Glucocorticoids Specifically Enhance L-Type Calcium Current Amplitude and Affect Calcium Channel Subunit Expression in the Mouse Hippocampus

Pascal Chameau,1 Yongjun Qin,1 Sabine Spijker,2 Guus Smit,2 and Marian Joëls1
1Swammerdam Institute for Life Science; Center for NeuroScience University of Amsterdam, Amsterdam; and 2Center for Neurogenomics and Cognitive Research, Vrije Universiteit, Amsterdam, The Netherlands

Submitted 7 August 2006; accepted in final form 27 September 2006

Glucocorticoids specifically enhance L-type calcium current amplitude and affect calcium channel subunit expression in the mouse hippocampus. J Neurophysiol 97: 5–14, 2007. First published October 4, 2006; doi:10.1152/jn.00821.2006. Previous studies have shown that corticosterone enhances whole cell calcium currents in CA1 pyramidal neurons, through a pathway involving binding of glucocorticoid receptor homodimers to the DNA. We examined whether glucocorticoids show selectivity for L- over N-type of calcium currents. Moreover, we addressed the putative gene targets that eventually lead to the enhanced calcium currents. Electrophysiological recordings were performed in nucleated patches that allow excellent voltage control. Calcium currents in these patches almost exclusively involve N- and L-type channels. We found that L- but not N-type calcium currents were largely enhanced after treatment with a high dose of corticosterone sufficient to activate glucocorticoid receptors. Voltage dependency and kinetic properties of the currents were unaffected by the hormone. Nonstationary noise analysis suggests that the increased current is not caused by a larger unitary conductance, but rather to a doubling of the number of functional channels. Quantitative real-time PCR revealed that transcripts of the Ca1 subunits encoding for the N- or L-type calcium channels are not upregulated in the mouse CA1 area; instead, a strong, direct, and consistent upregulation of the β4 subunit was observed. This indicates that the corticosteroid-induced increase in number of L-type calcium channels is not caused by a simple transcriptional regulation of the pore-forming subunit of the channels.

INTRODUCTION

Corticosterone, which is released in high concentrations from the mouse adrenal cortex after stress, can easily pass the blood–brain barrier and bind to discretely localized intracellular receptors in the brain (for review, De Kloet et al. 2005). On activation, these receptors translocate to the nucleus where they regulate the transcription of responsive genes (Zhou and Cidlowski 2005). After stress, particularly the glucocorticoid receptors (GRs) will be activated, against a background of higher-affinity mineralocorticoid receptors (MRs), which are already substantially occupied with corticosteroid levels that circulate under resting/basal conditions. Hippocampal CA1 neurons co-express both receptor types (Van Steensel et al. 1996). Earlier studies have shown that corticosteroid hormones exert a slow but lasting control over many properties of hippocampal CA1 neurons through this binary receptor system (for review, Joëls 2001).

In particular, calcium influx into CA1 neurons turned out to be very sensitive to shifts in corticosteroid receptor activation. Thus predominant MR activation—occurring with low levels of corticosterone—results in small amplitudes of calcium currents, as measured with whole cell voltage-clamp methods (Karst et al. 1994). After stress or in vitro GR activation, the amplitude of calcium currents increases (Joëls et al. 2003; Karst et al. 1994; Kerr et al. 1992) with a delay of 1–2 h, pointing to a gene-mediated mechanism. Accordingly, it was found that the GR-mediated enhancement of calcium currents requires protein synthesis (Kerr et al. 1992) and binding of GR homodimers to the DNA (Karst et al. 2000). The enhanced calcium influx most likely contributes to a more prominent spike frequency adaptation when CA1 cells are depolarized (Joëls and de Kloet 1989; Kerr et al. 1989), which would help to normalize activity in the CA1 region after a temporary stress-induced arousal. However, enhanced calcium influx also renders a higher degree of vulnerability in case CA1 cells are exposed to additional challenges such as epileptic or ischemic insults (Karst et al. 1999; Krugers et al. 1999; Smith-Swintosky et al. 1996).

Thus, although it is clear that corticosterone, through a gene-mediated pathway, increases the amplitude of whole cell calcium currents in CA1 pyramidal cells, many questions about this phenomenon are still unanswered. Here, we addressed two specific issues. First, it is at present unknown which calcium channel is sensitive to GR modulation. Earlier recordings with whole cell patch clamp indicated that sustained and peak currents (mostly reflecting L- and N-type currents, respectively) are affected by the hormone (Kerr et al. 1992). Further distinction, however, was difficult, given the poor space clamp of this technique and hence the questionable distinction between currents based on voltage and kinetic properties. Therefore in a first experimental series, we examined calcium currents 1–4 h after a brief application of corticosterone (100 nM) or vehicle, using nucleated patches, which offer excellent voltage control. We used pharmacological agents to isolate specific currents. By applying nonstationary noise analysis, this preparation also allowed a preliminary study of single channel properties, distinguishing between a GR-dependent increase of the unitary channel conductance or the available number of channels in the membrane. The electrophysiological recordings were followed by a second series of experiments in which we examined whether GRs directly target genes encoding for the calcium channel subunits that were studied in the first series.

