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Hippocampal glucocorticoid receptor activation enhances voltage-dependent Ca^{2+} conductances: Relevance to brain aging

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ABSTRACT **Glucocorticoids (GCs) activate several biochemical/molecular processes in the hippocampus through two receptor types. In addition, GCs influence cognitive behaviors and hippocampal neural activity and can also increase the rate of aging-dependent cell loss in the hippocampus. However, the ionic mechanisms through which GCs modulate hippocampal neuronal function are not well understood. We report here direct evidence that activation of cytosolic steroid receptors, specifically of the type II GC receptor, can enhance voltage-dependent Ca^{2+} conductances in brain neurons. Ca^{2+} current was assessed by current-clamp measures of Ca^{2+} action potentials and by sharp electrode voltage-clamp analyses of voltage-sensitive currents in cesium-, tetrodotoxin-, and tetraethylammonium-treated CA1 neurons in hippocampal slices. Both Ca^{2+} action potentials and voltage-activated Ca^{2+} currents (N- and L-like) were increased by 2-hr exposure to the synthetic GC receptor agonist, RU 28362. This effect of RU 28362 was blocked by coincubation with cycloheximide, indicating that the GC receptor–Ca^{2+} channel interaction depends on *de novo* protein synthesis. Dysregulated calcium homeostasis is also viewed as a candidate mechanism in brain aging. Thus, present results are consistent with the hypothesis that excessive GC-receptor activation and resultant increased Ca^{2+} influx may be two sequential phases of a brain-aging process that results initially in impairment of function and eventually in neuronal loss.**

Although it has been recognized for >20 yr that the brain, and in particular the hippocampus, is rich in specific corticosteroid receptors (1, 2), the cellular effects of activating these receptors are still poorly understood (3). Glucocorticoids (GCs) stimulate a number of biochemical and genomic processes in hippocampal neurons (3–5) and have been found by extracellular recording to alter brain neuronal excitability (6–8). Further, stress-related hormones substantially influence memory and other cognitive functions (9, 10). In addition to these normal functions, moreover, chronic exposure to GCs can exert neurotoxic actions on hippocampal pyramidal cells, particularly in conjunction with the brain aging process (11–15). Long-term adrenalectomy protects (12), whereas chronic administration of corticosterone (Cort) (13) or chronic stress (14, 15) accelerates, aging-related hippocampal neuronal loss (for review, see ref. 16).

Studies with synthetic steroids (17) have indicated that two types of brain receptors bind GCs—the type I, or mineralocorticoid receptor (MCR), and the type II, or glucocorticoid receptor (GCR)—both of which are found in CA1 hippocampal neurons (18–20). The MCR has higher affinity for Cort than does the GCR, and an emerging view (21–23) suggests that the MCR and GCR act coordinately and, in some cases, oppositely, to regulate neuronal homeostasis and the stress response (for a comprehensive review, see ref. 21).

Recent intracellular electrophysiological studies have provided insights into how brain GCRs may act to modify ionic conductances and neuronal function. Two studies (24, 25) found that Cort increases the well-defined (26) Ca^{2+}-dependent, K^+-mediated afterhyperpolarization (AHP) that normally follows Na^+ action potentials in CA1 hippocampal neurons. This effect of Cort on the AHP likely accounts for inhibitory actions of GCs on hippocampal neurons (24, 25) and is mediated by the GCR because it can be mimicked by the highly specific GCR agonist RU 28362 (24). Conversely, further studies have indicated that MCR activation increases hippocampal excitability by suppressing neurotransmitter-mediated hyperpolarization (for review, see ref. 27). These MCR and GCR effects on hippocampal excitability can be blocked by inhibition of protein synthesis (28).

However, it is still not clear how GCs modulate the AHP. Although the effect of GCs on the AHP was suggested to be mediated by an increase in voltage-activated Ca^{2+} conductance (25), the GC effect could also be mediated by actions on Ca^{2+} buffering/extrusion or K^+ channels, among others. Steroids have been shown to influence a number of K^+ and Cl^- conductances, through actions on membrane receptors (29–31), but to date there has been no evidence that steroids can also influence voltage-sensitive Ca^{2+} conductance.

