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Glucocorticoid Actions on Synaptic Plasma Membranes: Modulation of [^{125}I]Calmodulin Binding

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The effects of corticosterone on Ca^{2+}-dependent binding of [^{125}I]calmodulin to purified synaptic plasma membranes (SPM) from rats brain were characterized. The enhancement of [^{125}I]calmodulin binding was a sigmoidal function of steroid concentration, with the maximal increase (>55% above control) occurring at a steroid concentration of 1×10^{-6} M and EC_{50} estimated at 1–2 $\times 10^{-7}$ M. Other glucocorticoids including hydrocortisone, dexamethasone and triamcinolone produced similar effects, whereas steroids without glucocorticoid activity such as 11-deoxycortisol, 11-deoxycorticosterone and cholesterol were ineffective.The steroid-induced increase of binding was correlated with an increase of membrane affinity for [^{125}I]calmodulin as shown by Scatchard analysis, and a decrease of the rate of dissociation of [^{125}I]calmodulin from the membranes as shown by kinetic analysis. Arrhenius analysis indicates that [^{125}I]calmodulin binding was influenced by lipid transition of the membranes and that corticosterone resulted in a shift of membrane transition toward a higher temperature. Since a variety of biochemical processes associated with synaptic membranes are dependent upon calmodulin for their regulation, we hypothesize that the effects of glucocorticoids in promoting membrane binding of calmodulin may lead to a cascade of alterations in synaptic function.

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INTRODUCTION

Steroid hormones are known to act by binding to intracellular receptors, which function as ligand-dependent transcription factors in the regulation of gene expression [reviewed in 1]. Less well known is their interaction with cell membranes, although it has become evident even a decade ago that a variety of steroid elicited responses can be attributed to direct actions of the hormones on plasma membranes [reviewed in 2–4]. For example, glucocorticoids enhance the β-adrenergic agonist response in isolated thymocytes, and the effect occurs within 1–2 min after steroid preincubation [5]. More recently, progesterone has been shown to produce an increase of intracellular free Ca^{2+} in human sperm within 30 s after the addition of the steroid [6]. Furthermore, specific binding sites in plasma membranes have been identified for glucocorticoids in liver [7–12], anterior pituitary gland [13], placenta [14], skeletal muscle [15] and lymphoma cells [16]; for estrogens in uterus [17], liver [18] and breast tissue [19]; and for progesterone in oocytes [20] and ovary [21].

In neuronal cells, there has been a resurgence of interest in the membrane actions of hormonal steroids in recent years [reviewed in 22, 23]. Biochemical studies indicate that certain adrenal and gonadal steroids or their metabolites modulate $GABA_A$ receptors [24–30], σ receptors [31, 32], nicotinic acetylcholine receptors [33] and α-adrenergic receptors [34] of the brain, apparently by binding to membrane sites on or near these receptors. In earlier literature, there has been ample electrophysiological evidence that neuronal membrane potential can be altered by certain steroid hormones [reviewed in 2]. More recent studies have provided additional data along this line. For example, cortisol hyperpolarizes the membrane potential of celiac ganglion neurons with a latency of <2 min after steroid superfusion of the ganglion [35]. 17β-Estradiol, but not 17α-estradiol and testosterone, produced hyperpolarization and an increase of K^+ conductance

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Abbreviations: CaM, calmodulin; CORT, corticosterone; GABA, γ-aminobutyric acid; SPM, synaptic plasma membrane; PVP, polyvinylpyrrolidone; PMSF, phenylmethylsulfonyl flouride; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; SDS, sodium dodecyl sulfate; EC_{50}, effective concentration to produce 50% of maximal effect; K_d, dissociation constant; B_{max}, maximum binding; k, first-order rate constant; $t_{1/2}$, half-life; T_d, transition temperature; MgATP, magnesium adenosine triphosphate.

in medial amygdala neurons within a few minutes after the introduction of the hormone [36]. In a behavioral study, progesterone induces lordosis in female rats, in part through modulation of hypothalamic oxytocin receptors; this rapid and specific effect of the steroid is independent of *de novo* protein synthesis [37]. Taken together, these recent electrophysiological and behavioral data further indicate that in neurons, steroid hormones can have a rapid onset of action, which is accompanied by a high degree of hormone specificity.

