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STUDIES ON THE ONTOGENESIS OF THE DIFFERENT ISOENZYMES OF Na^+, K^+-ATPase IN RAT BRAIN *IN VIVO* AND *IN VITRO* IN RELATION TO THEIR REGULATION AND CELLULAR LOCALISATION

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Abstract—Na^+,K^+ ATPase isoenzyme activities ($\alpha(+)$-high ouabain affinity; α low ouabain affinity) were investigated in developing rat brain *in vivo* and in whole rat brain reaggregating cultures *in vitro*. The perinatal profile of the two isoenzyme forms *in vivo* revealed that, although α activity predominates in immature (P14.5-P16) brain, the activity $\alpha(+)$ form increases more increases more rapidly such that it is predominant at birth in both cerebellum and forebrain. No regional variation in the proportional activities of the two isoenzyme forms was seen perinatally to explain the previously reported, differential sensitivity of the cerebellar α isoenzyme to neonatally induced hypothyroidism. Whole rat brain reaggregating cultures seeded at P16 show a normal development of Na^+,K^+ ATPase isoenzyme activity if grown for 14 days in a serum supplemented medium (S+). Cultivation of whole rat brain reaggregates in serum deprived medium (S−) leads to a retarded development of α isoenzyme activity possibly due to the absence of T_3 from the medium. Hormonally-induced changes in the development of the brain Na^+, K^+-ATPase isoenzymes are discussed in relation to their possible function and cellular localization.

The plasma-membrane Na^+,K^+-ATPase large subunit in brain exists as two isoenzymes which have different biophysical properties [1], different antigenic determinants [2] and different affinities for the cardiac glycoside, ouabain ($\alpha(+)$: high ouabain affinity; and α: low ouabain affinity; see Refs 1 and 3). Although specific functional roles and cellular localizations for the two forms have not yet been defined, it would appear that central neurones can express one or both forms depending on their state of differentiation whilst astroglia express predominantly the α isoenzyme [1, 3].

The two Na^+,K^+ isoenzymes appear to have differential sensitivities to certain drugs and neurotoxins [4, 5] and also to hormones such as insulin and thyroid hormone [6, 7]. We have shown, for example, that experimental hypothyroidism in neonatal rats selectively impairs the development of cerebellar but not forebrain α form of Na^+, K^+-ATPase activity [7]. A possible reason for this difference was thought to be regional differences in the proportions of the α form at birth. Early prenatal rat brain contains a higher proportion of this molecular form [5, 8]. The profile in cerebellum, a predominantly postnatally developing area, is unknown, although immature cultured cerebellar granule neurones and astrocytes from postnatal cerebellum do possess high proportions of the α form [3]. It is also known that whole rat brain reaggregate cultures *in vitro* seeded from prenatal tissue and grown in a serum-free environment show a retarded development of total Na^+,K^+-ATPase activity compared with those grown in the presence of foetal calf serum [9]. Thyroid hormone supplementation restores development of activity and thus may be one of the essential serum components regulating enzyme activity [9].

In the present study, therefore, we have examined the development of the two Na^+, K^+-ATPases in serum supplemented (S+) and serum-free (S−) reaggregates of rat brain to see whether a differential retarded development of one particular form occurs as it does in the cerebellum from hypothyroid rats *in vivo*. In this context we have also addressed the question as to whether brain regions (cerebellum and forebrain) of the developing rat exhibit different Na^+,K^+-ATPase isoenzyme profiles pre- and perinatally.

MATERIALS AND METHODS

Materials. All cell culture materials were obtained from either Flow Laboratories or Gibco-Bio-Cult. [^{32}P]ATP, adenosine 5-[γ-^{32}P] triphosphate, triethylammonium salt (3 Ci/mmole; 1 mCi/ml) was obtained from Amersham International Ltd. Ouabain octahydrate was purchased from Sigma Ltd.