A clear answer to the question of a putative GC–Ca^{2+} channel linkage is also of particular relevance to present concepts on mechanisms of brain aging. Elevated intracellular Ca^{2+} can be neurotoxic, and there is increasing evidence of neuronal Ca^{2+} dysregulation in brain aging (see refs. 32–37). Because the AHP is increased with aging (25, 37), the recent findings that GCs modulate the Ca^{2+}-dependent AHP (24, 25) and, moreover, exert an increased impact in aged neurons (25) suggest that there may be a link between the GC and the Ca^{2+} dysregulation hypotheses of brain aging. That is, it has been proposed that excessive GC activation and Ca^{2+} dysregulation may be two sequential and causally linked phases of the same brain aging process (16, 25). However, this proposal depends upon clear evidence that GCs directly modulate Ca^{2+} conductance.

Consequently, the present study specifically tests this possibility. We report here direct evidence that GCs enhance hippocampal voltage-dependent Ca^{2+} currents and apparently do so by classical steroid mechanisms involving *de novo* protein synthesis.

MATERIALS AND METHODS

Preparations and Treatments. Hippocampal slices (450 μm thick) were obtained from 3- to 4-mo-old male Fischer 344 rats and maintained according to standard techniques (ref. 38; see refs. 25 and 37 for additional details). All animals were

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Abbreviations: AHP, afterhyperpolarization; GC, glucocorticoid; CHX, cycloheximide; GCR, glucocorticoid receptor; MCR, mineralocorticoid receptor; Cort, corticosterone; RU, synthetic glucocorticoid agonist RU 28362.

adrenalectomized 3–7 days before study, either by surgical removal of the adrenals under general anesthesia (xylazine at 4.4 mg/kg plus ketamine at 68 mg/kg i.m.; see ref. 25 for additional details) or by pharmacological blockade of corticosteroid biosynthesis by metyrapone (200 mg/kg s.c. administered twice per day for 4–7 days) (see ref. 39). In subsequent analyses, the mean values for electrophysiological measures of the pharmacological and surgical adrenalectomy conditions were extremely close, and the two conditions were combined.

The highly specific synthetic type II GCR agonist RU 28362 (RU; ref. 17; Roussel–Uclaf) was diluted in ethanol and bathing medium and applied directly by micropipette to hippocampal slices from rats adrenalectomized for a period of 3–7 days. The final concentration (in the well) of ethanol for both steroid or vehicle conditions was 0.015%; the final concentration of RU was 7 μM. Presumably, this is a saturating concentration, although gradients through the slice make it difficult to determine the final concentration seen by a neuron within the slice. Slices were exposed for at least 2 hr to either vehicle, RU alone, or RU and the protein synthesis inhibitor, cycloheximide (CHX, final concentration, 10 μM per well). The need for prolonged incubation of the slices in RU and CHX precluded using neurons as their own controls. CHX alone had no effect on vehicle values and, therefore, vehicle and vehicle plus CHX control groups were combined.

Electrophysiological Techniques. CA1 pyramidal cells were impaled and cesium (Cs)-loaded with CsCl-filled microelectrodes (75–100 MΩ, 2 M CsCl in 5 mM Tris, pH 7.15). The Cs-loading substantially blocks K^+ currents—particularly the afterhyperpolarization current—in hippocampal neurons (40). Tetrodotoxin was applied (1.0 μM) to block Na^+ spikes and allow the unmasking of relatively pure Ca^{2+} action potentials (e.g., refs. 41 and 42). Cells that showed at least 35 MΩ input resistance were selected for study. After acquisition of Ca^{2+} action potential data in current-clamp mode, tetraethylammonium (10 mM) was applied to block remaining repolarizing K^+ currents, which are relatively insensitive to Cs. Blockade was deemed effective once Ca^{2+} action potentials began to exhibit prolonged plateaus, with repolarization delayed beyond 1500 ms. A subpopulation of neurons (see below) was then studied in voltage-clamp mode.