Address for reprint requests and other correspondence: P. Chameau, SILS-CNS, Univ. of Amsterdam, Kruislaan 320, 1098 SM Amsterdam, The Netherlands (E-mail: pchameau@science.uva.nl).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
To this end, RNA was isolated from the CA1 area 1 h after a brief treatment with vehicle or a high dose of corticosterone (100 nM) sufficient to activate GRs (Karst et al. 2000). We studied, with quantitative real-time PCR, changes in the transcript number of the calcium channel subunits Ca_{1.2}, Ca_{1.3} and Ca_{2.2} (which will be translated to the pore-forming subunit of the N- (Ca_{2.2}) and L- (Ca_{1.2}, Ca_{1.3}) type calcium channels, respectively; for review, Catterall 2000), as well as transcripts of the auxiliary subunits Ca_{\beta}1–4 (review Dolphin 2003).

**METHODS**

**Animals and tissue treatment**

Male C57/B16 mice (Harlan), weighing 50–70 g, were housed (lights on between 8:00 A.M. to 8:00 P.M.) pairwise in standard cages, with food and water ad libitum, for at least 1 wk before the start of the experiment. At the day of the experiment, they were decapitated under isoflurane anaesthesia in the morning (~9:00 A.M.). We verified in trunk blood that circulating plasma corticosterone concentrations in all mice were well below 5 μg/dl, which supposedly leads to substantial MR but only limited GR activation (Reul and de Kloet 1985). After decapitation, the brain was dipped in ice-cold (4°C) artificial cerebrospinal fluid (ACSF) of the following composition (in mM): 126 NaCl, 1.5 KCl, 1.5 MgCl\(_2\), 2 CaCl\(_2\), 26 NaHCO\(_3\), 1.25 KH\(_2\)PO\(_4\), and 10 d-glucose. Next, the frontal lobes and cerebellum were removed and brain slices (300 μm thick) were prepared using a Leica VT1000S vibratome (Leica Microsystems, Nussloch, Germany); these slices were allowed to recover for at least 1 h in ACSF (32°C) before corticosterone treatments started.

For real-time PCR experiments, slices from each animal were randomly divided over either of the two treatment groups tested per animal, allowing paired statistical testing. The treatments consisted of 1) 20-min treatment with vehicle (≤0.01% ethanol in ACSF) or 100 nM of corticosterone (n = 12 mice); this protocol suffices to activate GRs (in addition to MRs) in vitro (Karst et al. 2000); or 2) treatment for 40 min with the protein synthesis inhibitor cycloheximide (100 μM) or vehicle, giving 100 nM corticosterone during the last 20 min of this treatment (n = 8); this could determine whether the gene under study is a primary or a downstream target of GRs. One hour after start of the corticosterone or vehicle treatment, the CA1 pyramidal cell layer was isolated from the remaining tissue under a binocular, using a sharp surgical knife. Tissue thus collected was kept on ice and immediately processed for RNA isolation.

For electrophysiological experiments, brain slices of mice were prepared and treated in a similar way, although here a paired approach was not used.

**Electrophysiology**

In brief, experiments were performed at room temperature (20–22°C) on 6- to 8-wk-old mice. Slices were superfused with ACSF equilibrated with 95% O\(_2\)-5% CO\(_2\). Whole cell patch-clamp recordings were obtained from hippocampal CA1 pyramidal cells (Karst et al. 2000). For each cell showing calcium currents in the whole cell recording mode, the nucleated outside out patch configuration was established as described previously (Karst et al. 2000; Sather et al. 1992), 10 min after the whole cell configuration was obtained. Calcium currents (\(I_{Ca_{L/S}}\)) were recorded with the following extracellular solution (in mM): 106 NaCl, 1.5 KCl, 1.5 MgCl\(_2\), 2 CaCl\(_2\), 26 NaHCO\(_3\), 1.25 KH\(_2\)PO\(_4\), 10 d-glucose, 10 TEA-Cl, 5 4-aminopyridine, and 0.5 TTX (pH 7.4, 300 mM). Patch pipettes had a resistance of 4–6 MΩ and were filled with the following solution (in mM): 135 TrisMeSO\(_4\), 10 HEPES, 10 EGTA, 4 NaATP, and 0.3 NaGTP (pH 7.3, 295 mOsm). All compounds were obtained from Sigma, with the exception of TTX (Latoxan, France).