We have previously identified the specific binding sites for glucocorticoids and gonadal steroids in highly purified synaptic plasma membranes (SPM) from rat brain [38, 39]. More recently, the binding sites for glucocorticoids have also been identified in neuronal membranes from an amphibian brain [40, 41], and the binding sites for progesterone have been further characterized in another neuronal membrane preparation from rat brain [42, 43]. These findings provide strong evidence that neuronal plasma membranes mediate steroid hormone actions through membrane receptors. However, the transduction of steroid signals by the membranes remains to be understood. In this study, we have found that in SPM, membrane binding of calmodulin is altered by glucocorticoids. Since calmodulin is involved in a variety of Ca^{2+}-dependent events in plasma membranes [44, 45], we propose that the action by glucocorticoids on membrane binding of calmodulin is among the mechanisms whereby these steroids regulate membrane function.

EXPERIMENTAL

Animals and materials

Male Sprague–Dawley rats (150–200 g) were supplied by a commercial breeder (Sasco King Animal Labs., Oregon, WI). [^{125}I]Calmodulin was prepared by ^{125}I-iodination of calmodulin (bovine brain; Sigma Chemical Co., St Louis, MO), by a precedure described previously [46] using Bolton and Hunter reagent (2000 Ci/mmol; Amersham Corp., Arlington Heights, IL). The recovered ^{125}I-labeled material exhibited a single radioactive protein band corresponding to calmodulin on SDS–polyacrylamide gel electrophoresis. In some experiments, commercially available [^{125}I]calmodulin (87 mCi/mg; NEN Research Products, Boston, MA) was used. Both preparations of [^{125}I]calmodulin yielded similar results. Polyvinylpyrrolidone (PVP; average M_w 10,000), phenylmethylsulfonyl fluoride (PMSF), steroids and other biochemicals were purchased from Sigma.

Preparation of SPM

The preparation of SPM was performed using a procedure described previously [47], with modifications by us [38]. The cerebral cortex was homogenized in 5 vol of 0.32 M sucrose–10 mM Tris–HCl, pH 7.4. Nuclei and other cellular debris were pelleted by centrifuging at 1000 *g* for 10 min. The synaptosomes remaining in the supernatant were pelleted by a subsequent centrifugation at 10,000 *g* for 20 min. The synaptosomal pellet was resuspended in the homogenization buffer and placed on a discontinuous gradient of 0.85, 1.0, and 1.2 M sucrose. After centrifuging at 90,000 *g* for 90 min, the synaptosomes were removed from the interface between the 1.0 and 1.2 M sucrose. The mitochondria formed a pellet in the 1.2 M sucrose. The synaptosomal fraction was diluted with 4 vol of 0.25 M sucrose–10 mM Tris–HCl, pH 7.4, and centrifuged for 20 min at 15,000 *g*. The synaptosomes were lyzed by resuspending the pellet in 10 mM Tris–HCl, pH 7.4. The osmotically lyzed synaptosomes were layered on a discontinuous sucrose gradient (0.85, 1.0 and 1.2 M) and centrifuged at 90,000 *g* for 90 min. The synaptosomal cytosol remained on the top layer of the gradient and the mitochondria from the nerve terminals were pelleted at the bottom. The band containing the SPM fraction at the interface between 1.0 and 1.2 M sucrose was removed and diluted with 5 vol of 10 mM Tris–HCl, pH 7.4. After centrifuging at 15,000 *g* for 20 min, the pellet containing the SPM was resuspended in 50 mM Tris–HCl, pH 7.4, containing 2 mM dithiothreitol and 50 μM PMSF. In one experiment where synaptic vesicles were used, the band on top of the 0.85 M sucrose layer was removed and diluted with 5 vol of 0.25 sucrose containing 10 mM Tris–HCl, pH 7.4. After centrifuging at 20,000 *g* for 20 min, the supernatant was centrifuged at 100,000 *g* for 30 min to pellet the vesicles. The purity of the SPM and synaptic vesicle fractions was monitored as described previously by us [38], using electron microscopy and marker enzymes (5′-nucleotidase for plasma membranes, fumarase for mitochondria, glutamate dicarboxylase for synaptic vesicles, and lactate dehydrogenase for cytosol).