Hippocampal neurons are known to have multiple types of voltage-activated Ca^{2+} channels (43–45), and previous work in this laboratory with sharp electrode discontinuous voltage-clamp techniques similar to those used here has identified several types of inward Ca^{2+} current in nondissociated hippocampal-slice neurons (46, 47). These currents differ in some, but not all, aspects from Ca^{2+} currents defined in cell cultures and dissociated cells. In hippocampal-slice neurons held at −70 to −80 mV, the Ca^{2+} current activated at the lowest voltage range (above −50 mV) is a low-amplitude current, which, unlike T, N, or L current (43–45), is completely noninactivating and cannot be modulated by L-channel dihydropyridine agonists or antagonists. We have termed this current R current (for resistant) (47), and this current appears similar in many ways to the recently described P current (48). An N-like (or T-like) current, which is subject to pronounced Ca^{2+}- and voltage-dependent inactivation, is activated at somewhat higher voltages (≈−40 mV). A third type of Ca^{2+} current, elicited at still higher voltage ranges (≈−30 mV), is partly sensitive to dihydropyridine modulators, is slowly inactivating, and appears analogous to L-type currents. The activation of this L-type current is associated with a long tail-like "aftercurrent." Our control studies indicate that the aftercurrent is not due to inadequate space clamp or to Ca^{2+}-dependent Cl^- current (37, 46, 47), but further studies will be required to establish this definitively.

Voltage and current traces were acquired and amplified by an Axoclamp model 2A discontinuous voltage-clamp amplifier, digitized, and stored on the hard-disk of a personal computer and analyzed off-line by ASYST-based software developed by one of the authors (L.W.C.). Analyses of variance (ANOVAs) using *post-hoc* Bonferroni procedures or *t* tests were used to assess Ca^{2+} action-potential and Ca^{2+}-current values. A probability level of $P = 0.05$ was used as the minimal criterion for statistical significance.

RESULTS

Current Clamp. Ca^{2+} action potentials recorded in tetrodotoxin-treated, CsCl-loaded hippocampal CA1 pyramidal cells (held at −70 mV) exhibit a characteristic shape (46, 49). In response to an intracellular depolarizing current pulse (40 ms, 150% of threshold), there is an initial, large-amplitude, fast-spike phase followed by a lower-amplitude, longer-lasting slow "hump" phase (Fig. 1, upper trace). For each cell studied in current clamp ($n = 61$), five Ca^{2+} action potentials were averaged and quantified (see below). Input resistance was assessed with a low-intensity (0.2 nA, 40 ms) hyperpolarizing-current pulse and was not affected by any of the treatments (means ± SEM in MΩ: vehicle, 48.5 ± 2.0; RU, 48.9 ± 1.4; RU plus CHX, 48.2 ± 3.6; $F_{2,58} = 0.03$, not significant).

The effects of RU and RU plus CHX on Ca^{2+} action potentials are illustrated in both Fig. 1 (representative examples) and Fig. 2 (quantitative results). Except for fast-spike amplitude, all measures of Ca^{2+} action-potential size were increased substantially by incubation with RU. Further, coincubation with the protein-synthesis inhibitor CHX blocked the RU-induced increase for all measures. Significant results by one-way ANOVA were as follows: slow-hump amplitude, $F_{2,58} = 9.70$, $P = 0.0002$; overall action-potential duration,

