.6$; $p = 0.04$) and CA3 ($F_{(1,10)} = 11.1$; $p = 0.008$) and increases that were not significant in the dentate gyrus and cortex ($p < 0.1$ and $p < 0.06$, respectively). Thus, rats that cannot respond to cold with the normal marked increase in B exhibited stimulus-induced increases in PMCA1, unlike adrenal-intact animals that persistently increase B secretion after this stressor. Taken together, these data suggest that stress has dual effects on PMCA1 expression: a non-B-dependent effect appears to increase PMCA1 expression, whereas B blunts or even reverses this effect.

Finally, we examined the effect of acute restraint stress superimposed on chronic cold stress on PMCA1 mRNA expression (experiment 5). In this stress paradigm, animals are maintained in the cold or at room temperature for 5 d followed by 0.5 hr of restraint stress, and substantially higher levels of B are achieved than in response to either 4 hr of restraint stress or chronic cold stress alone (Table 1; Akana and Dallman, 1997; Bhatnagar and Dallman, 1998). As shown in Table 1, no significant differences in PMCA1 expression were found between the two groups examined as a whole (similar to the findings of experiment 3). However, the within-group variation in B level was high (also as with experiment 3), and in view of the rapid *in vitro* response to B in hippocampal cultures (H19–7 cells) and the significant but modest negative relationship between B levels and PMCA1 mRNA 4 hr after restraint stress observed earlier (experiment 3; Fig. 6*c*), it seemed likely that a strong inverse correlation between B levels and PMCA1 expression would be found in this more robust stress paradigm. Indeed, as shown in Figure 9, when examined by linear regression, PMCA1 expression 30 min after the onset of acute restraint stress was strongly negatively correlated with B in cortex ($r^2 = 0.44$; $p < 0.01$), dentate gyrus ($r^2 = 0.49$; $p < 0.01$), CA1 ($r^2 = 0.61$; $p < 0.01$), and CA3 ($r^2 = 0.73$; $p < 0.01$). These data further support the idea that both acute and chronic elevations in B inhibit PMCA1 expression. The effect of different stressors on PMCA1 mRNA is summarized in Table 1.