[^{125}I]Calmodulin binding

The SPM were washed twice with 2 mM EGTA (in 50 mM Tris–HCl, pH 7.4) to deplete endogenously bound calmodulin, as described by others in previous binding studies [48]. The EGTA-washed membranes (10–12 μg protein) were equilibrated with corticosterone (30 min at 37°C) at indicated concentrations in 190 μl of binding buffer (50 mM Tris–HCl, pH 7.4, 0.1 mM $CaCl_2$, 2 mM dithiothreitol, 50 μM PMSF and 2 mg/ml bovine serum albumin). Since ethanol as low as 0.3% v/v (52 mM) was found to inhibit calmodulin binding, the steroid was dissolved in 1% PVP and the final concentration of PVP in the incubation mixture was 0.05%. In a standard assay, [^{125}I]calmodulin (10,000–15,000 cpm/pmol) in 10 μl was added to produce a final concentration of 50 nM. The binding of the labeled ligand to the membranes was allowed to reach equilibrium at 37°C (15 min). The membranes were separated by filtration on a Whatman GF/B filter and washed twice with 3 ml of ice-cold binding buffer. Radioactivity bound to the membranes was determined. Nonspecific binding was determined in the presence of 500-fold unlabeled calmodulin. Radioac-

tivity due to nonspecific binding was <5% of total binding. Statistic analysis of data including t-test and linear regression analysis is indicated under each experiment.

RESULTS

Effects of corticosterone

The binding of [^{125}I]calmodulin to SPM was determined after the membranes were equilibrated with corticosterone in the concentration range of 1×10^{-8}–2×10^{-6} M. As shown in Fig. 1, the steroid hormone enhanced the binding of the labeled ligand to the membranes. The increase of binding was a sigmoidal function of steroid concentration, with the maximal increase (>55% above control) reached at 5×10^{-7} M steroid and the EC_{50} estimated as $1–2 \times 10^{-7}$ M. It is noted that the log dose–response curve of the effect of corticosterone on [^{125}I]calmodulin binding observed here is in close resemblance to the saturation isotherm of the specific steroid binding to SPM, which was reported to have a K_d of 1.2×10^{-7} M [38].

Since the Ca^{2+}–calmodulin complex is the active form that binds, the possibility was considered that corticosterone could exert its effect on binding by increasing the availability of Ca^{2+} to the membranes. This possibility was examined by determining the effect of Ca^{2+} on binding in the presence of 1 μM steroid. As shown in Fig. 2, the profiles of [^{125}I]calmodulin binding as a function of Ca^{2+} concentrations paralleled each other, whether the steroid was present or absent. In both cases, half-maximal binding was obtained around 5 μM Ca^{2+}. Furthermore, the increase of binding by the hormone persisted even at Ca^{2+} as high as 1 mM. Thus, Ca^{2+} availability was not involved in the hormone enhancement of binding. Another possibility was considered that the effect of corticosterone was on the calmodulin molecule rather than on the membrane. However, this possibility was excluded by two lines of evidence. First, to compare with SPM, [^{125}I]calmodulin binding was determined in synaptic vesicles, a secretory granule that is known also to bind calmodulin [49]. As shown in Table 1, corticosterone as high as 5 μM was without an effect on binding in the vesicles, indicating that the effect of the steroid found in SPM was not due to an alteration of the calmodulin molecule. Second, the steroid at 5 μM did not alter the fluorescence spectra of dansylated calmodulin, as determined by a procedure described previously [50] (data not shown).

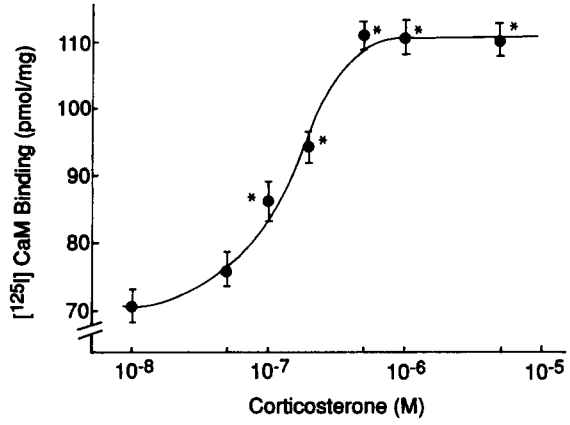

Fig. 1. Effects of various concentrations of corticosterone on [^{125}I]calmodulin binding as to SPM. Binding was performed with 50 nM [^{125}I]calmodulin as described in the text. Values are mean ± SD from 5–6 determinations. The control value (in the absence of the steroid) was 71.5 ± 2.8 pmol/mg. Significance of difference from control: *P < 0.001.