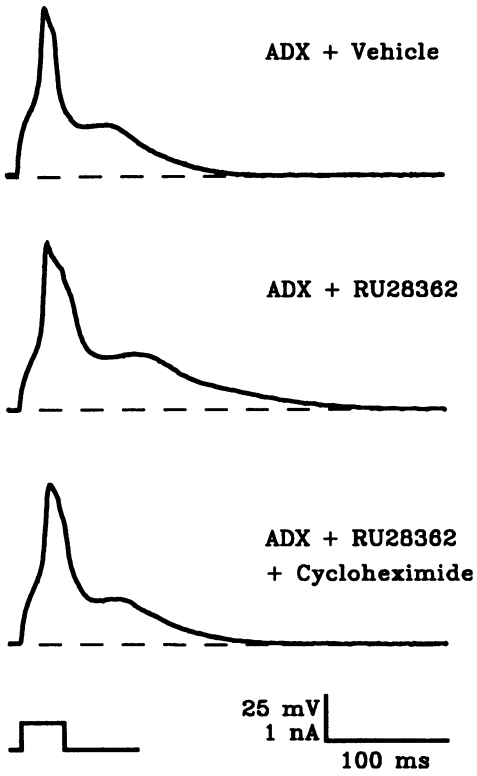

FIG. 1. Representative Ca^{2+} action potentials from CsCl-loaded, tetrodotoxin-treated neurons in hippocampal slices exposed to either vehicle, RU, or RU plus CHX. Action potentials were triggered by depolarizing constant-current pulses and have an initial large-amplitude, fast phase and a late slow phase. Neurons exposed to a saturating dose of RU exhibited wider initial phases and longer-duration and larger-amplitude slow phases than control neurons. Coincubating RU-treated slices with 10 μM CHX eliminates the effect of RU on the Ca^{2+} action potential.

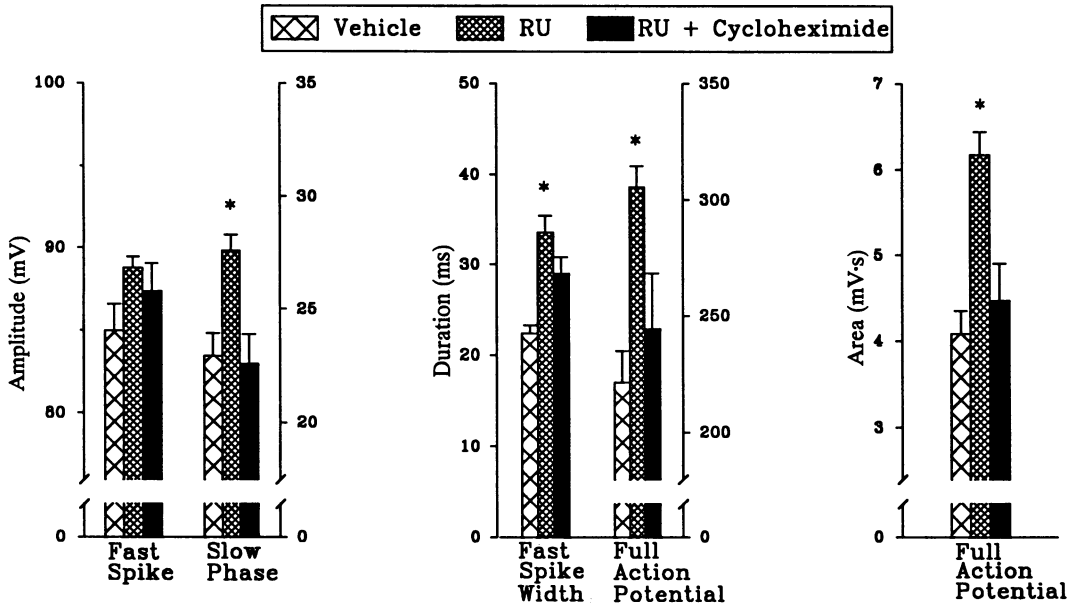

FIG. 2. Means ± SEM for measures on Ca^{2+} action potentials of neurons treated with either vehicle ($n = 19$), RU ($n = 30$), or RU plus CHX ($n = 12$). The width of the initial fast phase, the late slow-phase amplitude, overall duration, and overall area under the full action potential curve were significantly increased by treatment with RU alone. These effects were blocked by concurrent treatment with CHX. *, $P < 0.05$ in *post hoc* comparisons (Bonferroni) between vehicle control and RU groups.