Data acquisition was performed using an Axopatch 200 B amplifier (Axon Instruments, Foster City, CA). Signals in the whole cell configuration were filtered at 2 kHz and sampled at 4 kHz; in the nucleated patch configuration, signals were filtered at 10 kHz and sampled at 20 kHz. Cells and nucleated patches were voltage clamped at a holding potential of ~85 mV, and calcium currents were evoked by 200-ms voltage pulses to different potentials. When nifedipine was applied, nucleated patches were held during the first 5 min at ~40 mV, as the compound binds preferentially to channels when they are in the inactivated state (Sanguineti and Kass 1984); holding potential was brought to −85 mV, just before the test protocol was started. In agreement with the expectation, we observed that blockade of currents was indeed much more pronounced when cells were temporarily held at ~40 mV (data not shown). Although the effects of corticosterone with or without this period at ~40 mV were qualitatively comparable, we here only present the former because of the more complete block of L-type channels. Series resistance (\(R_s\)) was compensated for 60–70%. Data analysis was performed using Clampfit (Axon Instruments). Signals incorporated in further analysis were always leak-subtracted. All data in this study are represented as the means ± SE. Statistical comparison was done with an unpaired Student’s t-test (\(P < 0.05\)).

To exclude the influence of small variations in the size of the nucleated patches, all data in this study are expressed as current density, i.e., the current amplitude divided by the capacitance. Mean capacitances of the various treatment groups ranged from 1.5 to 2.5 pF. In none of the treatment groups (nifedipine, ω-conotoxin, agatoxin, or BayK) did we observe a significant difference in capacitance between the tissue treated with corticosterone and vehicle-treated tissue.

For nonstationary fluctuation analysis (Sigworth 1980), 20 current responses evoked by 200-ms depolarizing pulses to 0 mV per nucleated patch were averaged, and the variance around the mean was calculated. The relationship between the mean current amplitude (\(I\)) and the variance (\(\sigma^2\)) was fitted with the equation \(\sigma^2 = \mu - F/N\), where \(\mu\) is the single-channel current amplitude and \(N\) is the estimate of the number of channels open at the peak of the current. The maximum open probability (\(P_{o_{\text{max}}})\) was calculated according to the following equation: \(P_{o_{\text{max}}} = 1-(\sigma^2_{\text{peak}}/I_{\text{peak}})\), where \(\sigma^2_{\text{peak}}\) is the variance of the current at its peak, and \(I_{\text{peak}}\) is the peak current amplitude.

Quantitative real-time PCR

The dissected CA1 areas from each hippocampal lobe were pooled per animal. RNA was isolated with Trizol (Life Technologies) and subjected to DNase I (SU, Roche 776785) treatment. RNA quality and integrity was examined on an agarose gel (data not shown). Subsequently, the treated RNA (0.5–1 μg) was reverse-transcribed (200 U MMLV, Gibco) with random primers (25 pmol).

SYBR Green PCR measurements (10 μl; ABI PRISM 7900, Applied Biosystems) were performed with transcript-specific primers (300 nM) and SYBR green 2× master mix (Applied Biosystems) on cDNA corresponding to ~15 ng RNA, using standard cycle setting. Primers were designed using Primer Express 1.5 (Applied Biosciences). In some cases, duplicate primer sets were used to verify differential gene expression (Table 1). From a panel of six housekeeping genes [glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, neuronal specific enolase (NSE), hypoxanthine phosphoribosyl transferase (HPRT), Neurofilament-L (NFL) and (α-tubulin)], the pair of GAPDH and β-actin showed the most stable expression across all individual samples, and were therefore taken as reference. Expression levels were calculated with the Δ-ΔCt method (Spijker et al. 2004). For statistical analysis a paired t-test was applied.
RESULTS

Calcium currents in nucleated patches

The first question we addressed regarded the sensitivity of specific calcium current types to GR activation, using a preparation with optimal voltage control. To this end we recorded calcium currents in nucleated patches from CA1 pyramidal neurons. Because (with the exception of 1 brief report; Karst et al. 2000) calcium currents in nucleated patches had not been described, we first optimized the recording conditions and next determined their current properties.

Reliable currents in nucleated patches were obtained when recording with a TrisMeSO₃-based medium in the pipette. As depicted for an example in Fig. 1, the amplitude of total calcium currents in nucleated patches was small (mean maximal peak: 30 ± 11002 pA, n = 12). This is roughly 45-fold lower than seen for whole cell currents recorded in these cells (mean maximal peak current: 1.3 ± 0.3 nA, n = 12). Currents from nucleated patches displayed an inactivation that for the first 100 ms could be fitted with a single exponential decay (Fig. 1B; see also Fig. 3A). For each current trace, we determined the

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca₂.2</td>
<td>TCGCAGGATTCGATGTTTG</td>
<td>GAGCTGCTGCTGCTGCTGCTG</td>
</tr>
<tr>
<td>Ca₁.2</td>
<td>GAGCTGCTGCTGCTGCTGCTG</td>
<td>GAGCTGCTGCTGCTGCTGCTG</td>
</tr>
<tr>
<td>Ca₁.3</td>
<td>GAGCTGCTGCTGCTGCTGCTG</td>
<td>GAGCTGCTGCTGCTGCTGCTG</td>
</tr>
<tr>
<td>Caβ1</td>
<td>GAGCTGCTGCTGCTGCTGCTG</td>
<td>GAGCTGCTGCTGCTGCTGCTG</td>
</tr>
<tr>
<td>Caβ2</td>
<td>GAGCTGCTGCTGCTGCTGCTG</td>
<td>GAGCTGCTGCTGCTGCTGCTG</td>
</tr>
<tr>
<td>Caβ3</td>
<td>GAGCTGCTGCTGCTGCTGCTG</td>
<td>GAGCTGCTGCTGCTGCTGCTG</td>
</tr>
<tr>
<td>Caβ4 (1st)</td>
<td>GAGCTGCTGCTGCTGCTGCTG</td>
<td>GAGCTGCTGCTGCTGCTGCTG</td>
</tr>
<tr>
<td>Caβ4 (2nd)</td>
<td>GAGCTGCTGCTGCTGCTGCTG</td>
<td>GAGCTGCTGCTGCTGCTGCTG</td>
</tr>
</tbody>
</table>

