Pergamon

0960-0760(95)00178-6

J. Steroid Biochem. Molec. Biol. Vol. 55, No. 2, pp. 185–192, 1995

0960-0760/95 $9.50 + 0.00

Glucocorticoid Actions on Synaptic Plasma Membranes: Modulation of Dihydropyridine-sensitive Calcium Channels

Paul Y. Sze* and Byung H. Yu

Department of Pharmacology and Molecular Biology, The Chicago Medical School, North Chicago, IL 60064-3095, U.S.A.

We have previously shown that glucocorticoids accelerate depolarization-induced $^{45}Ca^{2+}$ influx in synaptosomes isolated from rat cerebral cortex, indicating that the steroids may modulate voltage-dependent Ca^{2+} channels. The present study was undertaken to characterize the biochemical action of glucocorticoids on dihydropyridine-sensitive voltage-dependent Ca^{2+} channels known to be present in brain synaptosomes. The [^{3}H]dihydropyridine labeled site was used as a marker to determine the levels of functional Ca^{2+} channels. No effect on equilibrium binding of [^{3}H]PN 200-110 was found when membranes from disrupted synaptosomes were incubated with corticosterone as high as 1 μM. However, when intact synaptosomes were first incubated with corticosterone at 37°C and then disrupted, a significant increase in [^{3}H]PN 200-110 binding was found. Steroid incubation of synaptosomes at 0°C was ineffective. It appears that metabolic processes requiring intracellular factors were involved in the steroid action. In examining this possibility, [^{3}H]PN 200-110 binding was activated in disrupted membranes by MgATP and Ca^{2+}–calmodulin, and corticosterone was found to enhance the activation in a concentration-dependent manner. [^{3}H]PN 200-110 binding to membranes was also activated by incubation with MgATP and cAMP-dependent protein kinase, but this activation was not enhanced by the steroid. These findings are consistent with the interpretation that the steroid promotes Ca^{2+} channel activity by enhancing calmodulin-dependent activation of the channels. The action on voltage-dependent Ca^{2+} channels in synaptic terminals may well be a mechanism whereby glucocorticoids modulate neuronal activity.

J. Steroid Biochem. Molec. Biol., Vol. 55, No. 2, pp. 185–192, 1995

INTRODUCTION

In neuronal cells, evidence has mounted in recent years that hormonal steroids and some of the other metabolites in steroidogenesis can act directly on the plasma membrane in altering membrane functions [1–3]. In biochemical studies, various steroids have been shown to modulate neuronal $GABA_A$ receptors [4–11], muscarinic and nicotinic acetylcholine receptors [12, 13], α_1-adrenergic receptors [14] and σ receptors [15] *in vitro*, apparently by binding to membrane sites at or near these receptors. The structural specificity and the rapid onset of the steroid actions on neuronal plasma membrane, an excitable membrane, can be illustrated eloquently by electrophysiological data. For example, 17β-estradiol, but not 17α-estradiol or testosterone, produces hyperpolarization and an increase of K^+ conductance in medial amygdala neurons within 2 min after steroid superfusion of the brain slice [16]. 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 [17]. Clearly, the membrane-mediated actions of steroids are emerging as important factors in the modulation of neuronal activity. In non-neuronal cells,

*Correspondence to P. Y. Sze.
Received 19 May 1995; accepted 28 Jul. 1995.

Abbreviations: GABA, γ-aminobutyric acid; SPM, synaptic plasma membranes; PN 200-110, isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-methoxycarbonylpyridine-3-carboxylate; PKA, cAMP-dependent protein kinase; CS/PKA, catalytic subunit of PKA; PVP, polyvinylpyrrolidone; PMSF, phenylmethylsulfonyl fluoride; CORT, corticosterone; B_{max}, maximum binding; K_d, dissociation constant; EC_{50}, effective concentration at 50% maximal response; CaM, calmodulin.

it became evident even a decade ago that a variety of steroid elicited responses can be attributed to direct actions of the steroids on the plasma membrane [18, 19]. Moreover, specific binding of hormonal steroids to the plasma membrane has been demonstrated persistently in several cell types, including liver, uterus and oocytes [summarized in 20].

In earlier studies, we identified the specific binding sites for glucocorticoids and gonadal steroids in highly purified synaptic plasma membranes (SPM) derived from rat brain synaptosomes [21, 22]. The specific binding sites for glucocorticoids were also characterized in SPM from an amphibian brain [23, 24]. These data provide evidence that neuronal plasma membrane mediates glucocorticoid actions through membrane receptors. In a recent study, we found that glucocorticoids stimulate $^{45}Ca^{2+}$ influx in synaptosomes upon membrane depolarization by high extracellular K^+ or veratridine, indicating that voltage-dependent Ca^{2+} channels are a target of the steroid actions on the synaptic membrane [25]. Such an action of glucocorticoids on Ca^{2+} transport across the membrane is obviously important and may well be a mechanism by which these steroids modulate synaptic activity in the brain. As an experimental model, synaptosomes (isolated synaptic terminals) do not contain the cell nucleus and are thus particularly useful for the analysis of non-genomic, membrane-mediated actions of steroids.

The present study was undertaken to investigate the biochemical events underlying the action of glucocorticoids on Ca^{2+} channels in synaptosomes. The slow voltage-dependent Ca^{2+} channels present in brain synaptosomes are sensitive to dihydropyridine agonists and antagonists, similar to those in skeletal and cardiac muscle [26]. It is generally agreed that in the analysis of these dihydropyridine-sensitive Ca^{2+} channels in disrupted (depolarized) membranes, there is a correlation between the number of high-affinity dihydropyridine binding sites and the number of functional Ca^{2+} channels (active channels that can open) [27, 28]. Thus, the [^{3}H]dihydropyridine labeled site has widely been used as a convenient marker to assess the relative number of functional Ca^{2+} channels in membrane preparations. This approach was used in our study.

