ELSEVIER

Biochimica et Biophysica Acta 1368 (1998) 161–166

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Rapid report

Steroid hormones partition to distinct sites in a model membrane bilayer: direct demonstration by small-angle X-Ray diffraction

G.A. Golden, R.T. Rubin, R.P. Mason *

Center for Neurosciences Research, MCP ♦ Hahnemann School of Medicine, Allegheny University of the Health Sciences, Pittsburgh, PA 15212, USA

Received 3 September 1997; accepted 8 September 1997

Abstract

The classical, genomic mechanisms of steroid hormone action cannot account for their rapid cellular effects. Membrane-bound steroid receptors have been partially characterized, but many rapid steroid effects occur in the absence of steroid-protein binding. Although it has been proposed that these effects could be due to steroid-induced biophysical alterations of the cell membrane, only indirect supporting evidence for this hypothesis has been forthcoming. In the present study, the ability of cortisol and estradiol (E_2), natural steroids of different lipophilicity, to induce alterations in a model membrane (lecithin) bilayer was examined directly by small-angle X-ray diffraction under physiologic-like conditions. Within minutes, both steroids partitioned to distinct sites in the membrane. With increasing membrane cholesterol content, cortisol was displaced toward the polar headgroup region of the phospholipid bilayer, whereas E_2 was displaced in the opposite direction, toward the nonpolar hydrocarbon core. Membrane-based partition coefficients ($Kp_{[mem]}$) for both steroids ($>100:1$) were highest at those cholesterol concentrations that displaced the steroids toward the headgroup region (high cholesterol for cortisol; low for E_2). Both steroids, when located in the headgroup region, increased overall bilayer width by 3–4 Å, a change that could modulate the structure and function of integral membrane proteins independent from steroid effects on the genome. © 1998 Elsevier Science B.V.

Keywords: Membrane bilayer; Cholesterol; Partition coefficient; Steroid; X-ray diffraction

The tertiary structures of integral membrane proteins are sensitive to changes in the composition and structure of the surrounding lipid matrix [1–3]. Modulation of lipid bilayer dynamics can alter membrane-bound protein function, resulting in the loss of normal membrane physiology [1,4]. Some rapid cellular effects of steroids may be related to their intercalation into the membrane lipid bilayer, but there is only indirect support for the hypothesis that steroids alter the biophysical properties of cell membranes. In this study, we determined whether two naturally occurring steroid hormones of different lipophilicity are capable of altering the biophysical properties of a model membrane bilayer. Membrane-based partition coefficients of the steroids were measured, and small-angle X-ray diffraction was used to directly determine the spatial distribution of the steroids in the membrane bilayer and changes in bilayer width.

Steroid hormones are derived from cholesterol, which readily partitions into the phospholipid bilayer, with the 3-hydroxyl group oriented near the surface of the bilayer in the polar phosphate headgroup re-

* Corresponding author. Fax: +1 412 359 6390.

PII S0005-2736(97)00227-7

gion and the sterol nucleus located in the upper hydrocarbon core region [5]. The sterol nucleus is a rigid, planar, fused-ring structure, which greatly hinders the intrinsic motion of membrane fatty acyl chains, thereby increasing membrane bilayer width and reducing lipid mobility. The presence of cholesterol in the membrane modulates bilayer width and lipid dynamics. Cholesterol also affects the ability of certain lipophilic molecules to partition into the membrane bilayer [6,7] and alters their energetically-favorable locations within the bilayer [7]. Considering the close structural homology between cholesterol and steroid hormones (Fig. 1), it is reasonable to predict that these hormones also can induce membrane structural changes and that their partitioning into the membrane is affected by cholesterol content. Heretofore, these interactions have not been tested directly.