TABLE 1. Primer sequences used in the qPCR experiments

Primers were designed with Primers Express software 1.5 (Applied Biosciences); the mRNA sequences were obtained from GenBank. Primers were preferably selected for the untranslated 3’ end of the mRNA with an amplicon between 75 and 125 bp.

FIG. 1. Voltage-dependent calcium currents recorded in CA1 hippocampal neurons. A: examples of calcium currents recorded in a CA1 pyramidal neuron in whole cell configuration (left). Currents were evoked by 200-ms depolarizing pulses from a holding potential of −85 mV to test pulses of various amplitudes (from −70 to 0 mV). Right: current-voltage relationship obtained from cell on left is shown (mean ± SE in control cells, n = 5). Only peak current is represented. B: examples of calcium currents recorded in the same cell, now in the nucleated patch configuration. Currents were evoked by 200-ms depolarizing pulses from a holding potential of −85 mV to test pulses of various amplitudes (from −70 to +20 mV). Arrows indicate positions where peak and sustained values of calcium current were measured (sustained current was measured as average current during last 10 ms). Right: mean ± SE current-voltage relationship is shown for vehicle-treated control cells (n = 16); both peak and sustained currents are represented. Note difference with A for x-axis. C: steady-state inactivation of I_Ca,2.2. Left: current inactivation was evaluated by a 2-s conditioning prepulse from a holding potential of −85 mV to different voltage pulses (from −100 to +10 mV), followed by a 200-ms test pulse to +10 mV. Right: level of steady-state inactivation (I/I_max) was plotted as a function of prepulse potential and fitted with a Boltzmann equation: I = I_max/[1 + exp(V_p - V_1/2/V_c)], where I_max is maximal current, V_p is potential of half-maximal current inactivation, and V_c is proportional to slope of curve. Data represent mean ± SE of 5 cells.
peak and sustained component as depicted in Fig. 1B. Maximal peak currents were seen around 0 mV. This is at a more depolarized potential than seen with whole cell recording in the same slices (Fig. 1A). The half-maximal voltage for steady-state inactivation in nucleated patches was around −50 mV (Fig. 1C).

The currents in nucleated patches were stable during the first 5–10 min, but gradually ran down (data not shown). Because the run-down would confound the interpretation of changes induced by calcium channel blockers during this period, the effect of such pharmacological treatments could not be reliably studied over time in one cell. Therefore we started the application of calcium channel blockers when the whole cell configuration of a cell was successfully established, so that blockers were present from the start of recording in the nucleated patches. Next, groups of cells recorded in the presence or absence of a particular blocker were compared, with unpaired statistical tests. All data shown here were obtained 5 min after the nucleated patch was isolated from the soma. To exclude the influence of small variations in the size of the nucleated patches, all data are expressed as current density, i.e., the current amplitude divided by the capacitance.

To determine the contribution of the various Ca-current types to the overall current we applied selective channel blockers. As a first step, nifedipine was applied, at a concentration (5 μM) that selectively blocks L-type over other types of calcium channels (Lorenzon and Foehring 1995). In the control group, application of nifedipine resulted on average in a peak current density that amounted to only 51% of the value in the absence of nifedipine (Fig. 2, A and B). The density of sustained currents was even reduced by 70% (Fig. 2C). Current density recorded in the presence of the N-type calcium channel blocker ω-conotoxin GVIA (500 nM) yielded peak values that were only 42% of the amplitude seen in untreated cells (Fig. 2, A and B). The density of the sustained current was reduced by 30%. This shows that the peak current is much more sensitive to ω-conotoxin than the sustained current. Neither the peak nor the sustained current was reduced at all by the P/Q channel blocker ω-agatoxin IVA (200 nM) (Fig. 2B). When nifedipine and ω-conotoxin were co-applied, a very small fraction of the peak (3%) and sustained (5%) current remained discernable.

Because the latter component was almost negligible, we will indicate in the following paragraphs currents recorded in the presence of nifedipine as N-type currents, and currents recorded in the presence of ω-conotoxin as L-type currents (see Discussion). The ratio between the sustained and peak components of this L-type current amounted to ~0.7, indicating that Ca current inactivation was limited in our nucleated patches. This differs from other preparations like HEK cells (Liang et al. 2003), stressing the importance of the recording conditions.