$F_{2,58} = 12.41$, $P < 0.0001$; fast-spike width, $F_{2,58} = 11.41$, $P = 0.0001$; overall action-potential area $F_{2,58} = 14.98$, $P < 0.0001$. *Post hoc* comparisons between the RU and vehicle groups differed significantly on each measure, whereas, none of the RU plus CHX measurements differed significantly from the vehicle group. The RU and RU plus CHX groups differed on each variable except fast-spike width.

Voltage Clamp. To reduce variability from differences in space-clamp efficacy and voltage control (50), only pairs of cells (one vehicle, one RU) from the same animal were used in a paired analysis. In addition, some cells were lost during these lengthy protocols. Therefore, voltage-clamp studies were limited to RU vs. vehicle comparisons in 16 neurons.

Protocols with steps from −70 mV to −30 mV were used to activate all three current types defined in this preparation. Current amplitude was measured for each cell ($n = 16$) at the peak of the current response (which reflects all three currents and, particularly, strong N currents), at the end of the depolarizing step (late pulse, at which N current is largely inactivated), and during the postpulse current (25 ms after the end of the depolarizing command pulse), which primarily reflects an L-type aftercurrent. In addition, protocols for Ca^{2+}- and voltage-dependent inactivation were done on each cell, and current–voltage relationships were examined in several cells for each condition.

Fig. 3*A* illustrates representative examples of the currents generated by voltage steps to −30 mV, and Fig. 4 shows the quantitative results for this protocol. Ca^{2+} currents in neurons incubated in RU were significantly larger than in vehicle controls (peak, $F_{1,7} = 5.58$, $P = 0.05$; late pulse, $F_{1,7} = 6.35$, $P = 0.04$; postpulse, $F_{1,7} = 9.73$, $P = 0.017$). R currents were not analyzed separately in these experiments, but preliminary observations indicate that they were not altered by RU treatment. Voltage- and Ca^{2+}-dependent inactivation, as well as current–voltage relationships (Fig. 3*B*) were not altered by RU.

DISCUSSION

The present studies demonstrate that selective activation of type II GCRs by RU results in a marked enhancement of Ca^{2+} action potentials and currents in hippocampal CA1 neurons. Because both peak current and late current showed significant effects of RU, the prolongation of the Ca^{2+} action potential by RU may be from increased current through both

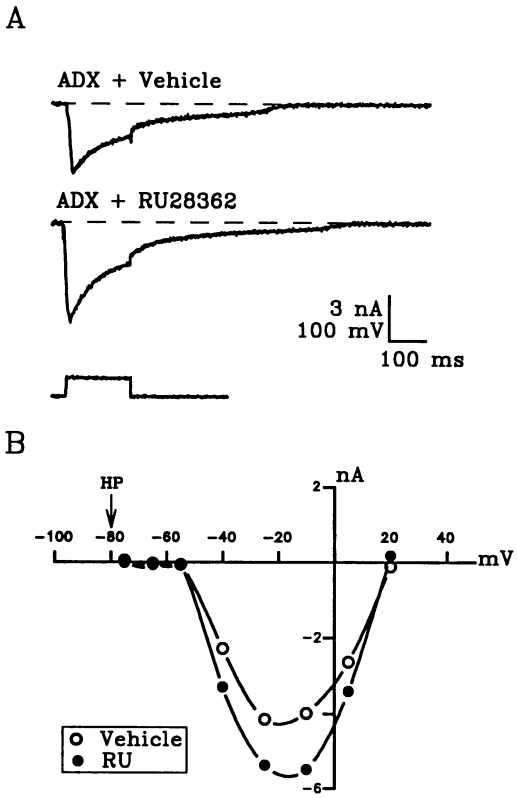

FIG. 3. (*A*) Representative voltage-clamp traces from CA1 neurons in slices from adrenalectomized animals treated with vehicle or 7 μM RU. Cells were stepped for 200 ms to approximately −30 mV, a command sufficient to elicit both a rapidly inactivating Ca^{2+} current and a slowly inactivating L-type Ca^{2+} current; the latter is also associated with a prolonged aftercurrent. Treatment with RU increased inward Ca^{2+} current. (*B*) Peak current–voltage curves for two representative neurons, indicating that RU increases inward current rather than shifting the activation curve. HP, holding potential.

