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EFFECTS OF PROGESTERONE, EPIPREGNANOLONE AND RU 38486 ON POTASSIUM UPTAKE IN CULTURED CORTICAL NEURONS

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Summary—It was previously reported that progesterone and its metabolites influence electrical properties of the CNS in many different ways. In the present study we elicited the effects of progesterone, its 5β reduced metabolite epipregnanolone and the anti-progestin compound RU 38486 on potassium uptake in cultured cortical neurons. K^+ was substituted by the tracer substance ^{86}Rb. When hormone treatment (10^{-9}–10^{-7} M/l) was performed for 3 days, addition of progesterone and epipregnanolone led to a significant decrease of ^{86}Rb uptake whereas treatment with RU 38486 markedly increased ^{86}Rb uptake. The effect of the anti-progestin could be reversed by the addition of increasing amounts of progesterone. Hormone actions were dose-dependent and most distinct when performed from the very first day of culture. Short-term (15 min) hormone treatment of neurons did not significantly alter ^{86}Rb uptake. These findings suggest a specific receptor mediated progestin action which, in a long-term course, controls potassium uptake across excitable membranes.

INTRODUCTION

Steroid hormones have been reported to exert a wide range of actions in the central nervous system (CNS), including regulation of cell growth and differentiation, neuronal and glial metabolism and sexual behavioral patterns [1–4]. Rapid neurotropic effects of steroids, which occur in milliseconds to seconds such as the alteration of neuronal excitability and rapid feedback control of the release of hypothalamic hormones are mediated at the plasma membrane level [5, 6] whereas persistent changes in neuronal functions result from changes in protein synthesis and alteration in chromatin structure. These effects usually involve binding of the steroids of intracellular receptors in the nucleus [6–8].

The brain contains substantial amounts of steroid precursors such as the 3β-hydroxysteroid pregnenolone [9–12] which is readily converted to progesterone in neural cells [13, 14]. The latter has often been regarded as "pre-hormone" in the CNS, exerting its effects by genomic as well as nongenomic mechanisms [15–18]. In this context progesterone has been described as a powerful modulatory steroid of presynaptic striatal dopaminergic terminals in the rat brain [19]. The CNS possesses the adequate enzyme system to convert progesterone into 5α and 5β reduced metabolites which are known to be highly active in modulating nervous functions. While 5α progestins have been reported to act mainly at genomic level, 5β progestins presumably influence directly excitable membranes by modifying membrane permeability and altering neuronal excitability [20].

To our knowledge little information exists about a specific action of these steroids on ion transport across neuronal plasma membranes. Therefore we examined the effects of progesterone, the 5β reduced progestin epipregnanolone and the potent progesterone antagonist RU 38486 [21] on potassium uptake in cultured cortical neurons using ^{86}Rb as the tracer substance.

EXPERIMENTAL

Reagents

Buffer salts (analytical grade), hormones, ouabain and furosemide were obtained from Sigma (Munich, F.R.G.). ^{86}Rb chloride (37 mBq) was purchased from Amersham International (England). Cell culture media were from Serva (Heidelberg, F.R.G.) and culture plastic were was purchased from Nunc (Denmark). Liquid scintillation fluid (Ecoscint) was used from National Diagnostics (U.S.A.)

Cell culture

Neurons from 14 day old mouse embryos (Balb/c) were prepared according to Bauer and Tontsch[22]. Cultures were grown in serum free standard medium [23] with and without progesterone (4×10^{-8} M/l) for 3 days. For the experiments various hormone concentrations, providing full occupation of receptor sites, were added from first day of

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culture. Hormones were dissolved in absolute ethanol and further diluted with culture medium. Control cultures were grown in parallel receiving the solvent without hormones. Media were changed once a day. Ouabain (1 mM/l final conc.) and furosemide (0.1 mM final conc.) were directly dissolved in influx buffer. Long-term (3 day) and short-term (15 min) hormone treatments were performed.

^{86}Rb-uptake

Neurons were washed twice at room temperature with influx buffer (buffer A) containing (in mM/l): 120 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 20 HEPES, 5.5 glucose. pH was adjusted to 7.5 with Tris. For ^{86}Rb-uptake buffer A containing 2 μCi/ml ^{86}Rb was used. Influx period lasted 20–30 min. Thereafter cells were rinsed rapidly 3 times with isotonic saline (0.15 M/l NaCl) at room temperature and 500 μl Triton X-100 (0.5%) were added to each well. After 12 h extraction time the supernatants were transfered to scintillation vials. 8 ml of scintillation fluid was added and radioactivity was determined in a liquid scintillation counter (Beckman).