In the presence of the dihydropyridine receptor agonist BayK8644 (5 μM), the current density was increased by 72 and 58% for the peak and sustained components, respectively (Fig. 2B).

Effect of corticosterone on calcium currents in nucleated patches

We next established the density of the total current in nucleated patches and the L-type and N-type currents, in slices pretreated during 20 min with 100 nM corticosterone 1–4 h before recording. As shown in Fig. 3, the total peak current was increased by 45% several hours after pretreatment with corticosterone (typical example in Fig. 3A). As expected, currents recorded after corticosterone administration in the presence of the GR-antagonist RU 486 (500 nM) were significantly smaller than those recorded in slices that were treated with corticosterone alone and did not differ from the control group (Fig. 3B). Corticosterone pretreatment did not affect other properties of the calcium currents in nucleated patches. Thus decay time was quite similar for the corticosterone treated and control cells (Fig. 3C). Both the steady-state activation and inactivation were not affected by the hormone treatment, with the exception of a small difference in the inactivation curve for voltages between −10 and 10 mV (Fig. 3D).

The corticosterone-induced increase in current density was even more pronounced for the sustained part of the total current (83%; Fig. 3B, right) than for the peak component (45%). In accordance, the density of L-type currents was markedly increased after corticosterone treatment (Fig. 4), both with re-
FIG. 3. Effect of corticosteroid on voltage-dependent calcium currents. A: examples of calcium currents in nucleated patches, evoked by 200-ms depolarization to 0 mV from a holding potential of −85 mV in control conditions (vehicle) and after corticosterone treatment. Solid line depicts fit with a single exponential. B: average current densities evoked by voltage pulses to 0 mV in control conditions, after corticosterone treatment and when corticosterone was applied in presence of GR antagonist (RU 486). Mean ± SE is given for peak (left) and sustained currents (right). Number of observations: vehicle = 16 cells; corticosterone (CORT) = 14; CORT + RU 486 = 7. C: time constant of inactivation in control conditions and after corticosterone treatment was comparable (mean ± SE). Decay of calcium current was fitted with a single exponential. Number of observations: vehicle = 16 cells; corticosterone = 14. D: voltage-dependent activation and inactivation of total calcium current in control conditions and after corticosterone treatment. Current was evoked by a 200-ms depolarizing pulse from a holding potential of −85 mV to different voltage potentials (from −50 to 0 mV). Conductance was calculated according to the equation $G(V) = I(V_m - V_r)/V_m$, where $I$ is current, $V_m$ is test pulse potential, $V_r$ is measured reversal potential. Averaged data were fitted with Boltzmann equation: $G = G_{max}/[1 + \exp(V - V_h)/V_c]$. Inactivation of calcium current was tested as described in Fig. 1. Following averaged values were obtained for activation curves in vehicle-treated slices: $V_h = -25 ± 1$ mV, $V_c = 6.6 ± 0.5$ (n = 16 cells); and 1–4 h after corticosterone treatment: $V_h = -21 ± 1$ mV; $V_c = 8.7 ± 0.8$ (n = 14). For inactivation curves we observed in vehicle-treated slices: $V_h = -49 ± 15$ mV, $V_c = 11 ± 3$ (n = 5); and in corticosterone-exposed slices: $V_h = -64 ± 5$ mV, $V_c = 15 ± 3$ (n = 5).

FIG. 4. Modulation of various calcium current components by corticosterone. A: average current densities evoked by a voltage pulse to 0 mV after corticosterone treatment. Bars represent mean ± SE of currents recorded under conditions where particular calcium channel blockers or BayK were present. Number of observations after corticosterone treatment: total = 14 cells; +Nif = 5; +αConoTx = 4; +AgaTx IV = 3; +Nif/αConoTx = 6; BayK = 11. B: comparison of current densities in the presence of specific calcium channel blockers, between control conditions (vehicle = veh; even bars; for more information see legend of Fig. 2), and after corticosterone treatment (CORT; striped bars). Currents in presence of nifedipine (supposedly representing N-type channel activity; light gray) are not affected by corticosterone, whereas currents in presence of α conotoxin GVI (most likely L-type currents; medium gray) are doubled in size. Small residual current seen when both nifedipine and α conotoxin were applied (dark gray) was not affected by corticosterone treatment. All bars in A and B represent mean ± SE of either peak (left) or sustained currents (right); *P < 0.05.
In the second series of experiments, we established to what extent Ca_	ext{a,1} and β subunits of mouse CA1 pyramidal cells are transcriptionally regulated by glucocorticoids. We focused on the subunits encoding the N- and L-type calcium currents (Ca_{2,2} and Ca_{1,2/1,3}, respectively), because these prevailed and from six different recordings under corticosterone-treatment (cort). The average peak current densities in these cells were $-6.9 \pm 0.9$ (vehicle) and $-13.7 \pm 1.6$ pA/pF (cort). The variance versus mean plot (Fig. 5B) could be fitted with a parabolic function (see METHODS) and gave an estimated single-channel amplitude of $0.879 \pm 0.278$ (vehicle) and $0.783 \pm 0.206$ pA (cort). The estimated single-channel conductances, assuming a reversal potential for evoked calcium currents of +125 mV were not significantly changed: 7.0 ± 2.2 (vehicle) and 6.2 ± 1.6 pS (cort) ($P = 0.783$, unpaired t-test). The numbers of channels open at the peak ($N$) were estimated to be $26 \pm 4$ ($n = 5$) (corresponding to a $P_{o,max}$ of $0.53 \pm 0.07$) in control conditions and $55 \pm 4$ ($n = 6$) (corresponding to a $P_{o,max}$ of $0.50 \pm 0.06$) after corticosterone. In our experiments, the $P_{o,max}$ values were not significantly changed ($P = 0.798$), but we observed a significant increase in the number of activated channels ($P < 0.001$). Considering a specific membrane capacitance of 0.01 pF/μm², the calculated channel densities were $0.129 \pm 0.016$ (vehicle) and $0.253 \pm 0.025$ (corticosterone) channels/μm². Based on these observations, it seems that corticosterone treatment does not markedly change single-channel conductance but instead doubles the number of (activated) channels in the membrane.