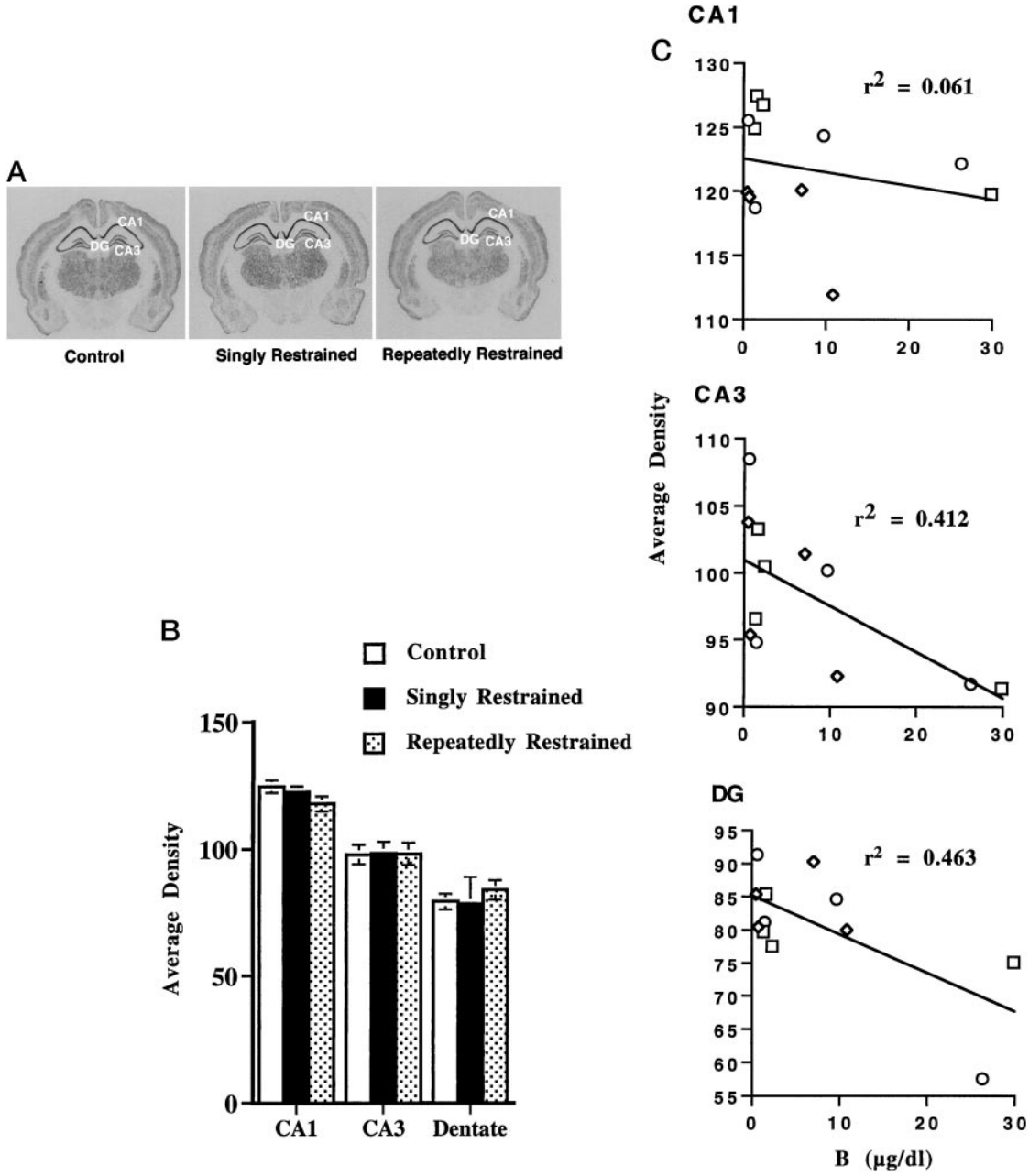
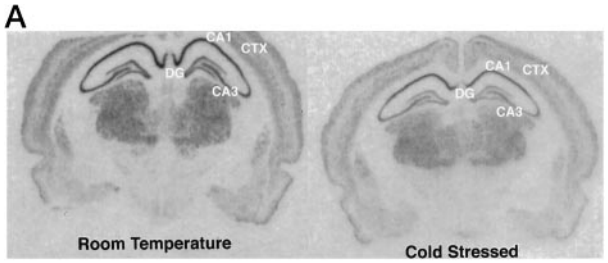


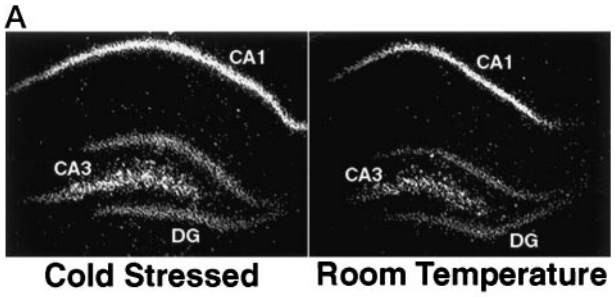
Figure 6. Effect of acute restraint stress on PMCA1 expression in rat brain. *a*, *In situ* hybridization was performed on rat brains: *left*, unstressed controls; *middle*, singly-restrained; *right*, repeatedly restrained. Significant repression of PMCA1 message was not observed. *b*, The above results were quantitated ($n = 4$ per group) as in Figure 3*b*. Statistically significant repression of PMCA1 message was not observed after restraint stress. *c*, PMCA1 exhibits an inverse correlation with terminal plasma B levels in CA3 and dentate gyrus regions of the hippocampus but not in the CA1 region when the three groups are treated as a single group (*open squares,* controls; *circles,* singly restrained; *diamonds,* repeatedly restrained).

DISCUSSION

Repression of PMCA1 gene transcription by corticosterone

We report in this study identification and characterization of PMCA1 as a gene repressed by corticosterone, probably through GR (or both GR and MR), in the hippocampus of adx rats. Corticosterone inhibits PMCA1 expression *in vitro* in a hippocampal cell line. Therefore, effects on PMCA1 in rat brain are likely to be caused by corticosterone actions in hippocampal cells and not mediated by actions of corticosterone on other cells elsewhere in brain. PMCA1 is downregulated *in vivo* in rat brain within 4 hr of administration of a single dose of B, and the dentate gyrus and cerebral cortex show the greatest degree of downregulation with repression also seen in pyramidal neurons of CA3 and CA1 regions. Predominant MR occupancy at low B levels results in small ionic conductance and transmitter responses (Joëls and de Kloet, 1994), decreased Ca conductance (Karst et al., 1994), and is involved in protective actions against stress. In contrast, at high B levels, GR occupancy has opposite effects on these parameters (Landfield and Eldridge, 1994). Thus, it appears that a balance in MR and GR-mediated effects is critical for neuronal excitability and stress responsiveness (de Kloet, 1991).