EXPERIMENTAL

Animals and materials

Male Sprague–Dawley rats (180–220 g) were supplied by a commercial breeder (Sasco King Animal Labs, Oregon, WI). (+)-[5-Methyl-^{3}H]PN 200-110 (70 Ci/mmol) was obtained from NEN Research Products (Boston, MA). Calmodulin (bovine brain), cAMP-dependent protein kinase (PKA) and its catalytic subunit (bovine heart), polyvinylpyrrolidone (PVP; average M_w 10,000), phenylmethylsulfonyl fluoride (PMSF), ATP, cAMP, nifedipine, steroids and other biochemicals were supplied by Sigma Chemical Co. (St Louis, MO).

Preparation of synaptosomes

All animals were sacrificed by decapitation at 9:00–10:00 a.m. and the cerebral cortex was homogenized in 5 vol of 0.32 M sucrose–10 mM Tris–HCl, pH 7.4. Synaptosomes were prepared as previously described [25]. Nuclei and 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 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 synaptosomal fraction was removed from the interface between the 1.0 M and 1.2 M sucrose. The fraction was diluted with 4 vol of 0.25 M sucrose–10 mM Tris–HCl, pH 7.4, and centrifuged at 15,000 *g* for 20 min to obtain the synaptosomal pellet.

Steroid treatments of synaptosomes and synaptic membranes

For experiments where intact synaptosomes were used, freshly prepared synaptosomes were suspended in an isotonic buffer (20 mM Tris–HCl, pH 7.4, 135 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 2.4 mM NaH_2PO_4, 10 mM glucose, and 2 mM dithiothreitol) to give a protein concentration of 2 mg/ml. Corticosterone (or other steroids) was dissolved in 1% PVP and the final concentration of PVP in the incubation mixture was 0.05%. The suspended synaptosomes were equilibrated with the steroid at 37°C for 15 min. The synaptosomes were then disrupted by a Polytron (Brinkman Instruments, Westbury, NY) and the membranes were pelleted at 48,000 *g* for 10 min for the determination of [^{3}H]PN 200-110 binding. In one experiment, the synaptosomes were disrupted before they were equilibrated with corticosterone.

Incubation of synaptic membranes (membranes from disrupted synaptosomes) with compounds that promote phosphorylation of Ca^{2+} channels was carried out by modifications of a procedure previously described [29]. After equilibration of the membranes with corticosterone (dissolved in PVP as described above) at 37°C for 15 min, the reaction by the calmodulin-dependent process was carried out in the presence of 50 mM Tris–HCl, pH 7.4, 10 mM $MgSO_4$, 0.5 mM ATP, 0.5 mM $CaCl_2$, 0.1 μM calmodulin and 0.05% β-mercaptoethanol. The reaction by the cAMP-dependent process was carried out in the presence of 50 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 2 mM EGTA, 0.5 mM ATP, 1 mM cAMP and 10 units/ml PKA. In both cases, the incubation (0.3 mg membrane protein per ml) was allowed to proceed at 37°C for 1–5 min (as specified) and stopped by adding 4 vol of ice-cold stop buffer (40 mM Na_2HPO_4, 10 mM KH_2PO_4, 50 mM

NaF, 20 mM EDTA, 1 mM iodoacetamide and 0.1 mM PMSF). The membranes were then pelleted at 48,000 *g* for 10 min for the determination of [^{3}H]PN 200-110 binding.

Assay for dihydropyridine binding

High-affinity binding of dihydropyridine to synaptic membranes was determined by the procedure described in a previous study [25], using [^{3}H]PN 200-110 as the ligand. The membranes (40–60 μg) were incubated with [^{3}H]PN 200-110 (0.5 nM or as indicated) in 0.2 ml of binding buffer (50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 20 mM NaF, 2 mM dithiothreitol, and 0.1 mM PMSF). The binding was allowed to reach equilibrium at 22°C (90 min in the dark). The membranes were separated by filtration on a Whatman GF/C filter and washed twice with 3 ml of ice-cold binding buffer. Radioactivity bound to the membranes was determined. Nonspecific binding was defined as 0.5 μM nifedipine (less than 10% of total binding). All values were shown as mean ± SEM derived from the indicated number of experiments (n). The difference between values was assessed by Student's t-test. EC_{50} values were determined from the dose–response plots by computerized nonlinear regression analysis (KaleidaGraph, Synergy Software, Reading, PA). Scatchard analysis of [^{3}H]PN 200-110 binding isotherms, using a one-site model, was performed by the same computer program.

RESULTS

[^{3}H]PN 200-110 binding in synaptic membranes

The effect of corticosterone on [^{3}H]PN 200-110 binding was examined in membranes from disrupted synaptosomes. The membranes were preincubated with the steroid (1 μM) at 37°C for 15 min, and [^{3}H]PN 200-110 binding was determined in a ligand concentration range of 0.02–1 nM. As shown in the Scatchard analysis in Fig. 1(A), corticosterone preincubation of disrupted membranes produced no effect on [^{3}H]PN 200-110 binding. When intact synaptosomes were preincubated with corticosterone (1 μM) at 37°C for 15 min and then disrupted for the determination of [^{3}H]PN 200-110 binding, the binding was found to be markedly increased [Fig. 1(B)]. Scatchard analysis shows that steroid stimulation of [^{3}H]PN 200-110 binding was due to an increase in B_{max} (426 fmol/mg as compared with 255 fmol/mg in the control) without a significant change in K_d for the ligand. Thus, in intact synaptosomes, but not in disrupted membranes, steroid preincubation produced an increase in the number of dihydropyridine binding sites. These data are in agreement with those from a preliminary experiment using [^{3}H]nitrendipine as the dihydropyridine ligand [25]. In a parallel experiment, intact synaptosomes were preincubated with corticosterone at 0°C instead of 37°C. No change in [^{3}H]PN 200-110 binding was found even after extended preincubation such as 30 min (data not shown). The fact that preincubation of intact synaptosomes at 37°C was a necessary condition for the increase of dihydropyridine binding to occur suggests that metabolic processes requiring intracellular factors were involved.