Protein determination

Cells were washed once with DPBS (Dulbecco's phosphate buffered saline) at room temperature and lysed with 500 μl 1 M/l NaOH for 24 h at 37°C. Aliquots were then taken and protein determined according to Lowry *et al.*[24].

Calculation

Na^+,K^+-ATPase was estimated as the ouabain inhibitible part of total ^{86}Rb-uptake. Activity of the Na/K/2 Cl^- cotransport [25] was estimated by furosemide inhibition. Influx rates are given as cpm/min/mg protein. Results are presented as mean ± standard deviation (SD).

RESULTS

Figure 1 shows ^{86}Rb uptake in cultured cortical neurons (3–5 days old). Initial uptake was linear for up to 2 h. Based on this finding an influx period of 20–30 min was chosen for all experiments.

Cortical neurons derived from 14 day old mouse embryos differentiated rapidly in culture during the first days. ^{86}Rb uptake increased markedly within the first 24 h but leveled after a few days (Fig. 2). Hormone treatment was most effective when it was performed from the very beginning of culture and lasted for 3 days.

^{86}Rb uptake was partly inhibited by ouabain (43 ± 8.8%) and by furosemide (38 ± 8.2%) (mean ± SD, $n = 8$).

Addition of progesterone and epipregnanolone (10^{-9}–10^{-7} M/l) led to a significant and dose-dependent suppression of initial ^{86}Rb uptake rate (Fig. 3a and b). The low uptake at higher concentration was

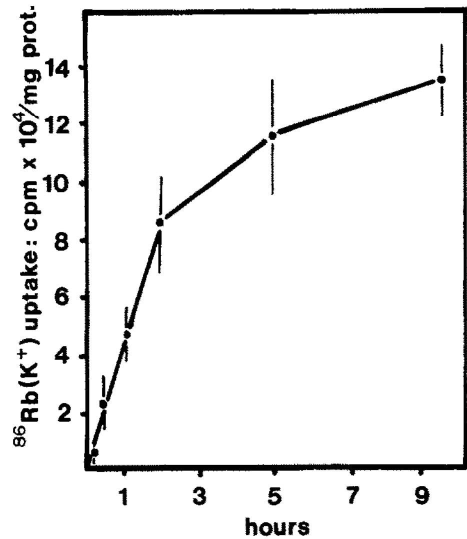

Fig. 1. Time dependence on ^{86}Rb uptake in 3–5 day old neurons cultured in 12 well multi plates. $n = 10$, means and SD are given.

due to cell damage as observed by microscopic inspection.

Short-term hormone treatment of neurons did not result in any significant effect on ^{86}Rb uptake (data not shown).

Addition of RU 38486 stimulated ^{86}Rb uptake exerting its maximum effect (about 95% increase) at concentrations of 10^{-9}–10^{-7} M/l (Fig. 3c). Higher concentrations again led to cell damage.

Percentages of ^{86}Rb uptake alloted to Na^+,K^+-ATPase activity and furosemide sensitive Na/K/2 Cl^- cotransport were not significantly influenced by hormone treatment.

When serum-free standard medium containing 4×10^{-8} M/l progesterone was used instead of progesterone deprived medium, a significant decrease in ^{86}Rb influx was observed (32 ± 4%) (mean ± SD, $n = 12$). The effect of RU 38486 was reversed when anti-progestin treated cultures were washed with progesterone containing standard medium and then

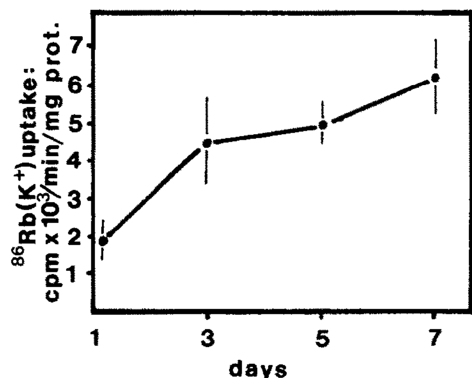

Fig. 2. Total ^{86}Rb uptake during neuronal differentiation *in vitro*. Cells were cultured in 35 mm ϕ plastic petri-dishes. $n = 5$, means and SD are given.