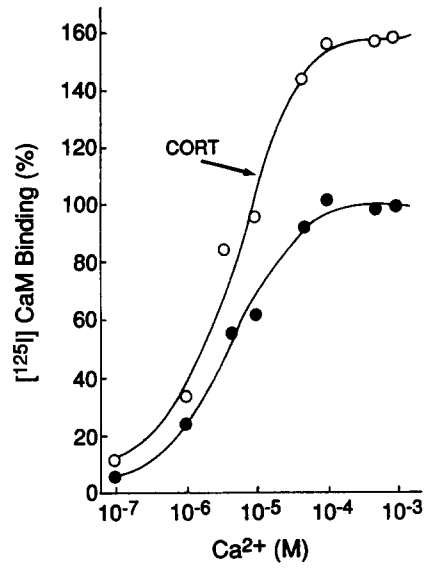

Fig. 2. Ca^{2+}-dependency of [^{125}I]calmodulin binding to SPM. Control: (●); addition of 1×10^{-6} M corticosterone: (○). Binding was performed with 50 nM [^{125}I]calmodulin as described in the text. Free Ca^{2+} concentrations below 10^{-4} M were controlled by Ca^{2+}–EGTA buffers [71]. All values are means from triplicate determinations, with SD < 5%.

Effects of various steroids

To determine hormone specificity, the effects of a variety of steroids on [^{125}I]calmodulin binding were compared (Table 2). Hydrocortisone, dexamethasone, and triamcinolone enhanced binding with an increase similar to that from corticosterone. At the concentration comparable to that of the hormonal steroids (1 μM), cholesterol was without an effect on calmod-

Table 1. Effects of corticosterone on equilibrium binding of [^{125}I]calmodulin to synaptic vesicles

Corticosterone (M)	[^{125}I]CaM binding (pmol/mg)
Control	32.7 ± 1.3
1×10^{-7}	30.2 ± 0.9
1×10^{-6}	31.0 ± 1.2
5×10^{-6}	33.5 ± 1.5

Binding was determined at 50 nM [^{125}I]calmodulin as described in the text except that synaptic vesicles (30–40 μg protein) were used. Values are mean ± SD from 5 determinations.

Table 2. Effects of various steroids on equilibrium binding of [^{125}I]calmodulin to SPM

Steroid	[^{125}I]CaM binding (pmol/mg)
Control	72.7 ± 3.1
Corticosterone	109.5 ± 3.4*
Cortisol	104.2 ± 3.3*
Dexamethasone	107.0 ± 3.9*
Triamcinolone	110.4 ± 3.3*
Cholesterol	71.5 ± 2.2
11-Deoxycortisol	70.1 ± 1.8
11-Deoxycorticosterone	73.0 ± 2.8
17β-Estradiol	82.6 ± 3.5
Progesterone	81.4 ± 4.2
Testosterone	79.7 ± 3.3

All steroids were 1×10^{-6} M. Binding was determined at 50 nM [^{125}I]calmodulin as described in the text. Values are mean ± SD from 5 determinations. *$P > 0.001$ from control.

ulin binding to SPM. 11-Deoxycortisol (cortexolone) and 11-deoxycorticosterone (cortexone), two nonglucocorticoid steroids which do not have specific binding with SPM [38], were also ineffective on binding. Progesterone, 17β-estradiol and testosterone at 1 μM produced only a small and insignificant effect on binding, and these small increases were not magnified by higher steroid concentrations (data not shown).