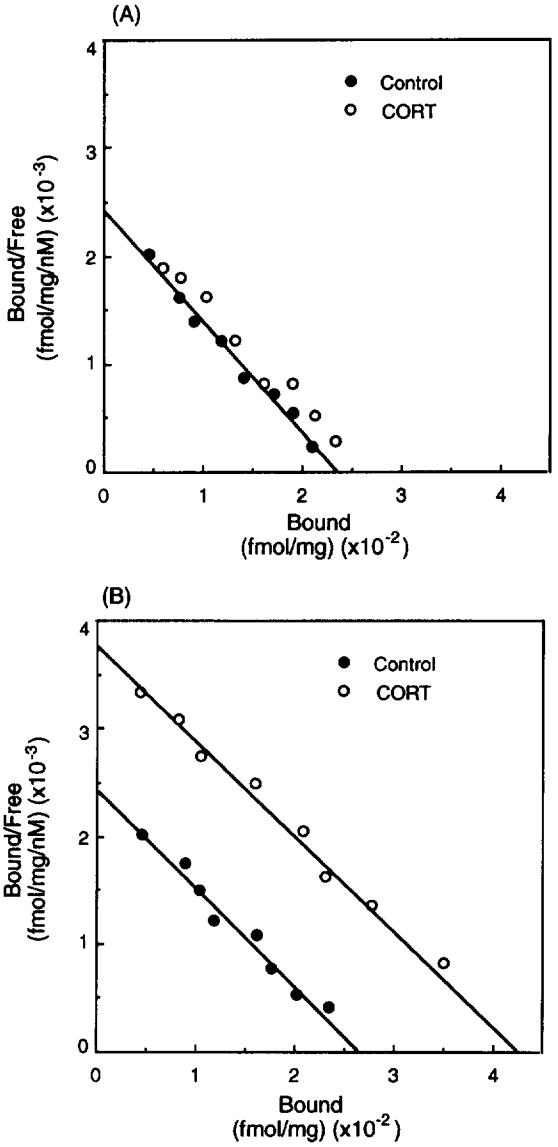

Fig. 1. Scatchard analysis of [^{3}H]PN 200-110 binding. Bound: bound [^{3}H]PN 200-110 in fmol/mg; Free: free [^{3}H]PN 200-110 in nM. Each value is the mean ± SEM (n = 4). (A) Preincubation of disrupted membranes. [^{3}H]PN 200-110 binding was determined after the membranes from disrupted synaptosomes were preincubated with 1 μM corticosterone (CORT). For control: B_{max} = 248 ± 12 fmol/mg; K_d = 0.110 ± 0.011 nM. For CORT-treated: B_{max} = 236 ± 13 fmol/mg; K_d = 0.106 ± 0.012 nM. The two lines are not significantly different (only the control line is shown). (B) Preincubation of intact synaptosomes. [^{3}H]PN 200-110 binding was determined in membranes after intact synaptosomes were preincubated with CORT and then disrupted. For control: B_{max} = 255 ± 14 fmol/mg; K_d = 0.114 ± 0.009 nM. For CORT-treated: B_{max} = 426 ± 15 fmol/mg; K_d = 0.107 ± 0.010 nM. The B_{max} in the two cases is significantly different (P < 0.001).

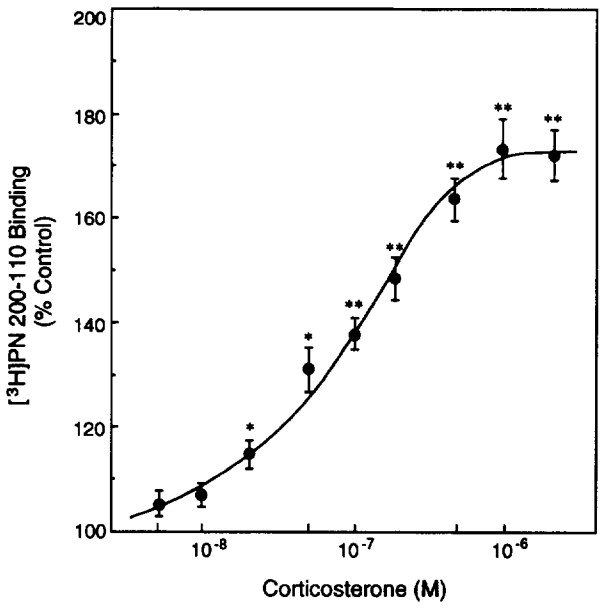

Fig. 2. Concentration effects of corticosterone on [^{3}H]PN 200-110 binding. Intact synaptosomes were preincubated with various concentrations of corticosterone and then disrupted for the determination of [^{3}H]PN 200-110 binding. At the ligand concentration used (0.5 nM), the control value (zero corticosterone) was 180 ± 12 fmol/mg (normalized to 100%). Each value is the mean ± SEM ($n = 6$). Significant difference from control: *$P < 0.05$, **$P < 0.001$. EC_{50} value was determined as 136 ± 31 nM from nonlinear regression analysis.

The concentration-dependency of the corticosterone effect on [^{3}H]PN 200-110 binding was determined after intact synaptosomes were preincubated with the steroid in the range of 5–2000 nM (Fig. 2). The stimulation of [^{3}H]PN 200-110 binding to the membranes was a sigmoidal function of steroid concentration in the log dose-response plot. The maximal increase (>70% above control) occurred at 1 μM steroid and the EC_{50} (effective concentration at 50% maximal response) was estimated at 136 nM.