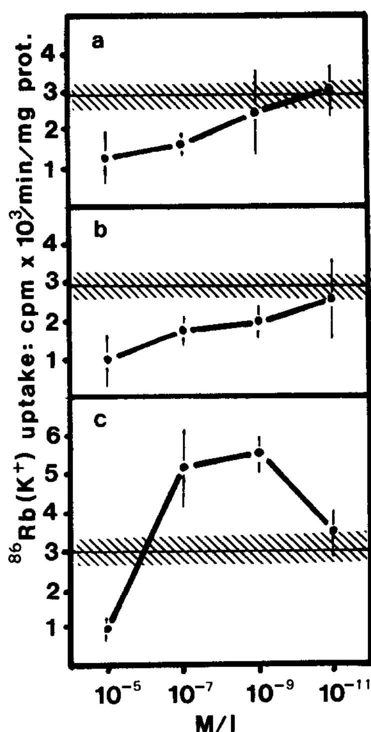

Fig. 3. Effects of progesterone (a), epipregnanolone (b) and RU 38486 (c) on total ^{86}Rb uptake in 3 day old neurons cultured in 12-well multi plates. ▨ represents ^{86}Rb uptake in control cells grown in serum-free medium without progesterone. $n = 9$, means and SD are given.

grown in this medium for 48 h (Fig. 4). ^{86}Rb uptake did not differ between controls grown in parallel with and without addition of solvent (maximum final concentration of 0.5% ethanol).

DISCUSSION

Results from our experiments indicate that ion transport across neuronal plasma membrane is to a certain extent under hormonal control. This is suggested by our data derived from ^{86}Rb influx studies

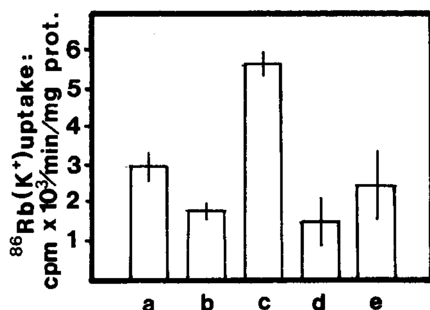

Fig. 4. Comparison of ^{86}Rb uptake in neurons cultured ion (a) progesterone-free medium (b) medium containing 4×10^{-8} M/l progesterone and (c) progesterone-free medium supplemented with 10^{-9} M/l RU 38486 for 3 days. (d) ^{86}Rb uptake in anti-progestin treated neurons [see (c)] cultured in medium containing 10^{-7} and 10^{-9} M/l (e) progesterone for 48 h.

demonstrating potassium entry into the cell via active and passive transport systems.

Since the brain is an extremely complex target organ for neurosteroid action the use of a culture system represents an adequate approach for detailed investigations. For our studies we used cultured cortical neurons derived from 14 day old mouse embryos which exhibited fully expressed K^+-transport properties (Na^+,K^+-ATPase, Na/K/2 Cl^- co-transport) after 3 days in culture.

^{86}Rb uptake was monitored as the final signal of hormone action. Addition of increasing concentrations of progesterone and epipregnanolone consequently led to a significant decrease of ^{86}Rb influx in long-term experiments (Fig. 3). Moreover, suppression was dose-dependent indicating that this effect was really subject to a specific progestin action.

It has to be kept in mind, that suppression of K^+ uptake generally leads to a decrease of internal K^+ concentration, inducing neuronal excitability whereas increase of K^+ uptake results in hyperpolarization and simultaneous depression of neuronal firing.

Our results are consistent with reports according to which progesterone and its 5β reduced metabolites increase neuronal activity at low doses in a concentration-dependent manner [5, 20].

Yet, since progesterone is readily metabolized in neural cells [26], any final hormone action may not necessarily be due to the parent compound. However, inhibition of progesterone metabolizing enzyme(s) is not an adequate approach to isolate specific progesterone action from a multihormonal one. This would lead to a general suppression of steroid synthesis, altering cell function and metabolism in many aspects.

So we were led to study the progesterone competing effect of the synthetic 19-nor-steroid compound RU 38486 [21, 27–31] which has been reported to act at receptor level.

Addition of RU 38486 (10^{-9}–10^{-7} M/l) resulted in a marked and dose-dependent increase of ^{86}Rb uptake when applied to neurons grown in serum-free standard medium without progesterone. Here we are tempted to assume that the antiprogestin completely removed endogenous progesterone from receptor sites thus eliminating basically progesterone action. In this respect it should be mentioned that neural cells in general contain endogenous progesterone since they are capable of synthesizing it from steroid precursors [13, 14]. Our hypothesis was once more confirmed by the finding that addition of increasing amounts of progesterone decreased RU 38486 effect on ^{86}Rb uptake.

Still, whether glucocorticoid receptor binding of RU 38486 and/or progesterone contributed to our results remain to be elucidated [32–34]. In fact, the mechanisms underlying final signals of steroid action on nervous tissue are particularly complex, including modulation of synaptic transmission 19 as well as interaction with the GABA receptor complex [5].

Based on our studies there is considerable evidence that a specific receptor mediated progestin action exists which, in a long-term process, changes potassium uptake across neuronal plasma membranes.

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