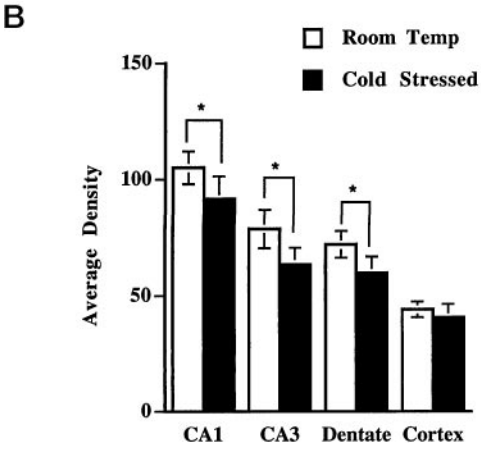


Figure 7. Chronic cold stress in intact animals results in repression of PMCA1 message in all regions of the hippocampus. *a*, A representative *in situ* hybridization showing an unstressed (room temperature) and cold-stressed animal probed for PMCA1. *b*, *In situ* hybridization results were quantitated as described above ($n = 4$). *Post hoc* analysis showed that significant effects were observed in CA1 ($F_{(1,6)} = 7.2$; $^*p = 0.04$), CA3 ($F_{(1,6)} = 12.8$; $^*p = 0.01$), and DG ($F_{(1,6)} = 11.3$; $^*p = 0.02$), whereas the difference in cortex did not attain statistical significance.

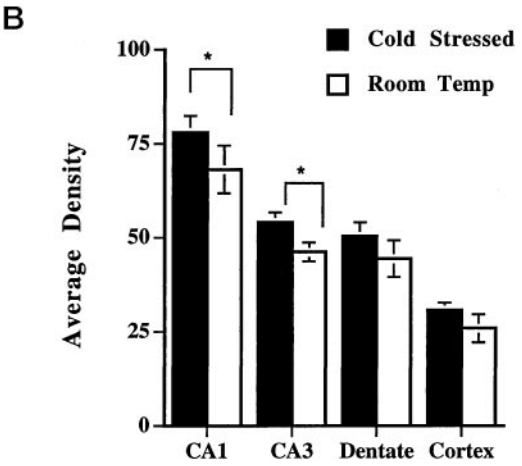


Figure 8. Chronic cold stress in B-clamped animals increases PMCA1 expression. Rats were adrenalectomized, and B was replaced by constant release pellet (see Materials and Methods). *a*, Representative *in situ* hybridizations were probed for PMCA1 as in Figure 7. *b*, PMCA1 expression was quantitated by densitometry ($n = 6$); by *post hoc* analysis, significant differences between room temperature and cold-stressed animals were observed in CA1 ($F_{(1,10)} = 5.6$; $p = 0.04$) and CA3 ($F_{(1,10)} = 11.1$; $p = 0.008$) subfields of hippocampus. Differences in the dentate gyrus and the cortex did not achieve statistical significance.

Differential regulation of PMCA1 mRNA by different models of stress

In adrenal-intact animals, PMCA1 also appears to be inhibited by B, however its regulation by stress appears to be complicated, encompassing both B and other neurochemical components. The mild stimulus of chronic or acute restraint results in no consistent change in PMCA1 expression, suggesting that the combination of restraint and corticosterone secretion cancelled any net effect on PMCA1 expression. However, the levels of B varied greatly within groups, and an inverse correlation was established between B levels and PMCA1 mRNA in CA3 and dentate gyrus regions. With the moderate stress of chronic cold, when corticosterone levels were significantly different between groups, PMCA1 mRNA levels were decreased in all hippocampal areas. In contrast, when corticosterone levels were relatively fixed (in B-replaced adx animals), PMCA1 mRNA increased in response to stress. It is notable that the change in B appears to be the critical parameter in relative PMCA1 expression because B levels were higher in adx + B than in stressed, adrenal-intact animals. The latter animals, however, had a more marked (23-fold) increase in plasma B, whereas B increased less than twofold in adx + B-replaced animals. Thus, the effects of stress on PMCA1 are complex, and B appears to regulate PMCA1 expression in concert with other noncorticosteroid neurohumoral factors. Together, these noncorticosteroid stress inputs appear to increase PMCA1 mRNA whereas the consequent corticosterone secretion represses it. It remains to be determined what pathways are implicated in the non-B-dependent stimulation of PMCA1. It is interesting to speculate that activation of PMCA1 is cFos-dependent. The differential repressive effects of GR and MR on cFos-stimulated gene transcription would then provide a potential explanation for the distinct effects of MR and GR on PMCA1 expression (Pearce and Yamamoto, 1993; Meijer et al., 2000).