To determine steroid specificity, the effects of a variety of steroids on [^{3}H]PN 200-110 binding were compared (Table 1). All the steroids were tested at 1 μM, the concentration that produced maximal stimulation by corticosterone. Cortisol, dexamethasone, and triamcinolone enhanced binding with an increase similar in magnitude to that from corticosterone. These synthetic glucocorticoids are known to bind to the same specific binding sites on SPM as corticosterone [21]. At the concentration comparable to that of the hormonal steroids (1 μM), cholesterol was without an effect on [^{3}H]PN 200-110 binding. Two structural analogs of corticosterone, 11-deoxycortisol (cortexolone) and 11-deoxycorticosterone (cortexone), which do not have glucocorticoid activity and which do not have specific binding with SPM, were ineffective on [^{3}H]PN 200-110 binding. Similarly, the gonadal steroids progesterone, 17β-estradiol and testosterone produced no effect on the binding. It should be noted that these gonadal steroids have specific binding sites on SPM, but their binding sites are distinguishable from those for glucocorticoids [21].

Calmodulin-dependent activation of [^{3}H]PN 200-110 binding

Since preincubation of intact synaptosomes with corticosterone at 37°C was a necessary condition for the increase of [^{3}H]PN 200-110 binding to occur, it appears that metabolic reactions were involved in the action of corticosterone. To examine this possibility, attempts were made to modulate dihydropyridine binding by endogenous factors that are known to phosphorylate and thereby activate Ca^{2+} channels. In this experiment, membranes from disrupted synaptosomes were incubated at 37°C in the presence of 0.5 mM ATP, 10 mM Mg^{2+}, 0.5 mM Ca^{2+} and 0.1 μM calmodulin ("calmodulin system"). The membranes were then pelleted for the determination of [^{3}H]PN 200-110 binding. As shown in the time course (Fig. 3), the activation of [^{3}H]PN 200-110 binding by the calmodulin system was rapid, reaching a near plateau at 5 min after incubation. At 5 min, the increase of binding was 170% above the zero-time value. The requirement for the four components (ATP, Mg^{2+}, Ca^{2+} and calmodulin) in the activation is summarized in Table 2. MgATP alone or Ca^{2+}–calmodulin alone was not sufficient to produce the activation. The addition of the Ca^{2+} chelator EGTA abolished the effect of the calmodulin system. In Scatchard analysis, the activation resulted in the increase of B_{max} without a significant change in K_d (data not shown), indicating an increase in the number of dihydropyridine binding sites. In membranes preincubated with corticosterone, the activation of [^{3}H]PN 200-110 binding was markedly enhanced. At the steroid concentration used (1 μM), the increase of binding after a 5 min incubation was 325% above the zero-time value, or nearly twice the amount of activation as in the absence of the steroid.

Table 1. Effects of various steroids on [^{3}H]PN 200-110 binding

Steroid	[^{3}H]PN 200-110 binding (fmol/mg)
None (control)	193 ± 10.5
Corticosterone	344 ± 20.0**
Cortisol	328 ± 22.0**
Dexamethasone	355 ± 17.8**
Triamcinolone	324 ± 19.2**
Cholesterol	188 ± 11.0
11-Deoxycortisol	201 ± 12.9
11-Deoxycorticosterone	182 ± 11.5
17β-Estradiol	208 ± 13.5
Progesterone	213 ± 10.4
Testosterone	198 ± 12.9

All steroids were 1 μM. After intact synaptosomes were preincubated with the steroid (5 min, 37°C), equilibrium binding was determined in disrupted membranes at 0.5 nM [^{3}H]PN 200-110.
Values are mean ± SEM ($n = 4$).
Significant difference from control: **$P < 0.001$.

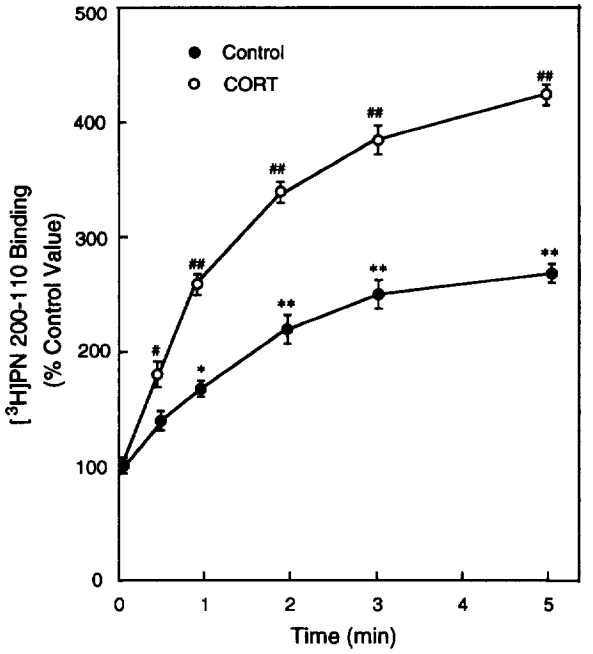

Fig. 3. Time-course of the activation of [³H]PN 200-110 binding in disrupted synaptic membranes by the calmodulin-dependent process. The membranes were preincubated in the presence and absence of 1 μM corticosterone (37°C, 15 min) and then incubated with the following: 0.5 mM ATP, 10 mM Mg^{2+}, 0.5 mM Ca^{2+} and 0.1 μM calmodulin (see text for details). The membranes were pelleted and [³H]PN 200-110 binding was detemined at 0.5 nM ligand. Each value is the mean ± SEM ($n = 6$). The zero-time value was 182 ± 10 fmol/mg (100%). Significant difference from zero-time value: *$P < 0.005$, **$P < 0.001$; from corresponding value in the control line: #$P < 0.01$, # #$P < 0.001$.