Kinetic analysis

The time course of [^{125}I]calmodulin binding to SPM was compared in the absence and presence of 1 μM corticosterone (Fig. 3). In both cases, a near plateau level of bound [^{125}I]calmodulin was reached at 37°C within 5 min, and maximal binding was reached within 10 min. These data demonstrate that the effect of the steroid was on equilibrium binding of the ligand rather

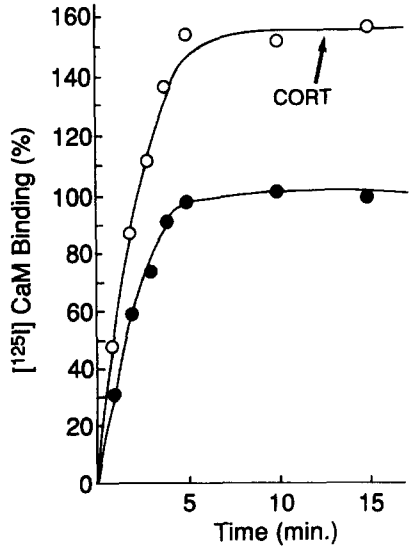

Fig. 3. Time course of [^{125}I]calmodulin binding to SPM. Control: (●); addition of 1×10^{-6} M corticosterone: (○). Binding was performed with 50 nM [^{125}I]calmodulin as described in the text. All values are means from triplicate determinations, with SD < 5%.

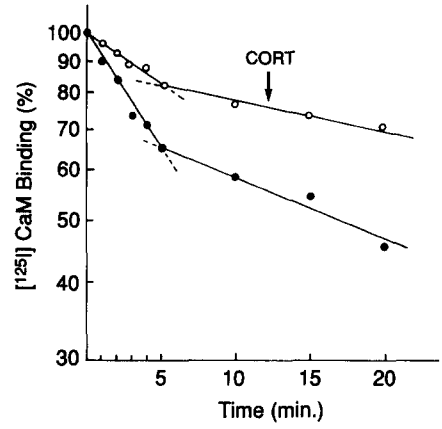

Fig. 4. Time course of [^{125}I]calmodulin dissociation from SPM. Control: (●); addition of 1×10^{-6} M corticosterone: (○). The membrane was first loaded with [^{125}I]calmodulin by preincubation with 200 nM [^{125}I]calmodulin at 37°C for 10 min. After the prelabeling, the [^{125}I]calmodulin–membrane complexes were chased by addition of 100-fold of unlabeled calmodulin (20 μM). At zero-time, the [^{125}I]calmodulin bound with the membrane was 176 pmol/mg for the control and 231 pmol/mg in the presence of corticosterone. Both zero-time values were taken as 100% to facilitate kinetic comparison. The amount of [^{125}I]calmodulin remaining with the membrane was determined at 37°C at indicated times. The data were analyzed in linear plots of ln ($[C_t/C_0] \times 100$) vs time. For control data: from linear regression analysis, the correlation coefficient is 0.985 (first line) and 0.983 (second line). The rate constant, k, calculated from the slope of the linear plot, is 8.9×10^{-2} min^{-1} for the first line and 2.3×10^{-2} min^{-1} for the second line. In the presence of 1 μM corticosterone: the correlation coefficient is 0.998 (first line) and 0.951 (second line). The slope of both lines is significantly different from that of the corresponding control ($P < 0.001$). The rate constant, k, calculated from the slope of the linear plot, is 3.9×10^{-2} min^{-1} for the first line and 9.2×10^{-3} min^{-1} for the second line.

than accelerating the time required to reach equilibrium.

To determine the effect of the steroid on the calmodulin–membrane complex, [^{125}I]calmodulin dissociation from SPM was kinetically analyzed. After loading the membranes with [^{125}I]calmodulin, 100-fold unlabeled calmodulin was added as the chaser to stop further labeling of the membranes. The rate of [^{125}I]calmodulin dissociation from prelabeled membranes was then followed by determining the amount of [^{125}I]calmodulin–membrane complexes remaining at various times. The data were analyzed by a first order reaction ($\ln [C_t/C_0] = t \times k$, where C_0 and C_t are the concentrations of [^{125}I]calmodulin–membrane complexes at t_0 and t_1 and k is the first order rate constant) and plotted accordingly. As shown in Fig. 4, the best fitting linear function consisted of two lines, indicating two dissociating components with different rates. Under the control condition, the fast dissociation component was estimated to have a dissociation rate constant (k) of 8.9×10^{-2} min^{-1} and a half-time ($t_{1/2}$) of 7.8 min, and the slow component gave a k of 2.3×10^{-2} min^{-1}, and a $t_{1/2}$ of 29.5 min. Corticosterone decreased the rate of dissociation of the

[^{125}I]calmodulin–membrane complexes for both the fast and slow component. At 1 μM corticosterone, the fast component gave a k of 3.9×10^{-2} min^{-1} and a $t_{1/2}$ of 17.7 min, and the slow component gave a k of 9.2×10^{-3} min^{-1} and a $t_{1/2}$ of 75.4 min. These kinetic date indicate that the steroid stabilizes the calmodulin–membrane complex from dissociation. Moreover, the fact that both the fast dissociating and the slow dissociating component were affected suggests that the alteration of membrane binding sites by the steroid is extensive rather than limited to particular sites.