Sample preparation: Lecithin/cholesterol multilamellar vesicles (MLV) were prepared from 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) in the presence and absence of cholesterol. POPC and cholesterol, both solubilized in chloroform, were obtained from Avanti Polar Lipids (Alabaster, AL) and stored in a desiccator at −20°C. Cortisol (Sigma, St. Louis, MO) or 17-β-estradiol (Research Biochemicals International, Natick, MA), solubilized in ethanol, was added to aliquots of cholesterol and phospholipid in mole ratios (C:P) of 0.2:1, 0.5:1, and 0.8:1 to yield a final concentration of 1:30 cortisol- or E_2-to-phospholipid mass ratio. The mixture was dried under N_2 gas and residual solvent was removed by vacuum. Buffer (154 mM NaCl, 0.5 mM HEPES, pH 7.4, 37°C) was added to each tube, yielding a final lipid concentration of 2.5 mg/ml, and the solutions were vortexed to form MLV [8]. Steroid-free MLV also were prepared as controls. Stacked membrane bilayers were formed by centrifugation of the vesicles at 35 000 × *g* for 90 min at 37°C. The supernatants were removed, and each bilayer pellet was mounted on a curved glass support and suspended in a humidity chamber overnight containing a saturated salt solution ($NH_4H_2PO_4$) in the vicinity of the sample, establishing a relative humidity of 93%. The oriented membrane samples then were placed in sealed brass canisters with thin aluminum foil windows in which temperature and relative humidity were controlled (as described above).

Small-angle X-ray diffraction: This technique can directly determine changes in membrane bilayer width and electron density distribution caused by the addition of small molecules to lipid bilayers [9]. It has

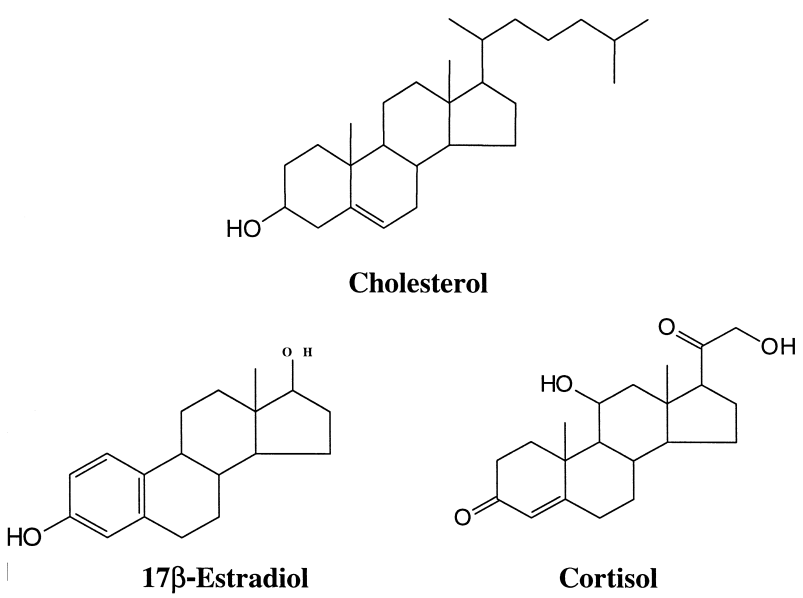

Fig. 1. Cholesterol is an amphipathic, planar molecule composed of four fused-rings (sterol nucleus). Various enzymes add, remove and modify constituent groups at different sites on the rings to form the steroid hormones. With only two polar hydroxyl constituent groups, E_2 is less polar (possesses greater lipophilicity) than cortisol.

been used to identify the membrane locations of such lipophilic molecules as cholesterol [10–13], calcium channel blockers [14], and anesthetics [5].

Structural analyses of membrane samples were carried out by aligning them at grazing incidence with respect to the X-ray beam. The radiation source was a collimated, monochromatic X-ray beam (CuK_α, $\lambda = 1.54$ Å) produced by a high-brilliance rotating anode microfocus generator (Rigaku Rotaflex RU-200, Danvers, MA). The fixed geometry beamline incorporated a single Franks mirror, providing nickel-filtered radiation ($K_{\alpha 1}$ and $K_{\alpha 2}$ unresolved) at the detection plane. The beam height at the sample was ~3 mm. The diffraction data were collected on a one-dimensional, position-sensitive electronic detector (Innovative Technologies, Newburyport, MA) calibrated with cholesterol monohydrate crystals. The sample-to-detector distance was 150 mm.

Fourier transformation of the diffraction data yielded the electron density distribution of the membrane. Each individual meridional diffraction peak was background-corrected by averaging the noise.