Different stressors activate different input pathways and result in varying magnitudes and duration of B secretion (Keller-Wood et al., 1984; Beaulieu et al., 1987; Palkovits, 1987) and thus the response of a gene to stress- and B-dependent effects may also depend on the type of stressor. In these studies, repeated re-

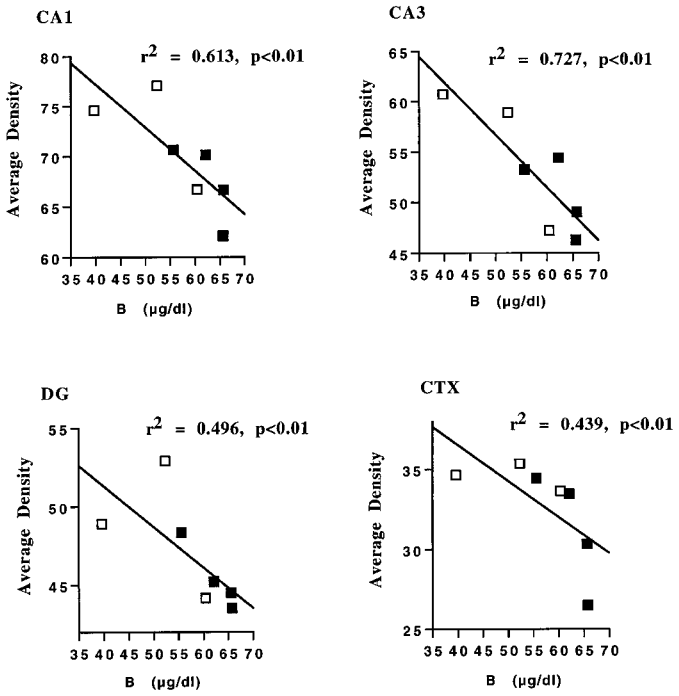


Figure 9. Linear regression of plasma B levels and PMCA1 expression (average optical density; average OD) demonstrates that 30 min acute restraint stress and cold stress in combination with 30 min acute restraint stress results in a strong inverse relationship between PMCA1 and B levels. Measurements were averaged from three brain sections per animal and $n = 7$ (*open squares,* 30 min acute restraint; *filled squares,* 5 d cold-stressed + 30 min acute restraint). The maximum relationship was observed in CA3 ($r^2 = 72\%$), followed by CA1 ($r^2 = 61\%$), dentate gyrus ($r^2 = 49\%$), and least but significant effect in the cortex ($r^2 = 43\%$).

straint, a transient stimulus that provokes markedly reduced adrenocortical responses with repetition (Lachuer et al., 1994), exerted no net effect on hippocampal PMCA1, although PMCA1 expression was inversely correlated with plasma B levels at the time of collecting the brains. In contrast to restraint, cold, which is known to persistently stimulate B secretion (Akana et al., 1996), resulted in diminished PMCA1 expression throughout the hippocampus, but not cortex. Whether there is an increase, decrease or no change in PMCA1 expression probably depends on the relative magnitudes of stimulation and B response. This dual regulation of PMCA1 coupled with interindividual variation in the magnitude of the elevation in B provides a plausible basis for the variability in pathological consequences of stress that have been described (Gilles et al., 1996; Adler et al., 1997).