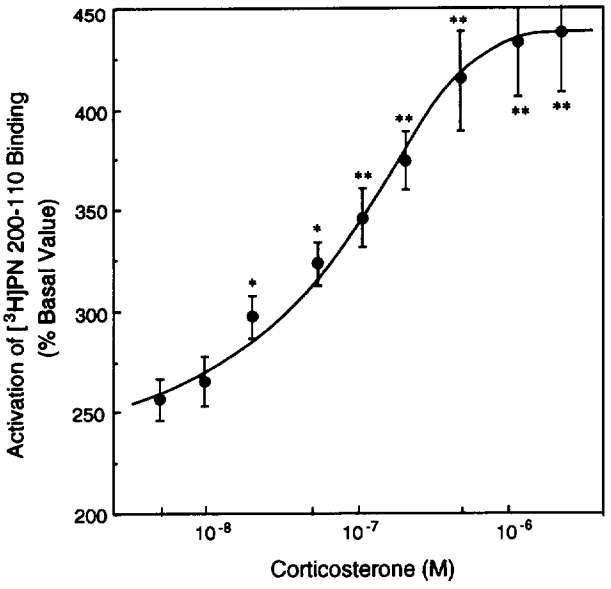

Fig. 4. Concentration effects of corticosterone on calmodulin-dependent activation of [³H]PN 200-110 binding. The experimental conditions were as described in Fig. 3, and the incubation time was 5 min. The activation at zero corticosterone was 255 ± 16% of basal value (the value before activation, see Fig. 3). Each value is the mean ± SEM ($n = 6$). Significant difference from zero corticosterone: *$P < 0.05$, **$P < 0.001$. EC_{50} value was determined as 145 ± 48 nM from nonlinear regression analysis.

The dose–response relationship of the corticosterone effects on the activation was determined (Fig. 4). In the absence of the steroid, the increase of [³H]PN 200-110 binding after a 5 min incubation with the calmodulin system was 155% above the zero-time value ("basal value"). Corticosterone enhanced the activation in a concentration-dependent manner. Within the steroid concentration range used (5–2000 nM), the enhancement of the activation was a sigmoidal function of the steroid concentration, with the maximal effect occurring at 1 μM and the EC_{50} estimated as 145 nM. The log dose–response curve obtained here is in striking resemblance to that from steroid preincubation of intact synaptosomes shown in Fig. 2, which gave an EC_{50} of 136 nM.

Table 2. Requirement in the calmodulin-dependent activation of [³H]PN 200-110 binding

Addition	[³H]PN 200-110 binding (fmol/mg)
None (control)	210 ± 9.5
ATP	208 ± 10.5
Ca^{2+} + CaM	221 ± 10.9
ATP + Ca^{2+} + CaM	625 ± 29.4**
ATP + Ca^{2+} + CaM + EGTA	199 ± 6.7

The basal buffer contained 10 mM $MgSO_4$.
Concentration of the addition: ATP, 0.5 mM; $CaCl_2$, 0.5 mM; calmodulin (CaM), 0.1 μM; EGTA, 2 mM. After incubation of disrupted membranes (5 min, 37°C), equilibrium binding was determined at 0.5 nM [³H]PN 200-110.
Values are mean ± SEM ($n = 4$).
Significant difference from control: **$P < 0.001$.

cAMP-dependent activation of [³H]PN 200-110 binding

[³H]PN 200-110 binding to synaptic membranes was also found to be activated by incubation with 0.5 mM ATP, 10 mM Mg^{2+}, 1 mM cAMP and 10 units/ml PKA ("cAMP system"). The time-course of this activation, shown in Fig. 5, was similar to that produced by the calmodulin system. At 5 min after the incubation, the increase of [³H]PN 200-110 binding was almost 200% above the zero-time value. The requirement for the four components (ATP, Mg^{2+}, cAMP and PKA) in the activation is summarized in Table 3. No activation occurred when cAMP or PKA was absent in the incubation. The activation was fully produced when cAMP and PKA were substituted by the catalytic subunit of PKA. Thus, the addition of exogenous PKA or its catalytic subunit was an absolute requirement for the activation to occur. It is possible that certain endogenous membrane-bound factors, such as PKA, are labile and could be lost during membrane isolation. In electrophysiological analysis, the addition of exogenous PKA and MgATP has been shown to prevent the rapid rundown of Ca^{2+} channel activity recorded

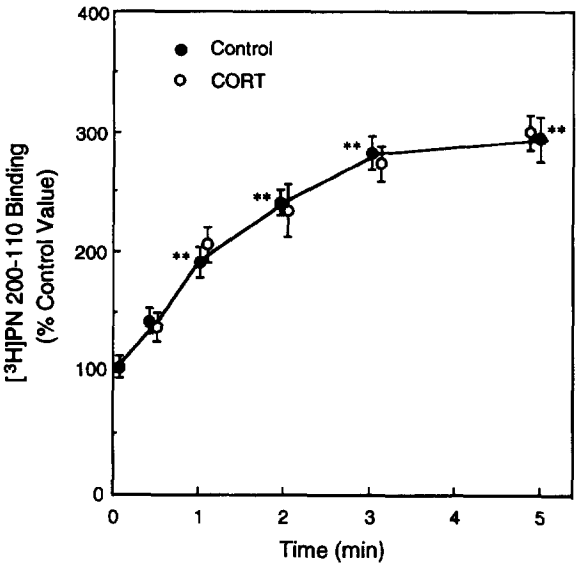

Fig. 5. Time-course of the activation of [^{3}H]PN 200-110 binding in disrupted synaptic membranes by the cAMP-dependent process. The experimental procedure was the same as in Fig. 3, except that the following incubation system was used: 0.5 mM ATP, 10 mM Mg^{2+}, 1 mM cAMP and 10 units/ml PKA (see text for details). Each value is the mean ± SEM ($n = 6$). The zero-time value was 205 ± 11 fmol/mg (100%). Significant difference from zero-time value: $^{**}P < 0.001$. Since there is no significant difference between all corresponding values in the absence and presence of CORT, only the control line is shown.

from cell-free membrane patches [30]. Of particular note is the effect of corticosterone on the activation. In contrast to the enhancement found in the calmodulin-dependent activation, the steroid did not affect the activation of [^{3}H]PN 200-110 binding by the cAMP-dependent process.