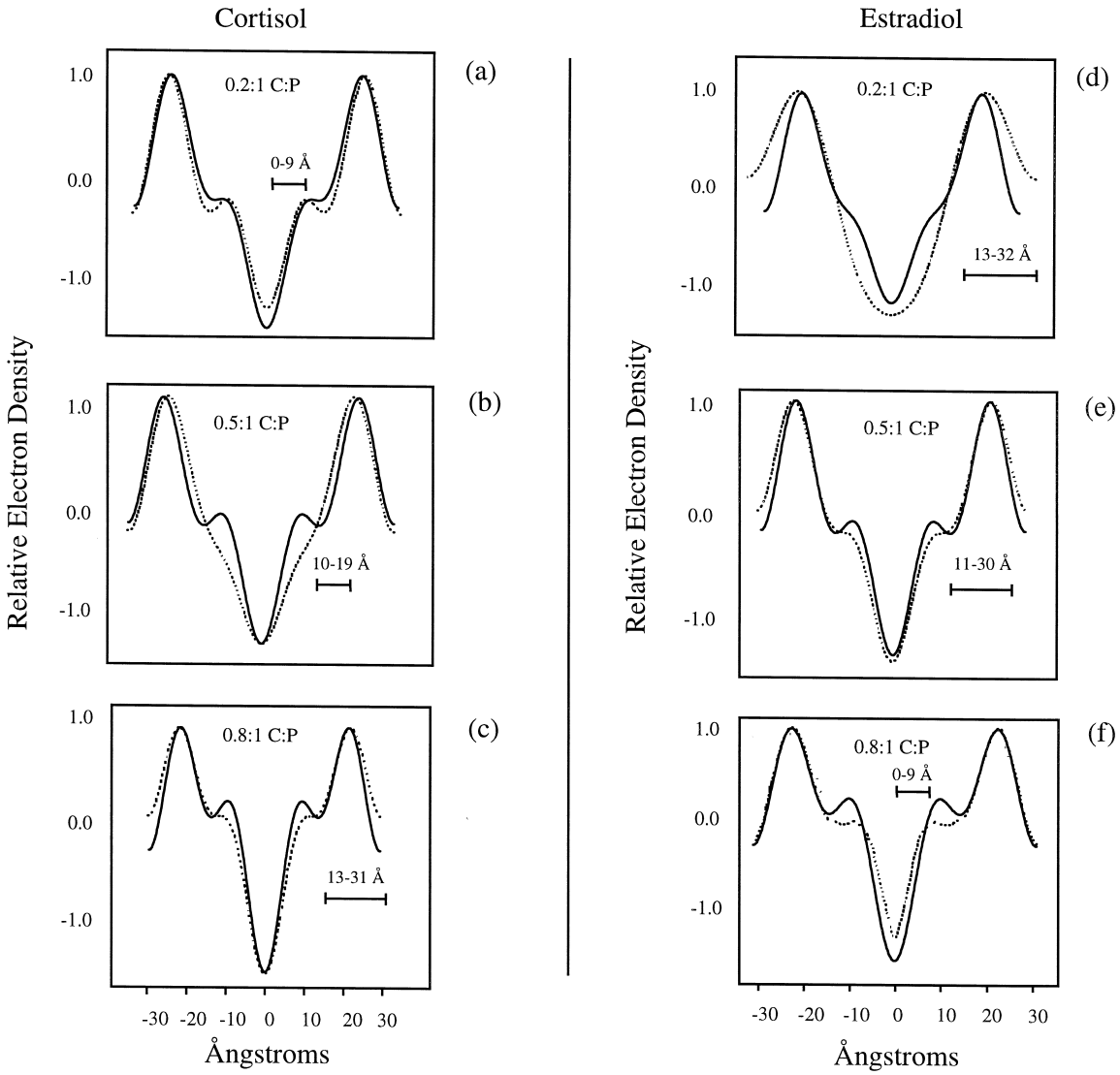

Fig. 2. Electron density profiles of model membrane bilayers at 0.2:1, 0,5:1, and 0.8:1 cholesterol:phospholipid ratios (C:P), with (- - -) and without (————) added cortisol (a, b, c) or estradiol (d, e, f). Increases in electron density correspond to the energetically favorable locations of the steroids. Regional increases in electron density are only labeled for one-half of each profile.