Repression of PMCA1 and other isoforms of this membrane calcium pump has been reported after kainic acid (KA)-induced seizures in rats (Garcia et al., 1997). PMCA1 expression was not reduced in hippocampus after injection of KA until 12 hr in CA3 and 24 hr in CA1; significant repression was not observed in the dentate gyrus. KA injection is a strong stimulus to the adrenocortical system and endogenous B secretion, and administration of B with KA blunts the rise in KA-induced immediate early genes such as AP-1 (Unlap and Jope, 1995a) and NFkB (Unlap and Jope,1995b). Thus, it appears that the rise in B inhibits PMCA1 stimulation by KA. It seems likely that the effects of KA are stimulatory on PMCA1 mRNA and are only slowly counteracted by persistent, endogenous B secretion.

PMCA1 mRNA was stimulated specifically in hippocampal regions CA1 and CA3, but not in the dentate gyrus by cold stress in B-clamped animals that are unable to hypersecrete corticosterone. Interestingly, high doses of B repress PMCA1 in all hippocampal areas, but stress, which results in endogenous B secretion, does not. These results re-emphasize the fact that elevated B levels and stress are not the same and that cellular responses are dependent on (or determined by) the context of stress, the neurochemical pathways activated by stressors, and the pattern and levels of circulating corticosteroids. In addition to the effects of sustained stress and B secretion on PMCA1 expression, we found evidence for acute modulation of PMCA1 by stress-induced B secretion. Thirty minutes after restraint, PMCA1 mRNA was strongly, inversely correlated to plasma B levels, particularly in hippocampal regions CA3 and CA1. Thus, it appears that B-dependent repression of PMCA1 is an important component of the stress response.

Context-dependent regulation of PMCA1 in neuronal cells

In H19–7 cells, we see repression of PMCA1 as early as 2 hr after treatment with B. This phenomenon is observed only in differentiated H19–7 cells and not in undifferentiated cells, although both express GR (Beaman-Hall et al., 1996; Meijer, unpublished data). These results emphasize that the action of B is state-dependent and that other factors play a key role in mediating the outcome. Interestingly, PMCA1 levels appear to be dynamic and rapidly repressed by GCs. Characterization of the PMCA1 promoter region and 5′ untranslated region will yield important insight into regulation of this gene by GCs. Elevated GCs compromise the ability of neural cells to withstand stresses such as ischemia or seizures (Sapolsky, 1996a,b). The deleterious effects of GCs are probably attributable to enhanced neuronal vulnerability to glutamate and calcium, which facilitate cell death. Short-term elevations in GCs cause reversible dendritic atrophy in CA3 (Woolley et al., 1990), but long-term elevation results in irreversible damage. It will be of interest to determine whether repression of PMCA1 in differentiated H19–7 cells does indeed result in an increased intracellular level of Ca^{2+}.

Our observation that PMCA1 is regulated by B in adx rats and differentiated H19–7 cells, and by different stressors to varying degrees, suggests state-dependent and cell-type-specific responses to the repressive action of GCs. It will interesting to determine which splice variant of PMCA1 is regulated by B in H19–7 cells. The b forms of all PMCAs exhibit 10-fold higher affinity for Ca^{2+}–calmodulin than the a form *in vitro* and with physiological concentrations of calmodulin (Enyedi et al., 1994). This suggests that PMCA1b can respond quickly to smaller changes in concentration of intracellular Ca^{2+} concentrations than other splice variants, as seen with the various treatments administered in this study. Electrophysiological data suggest that B also modulates Ca^{2+} currents by regulating Ca^{2+} influx in CA1 neurons via differential occupation of MR and GR (Nair et al., 1998). A role for Ca^{2+} in GR-mediated effects is demonstrated by the fact that high levels of B also affect Ca^{2+}-related membrane events (Kerr et al., 1989).

The change in concentration of intracellular Ca^{2+} level expected to result from repression of PMCA1 may lead to a variety of downstream effects, including changes in expression of genes such as NF-AT, Oct/OAP, and NF-kB (Dolmetsch et al., 1998; Li et al., 1998; Malviya and Rogue, 1998). In addition, Ca^{2+} is associated with increased synaptic vesicle exocytosis and neurotransmitter release (Goda and Südhof, 1997). Although it has

long been known that corticosteroids increase intracellular Ca^{2+} levels, the precise mechanisms regulating this process have remained unclear. Controlling the extrusion of Ca^{2+} ions by GC-mediated repression of PMCA is one possible mechanism by which this is achieved. Thus, repression of PMCA1 may be an important component of the physiological and pathophysiological effects of corticosteroids.

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