DISCUSSION

The increase of [^{3}H]dihydropyridine labeled sites by glucocorticoids shown in these experiments, together with the steroid stimulation of depolarization-induced $^{45}Ca^{2+}$ influx found in a previous study [25], has clearly established the voltage-dependent Ca^{2+} channels as a target of glucocorticoid actions in brain synaptosomes. The primary objective of our study was to investigate the biochemical events underlying the steroid action on these dihydropyridine-sensitive Ca^{2+} channels. There are three relevant findings. First, corticosterone does not appear to modulate Ca^{2+} channels by direct interaction with sites on or near the channels in altering their functional state. No change in [^{3}H]PN 200-110 binding occurred when disrupted membranes from synaptosomes were preincubated with the steroid. As mentioned in the Introduction, the number of high-affinity dihydropyridine binding sites on the Ca^{2+} channels is known to parallel the number of functional channels (i.e. active channels that can open) in membrane preparations [28]. Thus, in disrupted synaptic membranes exposed to corticosterone, the alteration of the functional state of Ca^{2+} channels was not evident from the data on [^{3}H]PN 200-110 binding. Instead, when intact synaptosomes were preincubated with the steroid at 37°C and then disrupted for the determination of [^{3}H]PN 200-110 binding, a significant increase of the dihydropyridine binding sites was found. Preincubation with the steroid at 0°C was ineffective. The fact that treatment of whole synaptosomes at 37°C was a necessary condition to produce the effect suggests that metabolic reactions requiring intracellular factors were involved.

Second, in examining the possibility that glucocorticoids promote metabolic reactions that regulate Ca^{2+} channel activity, we showed that dihydropyridine-sensitive Ca^{2+} channels in isolated synaptic membranes were activated by the addition of MgATP and Ca^{2+}–calmodulin, and that corticosterone enhanced this activation in a concentration-dependent manner. In cell-free membrane patches (i.e. isolated membranes), it has been demonstrated that voltage-dependent Ca^{2+} channels must be phosphorylated in order to open when the membrane is depolarized [30]. Dihydropyridine-sensitive Ca^{2+} channels in the membrane-bound state can be phosphorylated *in vitro* by calmodulin-dependent and cAMP-dependent protein kinases as well as by protein kinase C [29]. While all the three modes of phosphorylation have been implicated in the regulation of neuronal voltage-dependent Ca^{2+} channels [30–33], direct evidence is still lacking and the precise operation of phosphorylation by specific protein kinases in neuronal cells *in vivo* remains to be understood. If, however, calmodulin-dependent phosphorylation does operate *in vivo* in the regulation of voltage-dependent Ca^{2+} channels in synaptic terminals, the stimulation of this process by glucocorticoids would result in an increase of functional Ca^{2+} channels. The data on the increase of [^{3}H]PN 200-110 binding following steroid preincubation of intact synaptosomes are consistent with such a scenario. We further showed

Table 3. Requirement in the cAMP-dependent activation of [^{3}H]PN 200-110 binding

Addition	[^{3}H]PN 200-110 binding (fmol/mg)
None (control)	190 ± 8.1
ATP	186 ± 9.5
ATP + cAMP	201 ± 12.0
PKA + cAMP	180 ± 7.2
ATP + cAMP + PKA	589 ± 24.3**
ATP + CS/PKA	543 ± 22.2**

The basal buffer contained 10 mM $MgCl_2$.
Concentration of the additions: ATP, 0.5 mM; cAMP, 1 mM; PKA and its catalytic subunit (CS/PKA), 10 units/ml. After incubation of disrupted membranes (5 min, 37°C), equilibrium binding was determined at 0.5 nM [^{3}H]PN 200-110.
Values are mean ± SEM ($n = 4$).
Significant difference from control: $^{**}P < 0.001$.

that [^{3}H]PN 200-110 binding in synaptic membranes was also activated by the cAMP-dependent process, which required the addition of an exogenous protein kinase under our experimental conditions. This cAMP-dependent activation of [^{3}H]PN 200-110 binding was not affected by corticosterone. Although the effect of corticosterone on the possible activation by the protein kinase C process remains to be determined, we clearly demonstrated a selectivity in the stimulatory action of the steroid between the two processes examined. The selective stimulation of the calmodulin-dependent process may be related to the action of glucocorticoids on membrane binding of calmodulin (see below).

Third, the EC_{50} of corticosterone in increasing [^{3}H]PN 200-110 binding to synaptic membranes (138 nM) is almost identical to the EC_{50} in the stimulation of $^{45}Ca^{2+}$ influx in synaptosomes (130 nM) [25]. In both cases, the EC_{50} is similar to the K_d in specific binding of the steroid to the membranes (100 nM) reported earlier [21]. These data are consistent with the notion that binding of the steroid to the membrane is the initial and limiting step in the steroid action on Ca^{2+} channels. Several laboratories have agreed that in mammalian brain, the affinity of membrane binding for glucocorticoids and gonadal steroids is lower by one to two orders than the affinity of the nuclear receptors for these steroids [discussed in 22]. We have proposed that the lower affinity of the membrane binding allows rapid association and dissociation of the steroid, thus providing a rapid mechanism for eliciting and terminating a membrane response [21]. In this regard, it is relevant to note that in the action of cortisol on membrane potential in celiac ganglion neurons [34] and on antagonist binding in brain $GABA_A$ receptors [6], the maximal effect is produced only when the steroid concentration exceeds 100 nM.