The intensity functions were corrected by a factor of $s = 2\sin\theta/\lambda$, the Lorentz correction, in which λ is the wavelength of radiation (1.54 Å), and θ is the Bragg angle (one-half the angle between the incident beam and the scattered beam). Swelling analysis was used to assign a phase combination to the structure factors [15].

Sample preparation for Membrane Partition Coefficients ($K\mathrm{p}_{[\mathrm{mem}]}$): The ($K\mathrm{p}_{[\mathrm{mem}]}$) is an equilibrium value expressing the relative concentration of a substance in the membrane versus aqueous buffer and provides an accurate measure of a substance's affinity for the membrane bilayer. MLV were prepared at C:P mole ratios of 0:1, 0.2:1, 0.4:1, 0.6:1, 0.8:1, and 1.0:1 with added cortisol or E_2 (1:30 steroid:phospholipid mass ratio), as described above. Control samples contained steroid and buffer, but no phospholipid or cholesterol. The samples were centrifuged for 30 min at $35\,000 \times g$ and 37°C, and the supernatants were removed and assayed for steroid content. The steroid concentration of each sample was subtracted from the corresponding control value; the difference represented the amount of steroid contained within the MLV. $K\mathrm{p}_{[\mathrm{mem}]}$ were calculated at each cholesterol concentration as follows:

$$K\mathrm{p}_{[\mathrm{mem}]} = (\mathrm{g\ Steroid\ bound\ to\ membrane}/\mathrm{g\ Lipid})/(\mathrm{g\ Free\ steroid}/\mathrm{g\ Buffer}).$$

Assay procedures: Cortisol concentrations were determined in duplicate with a solid-phase (coated tube) radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA). The minimum detectable cortisol concentration was 1.0 ng/ml, and intra-assay and inter-assay coefficients of variation were $\leq 5.9\%$ and $\leq 5.4\%$, respectively. E_2 concentrations were determined in duplicate with a double-antibody radioimmunoassay kit (Diagnostic Systems Laboratories, Webster, TX). The minimal detectable E_2 concentration was 1.4 pg/ml, and intra-assay and inter-assay coefficents of variation were $\leq 3.9\%$ and $\leq 4.2\%$, respectively.

Representative electron density distribution profiles are shown in Fig. 2. They indicate the time-averaged spatial distribution of membrane phospholipids as a function of distance across a single bilayer. Regional increases in electron density associated with the addition of steroid indicate that cortisol and E_2 interacted at distinct sites in these model membrane bilayers. The locations of both steroids were affected by cholesterol content in a concentration-dependent manner (Table 1 and Fig. 2): Increasing cholesterol content displaced cortisol from the bilayer core to the membrane surface (phospholipid headgroup region), whereas the opposite occurred with E_2. Both steroids, when located in the headgroup region, increased overall bilayer width by 3–4 Å. This increase could deform transmembrane proteins [16] and suggests a structural mechanism by which steroids could influence cellular function independently from their genomic effects.

For the proposed mechanism to account for rapid steroid effects, the membrane structure changes induced by steroids must occur in the same short time. We therefore, also examined the kinetic and equilibrium binding properties of cortisol and E_2. The time required for both cortisol and E_2 to reach equilibrium binding was ≤ 5 min, since maximal binding occurred within 5 min, and no significant changes in $K\mathrm{p}_{[\mathrm{mem}]}$ occurred between 5 and 120 min ($p > 0.3$; data not shown). This time-frame is compatible with

Table 1
Energetically favorable equilibrium binding distribution (±distance from center of membrane) for cortisol and estradiol in POPC/cholesterol membranes at various cholesterol:phospholipid mole ratios (C:P), as determined by small angle X-ray diffraction. Changes in bilayer width (*d*-space) are indicated for each condition

	0.2:1 C:P		0.5:1 C:P		0.8:1 C:P	
	Location (Å)	*d*-Space change (Å)	Location (Å)	*d*-Space change (Å)	Location (Å)	*d*-Space change (Å)
Cortisol	±(0–9)	−0.2	±(10–19)	1.5	±(13–31)	2.9[a]
E_2	±(13–32)	4.0[a]	±(11–30)	1.5	±(0–9)	−0.7

[a] *d*-Space significantly different from control membrane bilayer ($p < 0.05$, $n = 3$).

rapid steroid effects that have been reported in cells and reasonably excludes early genomic effects.