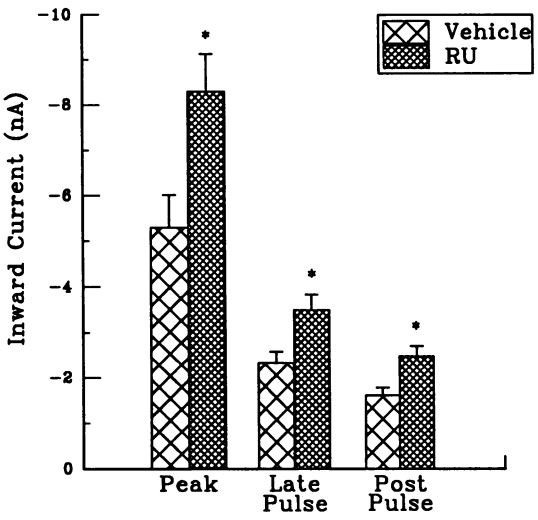

FIG. 4. Means (± SEMs) from paired CA1 neurons (vehicle, n = 8; RU, n = 8) for three measures on the Ca^{2+} current waveforms shown in Fig. 3. RU increased inward Ca^{2+} current at the current peak, at the end of the pulse (late pulse) where N-type current is largely inactivated, and during the postpulse period, which reflects an L-type aftercurrent. (*, $P \leq 0.05$.)

N-type and L-type voltage-sensitive Ca^{2+} channels. However, more detailed studies on isolated current types, at lower concentrations of RU, will be needed before we can clearly identify the specific channel type(s) modulated by GCs.

Thus, the present studies provide clear electrophysiological evidence that steroids, specifically GCs, directly modulate voltage-sensitive Ca^{2+} channels in brain neurons. As with the effect of GCs on the AHP (28), the GC–Ca^{2+} current linkage appears to depend on a classical steroid-protein synthetic action. Therefore, although the present data do not preclude the possibility that intracellular Ca^{2+} buffering or K^+ conductances are also modified by GCs, the modulation of voltage-activated Ca^{2+} channels by GCs appears the most parsimonious explanation of the previously observed (24, 25) effect of GCs on the AHP.

Several steroids have been reported to interact with membrane receptors that modulate K^+ and Cl^- currents (29–31). However, considering the critical role of Ca^{2+} in numerous cellular functions, the evidence of a direct, CHX-sensitive effect on Ca^{2+} conductance provides potentially important insights into the basic mechanisms of endocrine–brain interactions.

As noted above, moreover, the data may have important implications for brain aging processes. Although GCs clearly have protective effects on neurons, in addition to their deleterious actions (21–23), the present findings appear to provide insights into processes that could specifically underlie the age-associated neurotoxic actions of GCs. That is, the data reported here indicate that the previously described age-related increase in the Cort-dependent component of the hippocampal AHP (25) is based on an increase in voltage-activated Ca^{2+} influx. In turn, this enhanced influx of Ca^{2+} during the AHP after each Na^+ action potential might well contribute to cumulative Ca^{2+}-mediated neurotoxicity with aging. The direct modulation of Ca^{2+} influx by GCs is a key prediction of the hypothesis that prolonged/excessive GC activation and neuronal Ca^{2+} dysregulation are two sequential phases of a single brain aging process (16, 25). Consequently, the GC–Ca^{2+} current link observed here lends additional support to this hypothesis.

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