To determine the affinity of the membrane binding of calmodulin in the presence of corticosterone, the equilibrium binding of [^{125}I]calmodulin was measured in ligand concentration range of 25 to 500 nM. The binding was saturable, reaching a maximum at 500 nM calmodulin both in the absence and presence of the steroid. These data are shown as Scatchard plots in Fig. 5. Within the ligand concentration range used, the identity of multiple binding populations with distinguishable affinities was not apparent, since a single linear function was seen in the Schatchard alalysis. In this single-affinity model, the control line gave an apparent K_d of 131 nM and a B_{max} of 283 pmol/mg protein. When the steroid was present (1 μM), the apparent K_d was shifted to 73 nM, whereas the B_{max} was not significantly changed (280 pmol/mg). These results indicate that the effect of the steroid in increasing [^{125}I]calmodulin binding was due to an increased affinity of the membranes for the ligand.

Arrhenius analysis

A recent study [51] has demonstrated the presence of a biphasic linear function in Arrhenius analysis of the temperature-dependency of [^{125}I]calmodulin binding

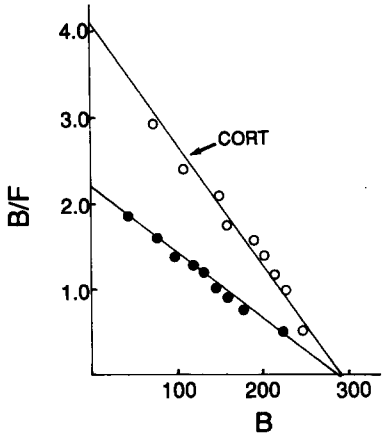

Fig. 5. Scatchard analysis of [^{125}I]calmodulin binding. B: [^{125}I]calmodulin bound in pmol/mg; F: free [^{125}I]calmodulin concentration in nM. Control: (●); addition of 1×10^{-6} M corticosterone: (○). Binding was performed in [^{125}I]calmodulin concentration range of 25–500 nM. Linear regression analysis for the control: correlation coefficient, 0.997; K_d, 131 ± 3.5 nM: and B_{max}, 283 ± 3.3 pmol/mg. In the presence of 1 μM corticosterone: correlation coefficient, 0.973; K_d: 73 ± 6.8 nM (significantly different from control, $P < 0.001$); and B_{max}, 280 ± 9.5 pmol/mg.

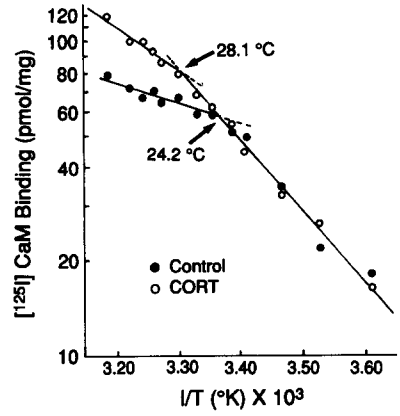

Fig. 6. Temperature dependency of [^{125}I]calmodulin binding. Control: (●); addition of 1×10^{-6} M corticosterone: (○). Binding was determined with 50 nM [^{125}I]calmodulin in temperature range of 4–40°C. The data were analyzed in Arrhenius plots of ln [binding] vs 1/temperature, T, in Kelvin. From linear regression analysis for the control, the correlation coefficient is 0.997 (first line) and 0.957 (second line), with the intercept of the two lines corresponding to 297.1 K or 24.2°C. In the presence of 1 μM corticosterone, the correlation coefficient is 0.987 (first line) and 0.981 (second line), with the intercept of the two lines corresponding to 301.2 K or 28.1°C. The slope of the first line is significantly different from that in the control ($P < 0.001$). The slope of the second line is not significantly different from the control line; therefore, no additional line is shown.