Animals and tissue preparation. SK&F Wistar rats (bred in our laboratories) were used in these studies and housed at 21 ± 1°, 40–60% relative humidity; and illuminated by fluorescent light (on 06.00 hr and off 18.00 hr GMT). For the *ex vivo* pre-and perinatal studies brains were removed at either P16 and used whole, or at P19 or L1 (postnatally) and divided into cerebellum and forebrain areas. The tissues were

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immediately placed in liquid nitrogen and stored at −70°. The tissue was thawed, weighed and homogenized (approx 4% w/v) in 20 mM Tris–Cl buffer (pH 7.2: 0–4° containing 5 mM $MgCl_2 . 6H_2O$), bath sonicated (30 sec) and centrifuged at 100,000 g for 45 min (5°) to give a crude membrane pellet. This pellet was then resuspended in buffer by homogenization and portions assayed for enzyme activity as described below.

Na^+,K^+-ATPase assay. A sensitive radiometric assay utilising [^{32}P]ATP was used to measure the two ouabain-sensitive components of Na^+,K^+-ATPase activity as described by Atterwill *et al.* [7]. Portions of the tissue suspension were measured for ATPase activity in the presence of different ouabain concentrations (OM, 5×10^{-3} M and 5×10^{-6} M) as described by Marks and Seeds [10] and Atterwill *et al.* [9]. This allows a differential assay of the activity of the two molecular forms of Na^+,K^+-ATPase assuming non-competitive inhibition by ouabain and that the ouabain K_i values for the two molecular forms do not change developmentally (See Refs 3 and 7; and confirmed Fig. 1). Data analysis was performed as described in the above publications.

Samples from tissue suspensions (and from cell culture preparations) were analysed in duplicate for protein by the method of Lowry *et al.* [11].

Reaggregate cell culture. Cultures from foetal rat brain at 16 days of gestation (P16) were grown according to the method of Honegger and Lenoir [12] and as described by Atterwill *et al.* [9]. Brains were dissected aseptically in sterile ice-cold isotonic Hanks D2 solution and washed extensively in Hanks DI solution (Ca^{2+} and Mg^{2+}-free). The tissue was then