Quantitative PCR

In the second series of experiments, we established to what extent Ca_{a,1} and β subunits of mouse CA1 pyramidal cells are transcriptionally regulated by glucocorticoids. We focused on the subunits encoding the N- and L-type calcium currents (Ca_{2,2} and Ca_{1,2/1,3}, respectively), because these prevailed

Nonstationary noise analysis

To determine whether the specific increase in L-type current amplitude induced by corticosterone treatment was caused by changes in the channel properties (change in conductance) or in the number of channels we performed nonstationary noise analysis. Because the currents recorded in the nucleated patch were mediated by both L-type and N-type channels, all experiments were done in the presence of ω-conotoxin (500 nM) to isolate L-type currents. We also pharmacologically increased the channels’ open probability by perfusing BayK (5 μM), because an indication of the number of channels can only be obtained when the peak of the parabola is approached (Sigworth 1980).

Under these conditions, a reasonable impression of the current fluctuations around the mean could be obtained (Fig. 5A). The nonstationary noise analysis was performed on stable nucleated patch recordings where the successive currents evoked by series of pulses from a holding potential of −85 to 0 mV did not shown run down. The data presented here result from five different recordings in control conditions (vehicle) and from six different recordings under corticosterone-treatment (cort). The average peak current densities in these cells were $-6.9 \pm 0.9$ (vehicle) and $-13.7 \pm 1.6$ pA/pF (cort). The variance versus mean plot (Fig. 5B) could be fitted with a parabolic function (see METHODS) and gave an estimated single-channel amplitude of $0.879 \pm 0.278$ (vehicle) and $0.783 \pm 0.206$ pA (cort). The estimated single-channel conductances, assuming a reversal potential for evoked calcium currents of +125 mV were not significantly changed: 7.0 ± 2.2 (vehicle) and 6.2 ± 1.6 pS (cort) ($P = 0.783$, unpaired t-test). The numbers of channels open at the peak ($N$) were estimated to be $26 \pm 4$ ($n = 5$) (corresponding to a $P_{o,max}$ of $0.53 \pm 0.07$) in control conditions and $55 \pm 4$ ($n = 6$) (corresponding to a $P_{o,max}$ of $0.50 \pm 0.06$) after corticosterone. In our experiments, the $P_{o,max}$ values were not significantly changed ($P = 0.798$), but we observed a significant increase in the number of activated channels ($P < 0.001$). Considering a specific membrane capacitance of 0.01 pF/μm², the calculated channel densities were $0.129 \pm 0.016$ (vehicle) and $0.253 \pm 0.025$ (corticosterone) channels/μm². Based on these observations, it seems that corticosterone treatment does not markedly change single-channel conductance but instead doubles the number of (activated) channels in the membrane.

Quantitative PCR

In the second series of experiments, we established to what extent Ca_{a,1} and β subunits of mouse CA1 pyramidal cells are transcriptionally regulated by glucocorticoids. We focused on the subunits encoding the N- and L-type calcium currents (Ca_{2,2} and Ca_{1,2/1,3}, respectively), because these prevailed
in the nucleated patches; moreover, we examined the auxiliary subunits Caβ1–4. All of these have been reported to be present in the CA1 hippocampal area (Ludwig et al. 1997). Cycle thresholds were in all cases below 30.

As is evident from Fig. 6A, a small but significant down-regulation of Ca,2.2 expression in the CA1 area was seen 1 h after a brief administration of 100 nM corticosterone. Neither the Ca,1.2 nor Ca,1.3 subunit expression was affected by corticosterone administration. A small but significant decrease in Ca,2.2 expression was observed compared with vehicle-treated control group (white bars). Observations are based on tissue from 12 mice. B: changes in Ca, β1–4 expression 1 h after corticosterone administration. Whereas expression of β1 subunit was decreased, a marked upregulation of β4 subunit was observed after corticosterone treatment. C: corticosteroid effects on β4 subunit expression in presence of cycloheximide (gray bar) are not different from effect of corticosterone (cort) in absence of cycloheximide (dark bar). Data are based on different animals (n = 8) than used for data in A and B. To allow comparison between the 2 sets of data, we normalized data shown in C to vehicle group in A and B. Effect of corticosterone on β4 subunit expression in the 2 sets of animals (dark bars in B and C) is comparable. All bars represent average and range of observed values. *P < 0.05; **P < 0.01; ***P < 0.001.