As mentioned, cholesterol is a major constituent of mammalian cell plasma membranes and modulates membrane structure [13] and function [17]. As shown in Fig. 3, $K\,p_{[mem]}$ for both cortisol and E_2 varied as a function of membrane cholesterol content over a range of 0:1–1.0:1 C:P. At low C:P levels, the E_2 $K\,p_{[mem]}$ was inversely and significantly correlated with cholesterol content ($p < 0.05$), whereas the cortisol $K\,p_{[mem]}$ decreased only slightly. In contrast, at higher C:P levels, E_2 binding was unaffected by further cholesterol increases, whereas cortisol binding was significantly increased.

There are several lines of experimental evidence which support the possibility that the concentrations of steroids used in these experiments are biologically relevant. The biological activity of a hormone generally is considered to be proportional to its free concentration in plasma [18]. Alternatively, it has been suggested that intracapillary protein-bound hormone dissociation is sufficiently rapid relative to tissue uptake and metabolism that the in vivo free-hormone level is maintained at its in vitro equilibrium value at all times [18]. Additionally, because of local steroid synthesis from cholesterol, it is likely that certain cells, including neurons [19–21], are exposed to steroid concentrations greater than those in the plasma. Finally, in consideration of the high membrane-binding affinities of steroids, as shown here and elsewhere [22], it is possible that the plasma membrane acts as a sink for steroid hormones, allowing them to attain membrane concentrations considerably higher than their circulating levels.

It is unclear exactly which factor(s) contributed to the pronounced effect of cholesterol content on steroid location in the membrane. The highest binding concentrations and greatest effects on membrane width occurred for both cortisol and E_2 when they were located in the headgroup region of the membrane bilayer, suggesting more favorable binding properties and/or greater volume availability in this region. This occurred at different C:P ratios for the two steroids and suggests a potential nongenomic mechanism for specificity of steroid action, based on the composition of a given membrane and the molecular structure of a given steroid (Fig. 1). Because plasma membrane C:P varies considerably among cell types [23–25], it is possible that the same steroid could have different membrane-based actions in vivo, depending on the lipid composition of the target cell membrane. The issue of membrane composition also is relevant for genomic steroid actions, since the ability of a substance to partition into the membrane is a critical determinant of its bioavailability to intracellular receptors.

In conclusion, these experiments provide additional insight into biochemical and biophysical steroid-membrane interactions which may contribute to rapid membrane-mediated steroid effects. These

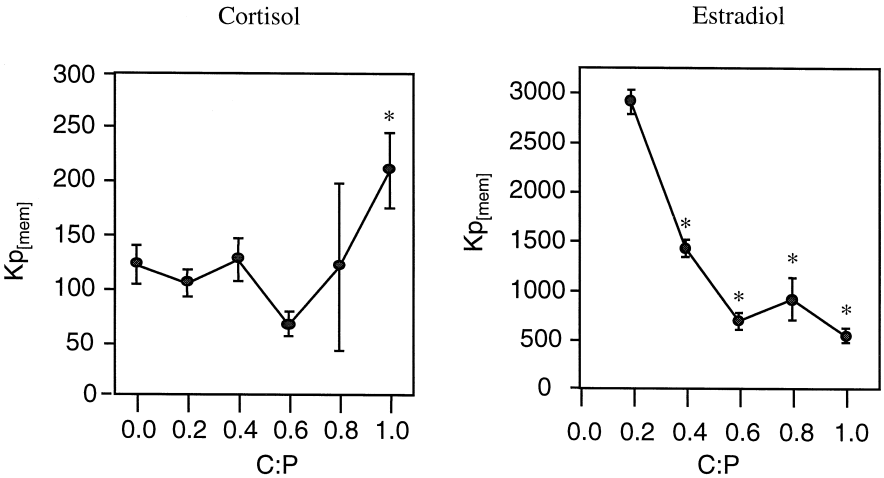

Fig. 3. Mean (±SEM) membrane-based partition coefficients ($K\,p_{[mem]}$) for cortisol ($n = 8$) and estradiol ($n = 8$) in POPC/cholesterol multilamellar vesicles. $K\,p_{[mem]}$ significantly different than $K\,p_{[mem]}$ at 0.2:1 C:P, ($p < 0.05$).

effects include, among others, modulation of calcium flux [26], augmentation of cAMP effects [27], and vasodilation of coronary and peripheral vascular beds [28].