Stimulation of Ca^{2+} influx by hormonal steroids has been shown in several other types of cells: in mouse thymocytes by dexamethasone [35], in rat endometrial cells by 17β-estradiol [36], and in *Xenopus* oocytes [37, 38] and human sperm [39–41] by progesterone. In these studies, the notion that the steroid action is independent of gene expression is based primarily on the rapid onset of the action. For example, the progesterone effect in sperm occurs in less than 30 s after exposure to the steroid [40]; the dexamethasone effect in thymocytes peaks as early as 5 min after incubation with the steroid [35]. It is generally assumed that such rapid effects cannot be mediated by a process involving gene transcription and protein synthesis, a relatively slow event. By using a subcellular preparation (synaptosomes) where the cell nucleus is absent, the genomic mechanism is unequivocally excluded. In brain synaptosomes, corticosterone does not stimulate the resting level of Ca^{2+} influx; rather, steroid stimulation occurs only upon depolarization of the membrane [25]. As shown in the present study, this effect is related to the activation of voltage-dependent Ca^{2+} channels (i.e. the increase of functional channels), and the action appears to be specific to glucocorticoids among the variety of steroids tested.

The selectivity of the glucocorticoid effect on calmodulin-dependent activation of Ca^{2+} channels is intriguing. We recently found that binding of a glucocorticoid to SPM increases the affinity of the membranes to bind calmodulin [20, 42]. A possible mechanism involving steroid binding sites and the alteration of lipid fluidity in specific subdomains of the membrane has been suggested [20]. Regardless of the underlying mechanism, the alteration of membrane binding of calmodulin is functionally important, since calmodulin regulates a variety of biochemical processes in synaptic membranes. Accordingly, we have postulated that the promotion of calmodulin binding by glucocorticoids may lead to a cascade of consequences in synaptic membrane function. One of the consequences may be the modulation of Ca^{2+} channels, the subject of this study. Glucocorticoids were found to enhance calmodulin-dependent activation of Ca^{2+} channels, and this event may underlie the steroid action on channel activity. Whether or not the calmodulin-dependent activation involves phosphorylation, which remains to be shown, binding of calmodulin to membrane sites would be the initial step in the activation, and steroid promotion of calmodulin binding would thus lead to the increase of the activation and hence an increase of functional Ca^{2+} channels. This scenario provides a working hypothesis for further investigation of the glucocorticoid action on neuronal Ca^{2+} channels.

Acknowledgements—The authors acknowledge the technical assistance of Hanna Sidorowicz. This study was supported in part by U.S. Public Health Service grant AA07230.

REFERENCES

1. Baulieu E.-E. and Robel P.: Neurosteroids: a new brain function? *J. Steroid Biochem. Molec. Biol.* 37 (1990) 395–403.
2. Paul S. M. and Purdy R. H.: Neuroactive Steroids. *FASEB J.* 6 (1992) 2311–2322.
3. McEwen B. S.: Non-genomic and genomic effects of steroids on neural activity. *Trends Pharmac. Sci.* 12 (1991) 141–147.
4. Majewska M. D., Harrison N. L., Schwartz R. D., Barker J. L. and Paul S. M.: Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. *Science (NY)* 231 (1986) 1004–1007.
5. Majewska M. D. and Schwarts R. D.: Pregnenolone-sulfate: an endogenous antagonist of the γ-aminobutyric acid receptor complex in brain? *Brain Res.* 404 (1987) 355–360.
6. Majewska M. D.: Antagonist-type interaction of glucocorticoids with the GABA receptor-coupled chloride channel. *Brain Res.* 418 (1987) 377–382.
7. Gee K. W., Bolger M. B., Brinton R. A., Coirini H. and McEwen B. S.: Steroid modulation of the chloride ionophore in rat brain: structure–activity requirements, regional dependence and mechanism of action. *J. Pharmac. Exp. Ther.* 248 (1988) 803–812.
8. Morrow A. L., Pace J. R., Purdy R. H. and Paul S. M.: Characterization of steroid interactions with γ-aminobutyric acid receptor-gated chloride ion channels: evidence for multiple steroid recognition sites. *J. Pharmac. Exp. Ther.* 37 (1990) 263–270.