**FIG. 6.** Effect of corticosterone treatment on calcium channel subunit expression. A: changes in Ca,2.2, 1.2 and 1.3 expression 1 h after corticosterone administration (dark bars). A small but significant decrease in Ca,2.2 expression was observed compared with vehicle-treated control group (white bars). Observations are based on tissue from 12 mice. B: changes in Ca, β1–4 expression 1 h after corticosterone administration. Whereas expression of β1 subunit was decreased, a marked upregulation of β4 subunit was observed after corticosterone treatment. C: corticosteroid effects on β4 subunit expression in presence of cycloheximide (gray bar) are not different from effect of corticosterone (cort) in absence of cycloheximide (dark bar). Data are based on different animals (n = 8) than used for data in A and B. To allow comparison between the 2 sets of data, we normalized data shown in C to vehicle group in A and B. Effect of corticosterone on β4 subunit expression in the 2 sets of animals (dark bars in B and C) is comparable. All bars represent average and range of observed values. *P < 0.05; **P < 0.01; ***P < 0.001.

Calcium currents in nucleated patches

While it has been reported earlier—using patch clamp or sharp electrodes in CA1 hippocampal cells—that GR activation leads to enhanced calcium (Karst et al. 1994; Kerr et al. 1992), poor voltage control in these studies prohibited definite conclusions about the effect of steroids on voltage dependency or kinetic properties. Moreover, it remained unresolved whether corticosterone affects L-, N-, or both types of currents (Kerr et al. 1992).

To further address these issues we optimized calcium current recording in nucleated patches, a preparation with optimal voltage control, yet not exposed to enzymatic dissociation procedures. No reliable currents were recorded with Cs-salt based pipette solutions that do yield large currents in the whole cell configuration; this most likely is explained by Cs-carried currents, which confound the small Ca-currents from nucleated patches. Even with the Tris-based solution that did allow reliable recording in nucleated patches, a preparation with optimal recording in nucleated patches, a preparation with optimal voltage control, yet not exposed to enzymatic dissociation procedures. No reliable currents were recorded with Cs-salt based pipette solutions that do yield large currents in the whole cell configuration; this most likely is explained by Cs-carried currents, which confound the small Ca-currents from nucleated patches. Even with the Tris-based solution that did allow reliable recording of currents, run-down was observed. To avoid any confounding effect of this run-down, we chose to analyze all currents 5 min after isolation of the patch from the soma, i.e., at a moment that run-down was not yet discernable. As a consequence, all effects had to be compared with an unpaired protocol; this is a considerable but unavoidable drawback of the selected preparation.

Calcium currents in nucleated patches were found to consist nearly completely of N- and L-type currents. The high abundance of L-type channels in nucleated patches is in agreement with the immunocytochemical localization of particularly the Ca,1.2 subunit; clusters of Ca,1.3 subunits were encountered at the soma (Bowden et al. 2001) as well as more distally (Hell et al. 1993). The relatively large contribution of N-type channels was somewhat surprising, because antibody staining showed rather low immunoreactivity of Ca,2.2 subunits in
L-type channels that contain a Cav1.2 subunit have somewhat different properties from channels containing a Cav1.3 subunit; these differences (Koschak et al. 2001). Pharmacologically, it is hard to distinguish between these two categories. Based on immunocytochemical localization of the subunits, however, it can be concluded that Ca_{1,3} compared with Ca_{1,2} subunits are localized at more distal parts of the dendritic tree and therefore by far underrepresented in somatic patches (Westenbroek et al. 1992, 1995; but Bowden et al. 2001). The second requirement is that the open probability of the channels is quite high, ideally approaching 1. To achieve this, we applied BayK8644, which favors mode 2 of channel gating (long openings), thus increasing the open probability (Hess et al. 1984; Thibault and Landfield 1996). Even in the presence of BayK8644, however, the full Sigworth’s parabola could not be achieved, indicating that open probability was not close to 1. It has been argued that under such circumstances, a relatively reliably estimate of the unitary conductance can be made. However, the number of channels needs to be interpreted with care (Alvarez et al. 2002), although the relative change in number of channels after corticosterone treatment does not depend on the correct estimation of the unitary conductance. We therefore tentatively conclude from these data that GR activation does not influence the unitary conductance. Because the overall current amplitude was nevertheless enhanced, the data strongly suggest that glucocorticoids double the number of channels in the membrane or the open probability of existing channels. Because BayK8644 was still able to increase the current after treatment with corticosterone, indicating that the two treatments do not fully share the same pathway, it can be tentatively concluded that GR activation most likely increases the number of L-type channels in the membrane.