R. Kenneth Czambel kindly performed the hormone assays, and Pamela E. Mason provided helpful technical and editorial advice. The authors acknowledge the helpful discussions provided by Thomas N. Tulenko, Ph.D. related to this work. This work was supported in part by an Allegheny University of the Health Sciences intramural grant to GAG, and NIMH grant MH28380 and NIMH Research Scientist Award MH47363, both to RTR.

References

[1] H.M. Chang, R. Reitstetter, R.P. Mason, R. Gruener, J. Membr. Biol. 143 (1995) 51–63.
[2] G. Lenaz, G. Curatola, L. Mazzanti, G. Parenti-Castelli, Mol. Cell. Biochem. 22 (1978) 3–32.
[3] C. Tanford, J. Am. Chem. Soc. 84 (1962) 4240–4247.
[4] H.M. Chang, R. Reitstetter, R. Gruener, J. Membr. Biol. 145 (1995) 13–19.
[5] N.P. Franks, in: E. Grell (Ed.), Membrane Spectroscopy, Springer-Verlag, Berlin, 1981, pp. 437–487.
[6] M.K. Jacobsohn, S. Bauder, S.R. Pine, G.M. Jacobsohn, Biochim. Biophys. Acta 1195 (1994) 131–140.
[7] T. Mavromoustakos, D.P. Yang, A. Makriyannis, Biochim. Biophys. Acta 1194 (1994) 69–74.
[8] A.D. Bangham, M.M. Standish, J.C. Watkins, J. Mol. Biol. 13 (1965) 238–252.
[9] S.M. Gruner, in: P.L. Yeagle (Ed.), The Structure of Biological Membranes, CRC Press, Boca Raton, 1992, pp. 211–250.
[10] R.P. Mason, W.J. Shoemaker, L. Shajenko, T.E. Chambers, L.G. Herbette, Neurobiol. Aging 13 (1992) 413–419.
[11] M. Chen, R.P. Mason, T.N. Tulenko, Biochim. Biophys. Acta 1272 (1995) 101–112.
[12] T.J. Mcintosh, Biochim. Biophys. Acta 513 (1978) 43–58.
[13] N.P. Franks, J. Mol. Biol. 100 (1976) 345–358.
[14] R.P. Mason, M.W. Trumbore, Biochem. Pharmacol. 51 (1996) 653–660.
[15] M.F. Moody, Science 142 (1963) 1173–1174.
[16] Y.P. Zhang, R.N.A.H. Lewis, R.S. Hodges, R.N. McElhaney, Biochemistry 31 (1992) 11579–11588.
[17] L.M. Gordon, P.W. Mobley, in: R.C. Aloia, J.M. Boggs (Eds.), Membrane Fluidity in Biology, vol. 4, Academic Press, Orlando, 1985, pp. 1–47.
[18] R. Ekins, Endocr. Rev. 11 (1990) 5–46.
[19] D.L. Martin, Glia 5 (1992) 81–94.
[20] S.H. Mellon, C.F. Deschepper, Brain Res. 629 (1993) 283–292.
[21] R.H. Purdy, A.L. Morrow, P.H. Moore, S.M. Paul, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 4553–4557.
[22] R.B. Heap, A.M. Symons, J.C. Watkins, Biochim. Biophys. Acta 218 (1970) 482–495.
[23] I.D. Kholodova, Bkr. Biokhim. Sh. 53 (1981) 114–130.
[24] J.B. Eggesbo, T.A. Hagve, K. Borsum, A.T. Hostmark, I. Hjermann, P. Kierulf, Scand. J. Clin. Lab. Invest. 56 (1996) 199–210.
[25] R.O. Calderon, B. Attema, G.H. DeVries, J. Neurochem. 64 (1995) 424–429.
[26] J.M. ffrench-Mullen, P. Danks, K.T. Spence, J. Neurosci. 14 (1994) 1963–1977.
[27] N. Petitti, A.M. Etgen, Neuroendocrinology 55 (1992) 1–8.
[28] M.Y. Farhat, S. Abi-Younes, P.W. Ramwell, Biochem. Pharmacol. 51 (1996) 571–576.