to SPM, indicating that membrane binding of calmodulin is dependent upon lipid phase transition of the membranes. To determine the effect of corticosterone on calmodulin binding in relation to the membrane transition, binding was determined in the temperature range of 4–40°C, and the data were analyzed in Arrhenius plots. As shown in Fig. 6, the presence of a biphasic linear function was evident in the temperature-dependency of [^{125}I]calmodulin binding to SPM. Under the control condition, the T_d, as indicated by the abrupt change in the slope of the linear function, was estimated as 24.2°C. In SPM preincubated with 1 μM corticosterone, a shift in the slope of the first line was apparent, with a consequent shift of the T_d to 28.1°C. The effect of the steroid in shifting membrane transition is consistent with the notion that the increase of calmodulin binding is related to steroid-induced alteration of the lipid environment surrounding the membrane binding sites.

DISCUSSION

Methodologically, two features of our study should be emphasized: the use of highly purified plasma membranes from synaptic terminals, and the pretreatment of the membranes with EGTA to deplete endogenously bound calmodulin. In earlier studies on calmodulin binding to brain membranes [48, 52, 53], crude particulate preparations were used and such preparations consisted of heterogeneous subcellular components from neuronal as well as glial cells. In a more recent study [54] that reported Scatchard charac-

teristics of calmodulin binding to purified SPM, the membranes were not washed with EGTA to fully expose calmodulin binding sites. Therefore, the binding characteristics reported in the previous studies cannot be compared with those found in the present study.

It should be mentioned that in the SPM, total endogenous content of calmodulin, as estimated by radiommunoassays in our laboratory, is 3.2 μg/mg membrane protein, of which 2.5 μg is EGTA-dissociable [51]. The other 0.7 μg, which is resistant to dissociation by Ca^{2+} chelators and requires a detergent for its extraction, represents Ca^{2+}-independent calmodulin binding with the membranes, perhaps involving a neurospecific protein [55]. In ongoing experiments in our laboratory, we have found that calmodulin tightly associated with SPM in the Ca^{2+}-independent manner is not affected by glucocorticoids [56]. Thus, the effect of corticosterone described in the present study is related to Ca^{2+}-dependent binding of calmodulin.

The main objective of our study is to characterize the effect of corticosterone in promoting calmodulin binding to the synaptic membranes. The steroid does not interfere with Ca^{2+} availability to the membranes or the formation of the Ca^{2+}–calmodulin complex, as shown by the lack of a Ca^{2+} effect in the steroid enhancement of binding. Kinetic data on the dissociation of bound [^{125}I]calmodulin from the membranes reveal that when the membranes are saturated with the steroid (at 1 μM), the dissociation rate constant for both the fast and slow dissociating component is markedly decreased. Thus, the steroid increases calmodulin binding by stabilizing the calmodulin–membrane complex from dissociation. The concentrations of corticosterone required to increase calmodulin binding ($> 10^{-8}$ M) are parallel to those in specific binding of the steroid to SPM reported in our earlier study [38]. The EC_{50} found here, 1–2 $\times$ 10^{-7} M, is almost identical to the K_d of corticosterone binding to SPM (1.2 $\times$ 10^{-7} M). We have previously proposed [38] that the lower affinity of the membrane binding of glucocorticoids, as compared to the affinity of intracellular receptor binding (with a K_d on the order of 10^{-9} M), allows rapid association and dissociation of the steroid, thus providing a rapid mechanism for eliciting and terminating a membrane response. In this regard, it is interesting to note that the hyperpolarizing effect of cortisol on celiac ganglion neurons was produced only when the steroid concentrations approached 1 $\times$ 10^{-7} M [35].