**Molecular changes**

If the number of calcium channels in the membrane is indeed doubled, this could be caused by increased synthesis of essential channel subunits and/or enhanced surface expression of pre-existing channels. To test this, we analyzed transcriptional regulation by corticosterone of 1) Ca_{1,1} subunits, which form the pore of L- (or N-) type calcium channels, and 2) Ca_{1,3} subunits, which are among other things involved in the surface expression of calcium channels (Dolphin 2003). Transcriptional regulation was examined 1 h after treatment with a high dose of corticosterone. We selected this time-point because functional changes through GRs in hippocampal CA1 neurons are seen already within 1–2 h (Joëls and de Kloet 1992). Any transcriptional change underlying these functional alterations is therefore expected to occur earlier. However, there should be sufficient time for genomic effects to develop, so a delay of 30–60 min is warranted. The 1-h time-point indeed exhibits substantial changes in overall gene expression pattern as shown by microarray studies (Morsink et al. 2006), which focus on moderately to high abundant transcripts.

Here, we found in mice that the pore-forming subunits of L-type calcium channels, i.e., Ca_{1,2/1,3}, are not transcriptionally regulated by high doses of corticosterone. This is different from what has been reported for rat amygdalar cells, using single cell RNA amplification (Karst et al. 2003). In a parallel qPCR study in rats (data not reported in this study), we confirmed that in rats—as opposed to mice—Ca_{1,2} subunits indeed seem to form a primary target for GRs. This apparent difference may be explained by ample interspecies differences between the rat and mouse amino acid sequences of both the GRs and GR-binding elements of the Ca_{1,2} subunit genes. We
did, however, find a strong up-regulation of the β4 subunit, which was consistent between experiments, primers, and species (data from rats not included in this study). In addition, a smaller down-regulation of the β1 and Caβ2,2 subunits was observed, which was not consistent between species. These latter effects therefore—as the data on the Caβ1,2 subunit—seem more complex and are less likely to explain the enhanced calcium currents, which is consistent in rats and mice (cf. Karst et al. 1994, 2000; Kerr et al. 1992). Interestingly, strong potentiation by corticosterone—albeit in a rapid manner—was very recently also observed for the β4 but not β2 subunit of BK channels expressed in HEK cells (King et al. 2006).

The Caβ β4-subunit is abundantly present in hippocampal cells (Pichler et al. 1997) and is thought to determine gating properties as well as trafficking of calcium channels from the endoplasmatic reticulum to the plasma membrane (Arikkath and Campbell 2003; Dalton et al. 2005). Our data do not support that gating properties are affected by GR activation. However, a role of β4 subunits in channel trafficking is highly compatible with the notion that, after corticosterone, the number of (functional) channels in the membrane is increased. Recent evidence suggests that such a selective effect on trafficking through a β SH3/guanylate kinase domain interaction is indeed possible (Takahashi et al. 2005). Although in cell lines, β4 subunits do not seem to interact preferentially with any of the Caβ1-subunits (Walker et al. 1998), local cellular components and/or orientation of the β subunit core may still confer such specificity (Maltez et al. 2005). In accordance it was found—albeit based on current properties—that in CA1 pyramidal cells, L-type channels form a complex with β4 rather than β3 subunits (Schjott et al. 2003), despite the clear presence of the latter. We therefore tentatively propose that GR homodimers transcriptionally regulate β4 subunits, thus increasing the trafficking of L-type calcium channels to the plasma membrane. In theory, this could be further tested in β4 subunit mutants, like the leghartic mice (Burgess et al. 1997). Unfortunately, these animals are reported to exhibit a hyperactive hypothalamo-pituitary-adrenal system, which in itself impairs the responsiveness to corticosterone with respect to calcium currents (Karst and Joëls, unpublished observations). Reduced effects of corticosterone in leghartic mice could therefore incorrectly be regarded as proof of a crucial role for the β-subunit. The alternative, i.e., transfecting relevant key factors in an expression system, is also difficult to interpret, because glucocorticoid effects on calcium current seem to depend critically on the intracellular environment (Joëls 2006; N. G. van Gemert, H. Karst, M. Joëls, unpublished observations). In the absence of these experiments, it remains unproven that the upregulation of the β4 subunit is the actual cause of the enhanced calcium current. Finally, it should be noted that we presently focused on transcriptional regulation of calcium channel subunits, yet more indirect/higher-order transcriptional effects of GRs on calcium channel function cannot be excluded. However, these phenomena probably would require more time to develop and hence are more likely to play a role under different experimental conditions, e.g., after a more prolonged period of stress.

**References**


Bowden SE, Fletcher S, Loane DJ, Marrion NV. Somatic colocalization of rat SK1 and D class (Ca(V)1.2) L-type calcium channels in rat CA1 hippocampal pyramidal neurons. *J Neurosci* 21: RC175, 2001.


**Grants**

This work was supported by the Netherlands Organization for Scientific Research.