9. Lan N. C., Chen J.-S., Belelli D., Pritchett D. B., Seeburg P. H. and Gee K. W.: A steroid recognition site is functionally coupled to an expressed $GABA_A$-benzodiazepine receptor. *Eur. J. Pharmac.* **188** (1990) 403–406.
10. Gee K. W. and Lan N. C.: γ-Aminobutyric $acid_A$ receptor complexes in rat frontal cortex and spinal cord show differential responses to steroid modulation. *Molec. Pharmac.* **40** (1992) 995–999.
11. Lopez-Colome A. M., McCarthy M. and Beyer C.: Enhancement of [^{3}H]muscimol binding to brain synaptic membrane by progesterone and related pregnanes. *Eur. J. Pharmac.* **176** (1990) 297–303.
12. Klangkalya B. and Chan A.: Inhibition of hypothalamus and pituitary muscarinic receptor binding by progesterone. *Neuroendocrinology* **47** (1988) 294–302.
13. Valera S., Ballivet M. and Bertrand D.: Progesterone modulates a neuronal nicotinic acetylcholine receptor. *Proc. Natn. Acad. Sci. U.S.A.* **89** (1992) 9949—9953.
14. Petitti N. and Etgen A. M.: Progesterone promotes rapid desensitization of α_1-adrenergic receptor augmentation of cAMP formation in rat hypothalamic slices. *Neuroendocrinology* 55 (1992) 1–8.
15. Su T.-P., London E. D. and Jaffe J. H.: Steroid binding at σ receptors suggests a link between endocrine, nervous, and immune systems. *Science (NY)* **240** (1988) 219–221.
16. Nabekura J., Oomura Y., Minami T., Mizuno Y. and Fukuda A.: Mechanism of the rapid effects of 17β-estradiol on medial amygdala neurons. *Science (NY)* **233** (1986) 226–228.
17. Schumacher M., Coirini H., Pfaff D. W. and McEwen B. S.: Behavioral effects of progesterone associated with rapid modulation of oxytocin receptors. *Science (NY)* **250** (1990) 691–694.
18. Duval D., Durant S. and Homo-Delarche F.: Non-genomic effects of steroids: interactions of steroid hormone molecules with membrane structures and functions. *Biochim. Biophys. Acta* 737 (1983) 409–442.
19. Szego C. M. and Pietras R. J.: Lysosomal functions in cellular activation: propagation of the actions of hormones and other effectors. *Int. Rev. Cytol.* **84** (1984) 1–302.
20. Sze P. Y. and Iqbal Z.: Glucocorticoid actions on synaptic plasma membranes: modulation of [^{125}I]calmodulin binding. *J. Steroid Biochem. Molec. Biol.* **48** (1994) 179–186.
21. Towle A. C. and Sze P. Y.: Steroid binding to synaptic plasma membrane: differential binding of glucocorticoids and gonadal steroids. *J. Steroid Biochem.* **18** (1983) 135–143.
22. Sze P. Y. and Towle A. C.: Developmental profile of glucocorticoid binding to synaptic plasma membrane from rat brain. *Int. J. Dev. Neurosci.* **11** (1993) 339–346.
23. Orchinik M., Murray T. F. and Moore F. L.: A corticosteroid receptor in neuronal membranes. *Science (NY)* **252** (1991) 1848–1851.
24. Orchinik M., Murray T. F., Franklin P. H. and Moore F. L.: Guanyl nucleotides modulate binding to steroid receptors in neuronal membranes. *Proc. Natn. Acad. Sci. U.S.A.* **89** (1992) 3830–3934.
25. Sze P. Y. and Iqbal Z.: Glucocorticoid action on depolarization-dependent calcium influx in brain synaptosomes. *Neuroendocrinology* **59** (1994) 457–465.
26. Tsien R. W., Lipscombe D., Madison D. V., Bley K. R. and Fox A. P.: Multiple types of neuronal calcium channels and their selective modulation. *Trends Neurosci.* **11** (1988) 431–438.
27. Hosey M. M. and Lazdunski M.: Calcium channels: molecular pharmacology, structure and regulation. *J. Membr. Biol.* **104** (1988) 81–105.
28. Triggle D. J. and Janis R. A.: Calcium channel ligands. *A. Rev. Pharmac. Toxic.* **27** (1987) 347–369.
29. Chang C. F., Gutierrez L. M., Mundina-Weilenmann C. and Hosey M. M.: Dihydropyridine-sensitive calcium channels from skeletal muscle: II. Functional effects of differential phosphorylation of channel subunits. *J. Biol. Chem.* **266** (1991) 16,395–16,400.
30. Armstrong D. and Eckert R.: Voltage-activated calcium channels that must be phosphorylated to respond to membrane depolarization. *Proc. Natn. Acad. Sci. U.S.A.* **84** (1987) 2518–2522.
31. Chad J. E. and Eckert R.: An enzymatic mechanism for calcium current inactivation in dialyzed *Helix* neurons. *J. Physiol.* **378** (1986) 31–51.
32. Narahashi R., Tsunoo A. and Yoshii M.: Characterization of two types of calcium channels in mouse neuroblastoma cells. *J. Physiol.* **383** (1987) 231–249.
33. Greenberg D. A., Carpenter C. L. and Messing R. O.: Interaction of calmodulin inhibitors and protein kinase C inhibitors with voltage-dependent calcium channels. *Brain Res.* **404** (1987) 401–404.
34. Hua S.-Y. and Chen Y.-Z.: Membrane receptor-mediated electrophysiological effects of glucocorticoid on mammalian neurons. *Endocrinology* **124** (1989) 687–691.
35. Homo F. and Simon J.: Early effect of steroids on ^{45}calcium uptake by mouse thymocytes. *Biochem. Biophys. Res. Commun.* **102** (1987) 458–465.
36. Pietras R. J. and Szego C. M.: Endometrial cell calcium and oestrogen action. *Nature* **253** (1975) 357–359.
37. Wasserman W. J., Pinto L. H., O'Connor C. M. and Smith D.: Progesterone induces a rapid increase in $[Ca^{2+}]_{in}$ of *Xenopus laevis* oocytes. *Proc. Natn. Acad. Sci. U.S.A.* **77** (1980) 1534–1536.
38. Moreau M., Vilain J. P. and Guerrier P.: Free calcium changes associated with hormone action in amphibian oocytes. *Dev. Biol.* **78** (1980) 201–214.
39. Thomas P. and Meizel S.: Phosphatidylinositol 4,5-bisphosphate hydrolysis in human sperm stimulated with follicular fluid or progesterone is dependent upon Ca^{2+} influx. *Biochem. J.* **264** (1989) 539–546.
40. Blackmore P. F., Beebe S. J., Danforth D. R. and Alexander N.: Progesterone and 17α-hydroxyprogesterone: novel stimulators of calcium influx in human sperm. *J. Biol. Chem.* **265** (1990) 1376–1380.
41. Blackmore P. F., Neulen F., Lattanzio F. and Beebe S. J.: Cell surface-binding sites for progesterone-mediated calcium uptake in human sperm. *J. Biol. Chem.* **266** (1991) 18,655–18,659.
42. Sze P. Y. and Iqbal Z.: Regulation of calmodulin content in synaptic plasma membranes by glucocorticoids. *Neurochem. Res.* **19** (1994) 1335–1341.