From Scatchard analysis of the Ca^{2+}-dependent [^{125}I]calmodulin binding, there are at least two points to be made. First, the best fitting plot from the Scatchard analysis appears to be a single straight line. However, this apparent single-affinity model most likely underestimates the complexity of calmodulin binding sites in the membranes and must be interpreted with caution. In synaptic membranes, in addition to several enzymes that are known to bind calmodulin for their activation, other calmodulin-binding proteins such as calcineurin and troponin I are known [57]. Thus, hetrogeneous groups of calmodulin binding sites are present in the membranes. In a Scatchard analysis, if the binding population consists of heterogeneous sites with closely related affinity for the ligand, the statistically derived single line may very well represent only the average affinity of the total binding population. Nonetheless, the estimated K_d, which is on the order of 10^{-7} M, suggests that most binding sites on the synaptic membranes do not bind calmodulin with a high affinity and thus can associate or dissociate calmodulin readily. Second, despite the presence of heterogeneous binding sites, the fact that a shift of the K_d by corticosterone is visible in the Scatchard analysis indicates that extensive changes can occur in the binding affinity of the membranes, at least under the *in vitro* experimental condition where the membranes are saturated with the steroid. This increase in the membrane affinity for calmodulin by the steroid is also reflected in the kinetic data showing decreased rates of the dissociation of bound [^{3}H]calmodulin from the membranes in the presence of the steroid.

The data from Arrhenius analysis provide information about the nature of the glucocorticoid action in modulating membrane binding of calmodulin. It is well known that for membrane-bound enzymes, the transition temperature, T_d, of the enzyme obtained from Arrhenius analysis is correlated with lipid phase transition of plasma membranes [58–61]. Moreover, substances that alter fluidization of the lipid domains surrounding the enzyme, such as ethanol, produce a shift of T_d of the enzyme [62–64]. Membrane lipid fluidity is known also to influence receptor binding and transport processes, including serotonin binding to brain synaptic membranes [65] and glucose transport across erythrocyte and fibroblast membranes [66]. Using a similar approach, we have demonstrated the presence of a biphasic linear function in Arrhenius analysis of [^{125}I]calmodulin binding to SPM, and a shift of the T_d from 24.2 to 28.1°C following the saturation of the synaptic membranes with corticosterone. The shift of the T_d to a higher value is consistent with the notion that the steroid increases membrane binding of calmodulin by reducing lipid fluidity of the membranes. From the hormone specificity of the glucocorticoid action, it appears that glucocorticoid binding sites on the membranes are involved in the action. Steroids that do not have glucocorticoid activity and that do not bind to specific glucocorticoid sites on the membranes are ineffective in altering calmodulin binding. Thus, cholesterol, a steroid known to reduce membrane fluidity at high concentrations, is without an effect on calmodulin binding at concentrations comparable to those of glucocorticoids. It is interesting to note that corticosterone is more efficient than cholesterol by at least three orders in stabilizing dipalmitoylphosphatidylcholine bilayers in an artificial membrane model [67]. Therefore, it is tempting to speculate that the specific binding site for glucocorticoids, possibly a phospholipoprotein [38], determines hormone specificity and the site of hormone action on

the membrane, whereas a primary action of glucocorticoids is on the conformation of lipid bilayers.

In a recent study [41], the specific binding of corticosterone in neuronal membranes from an amphibian brain is enhanced by Mg^{2+} and inhibited by guanyl nucleotides, suggesting that the steroid binding site is coupled to a guanyl nucleotide-binding protein. However, specific glucocorticoid binding to SPM from rat brain is not affected by Mg^{2+} or guanyl nucleotides (unpublished from our laboratory). The present findings indicate that glucocorticoid binding to mammalian SPM results in an alteration of membrane binding of calmodulin. Since a variety of biochemical events in plasma membranes are dependent upon calmodulin for their activation, an increase of membrane binding of calmodulin could lead to a cascade of alterations in membrane processes. In a recent study, we have found that depolarization-induced synaptosomal Ca^{2+} influx is enhanced by glucocorticoids [68]. There is considerable evidence that voltage-sensitive Ca^{2+} channels are activated by calmodulin-dependent phosphorylation [69]. Accordingly, we have found that although the basal level of [^{3}H]dihydropyridine binding to the synaptic membranes is not affected by glucocorticoids, the steroids enhance the activation of the radioligand binding by a mechanism involving Ca^{2+}–calmodulin and MgATP [70]. We are presently testing the hypothesis that the steroids increase synaptosomal Ca^{2+} influx by enhancing calmodulin-dependent activation of voltage-sensitive Ca^{2+} channels. This hypothesis is part of our overall proposal that promotion of calmodulin binding is a mechanism whereby glucocorticoids modulate membrane processes.

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