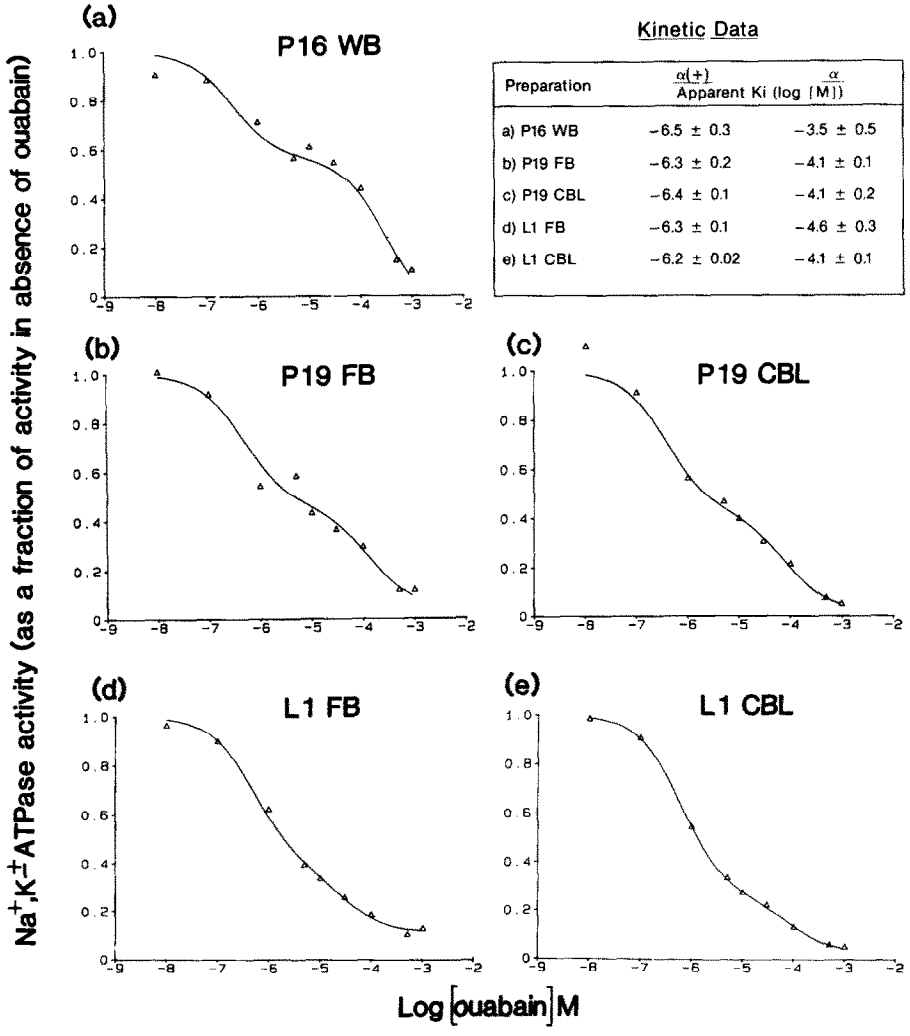

Fig. 1. Inhibition of Na^+,K^+-ATPase by ouabain in rat brain preparations at different developmental stages (P = Prenatal; L = Postnatal; WB = Whole Brain, FB = Forebrain; CBL = cerebellum). Enzyme activity was measured in the presence of various concentrations of ouabain as described in text and is expressed as a fraction of activity measured in the absence of ouabain after subtraction of ouabain-resistant (Mg^{2+}-ATPase; activity measured at 5 mM ouabain. Lines of best fit were calculated as described in [3] on the basis of a two-component model. Each mean curve is derived from 3-4 separate experiments. Numerical estimates of kinetic binding parameters are given in the inset.

extruded through a nylon gauze bag (Nitex-pore size 210 μm). The tissue "suspension" was then filtered through a smaller pore-size Nitex sheet (130 μm) and centrifuged at 1000 *g* for 5 min (5°).

Cells were then triturated (ten passes – pasteur pipette), recentrifuged, and this whole procedure repeated twice. The final cell pellet was then resuspended in either Dulbecco's modified Eagles medium (DMEM) containing 10% FCS (S+medium) and 2 mM glutamine + gentamicin, or in a chemically-defined, serum-free (S−) medium comprising 3 parts DMEM + 1 part Ham's F12 medium containing the N2 supplement of Bottenstein and Gordon [13].

Cell suspensions (10^7 cells/ml, 3.5 ml) were cultured in 25 ml Delong flasks (Bellco) and incubated in humidified air/9% CO_2 at 37° with orbital rotation at 70 rpm (Luckham Rotatest Shaker) for 3 days. The cells were then transferred to 50 ml Delong flasks 5 ml of fresh S+ or S− medium added, and culture continued for a total of 14 days *in vitro* (14 DIV). Five millimetres of the medium was exchanged every 48 hr.

Reaggregates were harvested by sedimentation through phosphate-buffered saline (×3) and the final cell pellet treated as described for tissue above to give a crude reaggregate membrane pellet. Portions of this (20–50 μg protein) were assayed for Na^+,K^+-ATPase activity as described.

For morphological and immunocytochemical characterization of the reaggregate cultures, see Ref. 9. One notable difference between aggregates grown in S+ and S− media was the absence of the single cell layer around the outside of aggregates grown in S− medium which is present in those supplemented with serum. When aggregates were immuno stained for glial fibrillary acidic protein (GFAP) this layer was found to comprise astrocytes – a "glia-limitans" (see [14]).

RESULTS

Analysis of different Na^+,K^+-*ATPase molecular form activities in pre- and perinatal rat brain*

Distinct developmental changes occurred in the two Na^+, K^+-ATPase isoenzymes in both rat brain regions in terms of both specific and the proportional activity of the two isoenzymes.

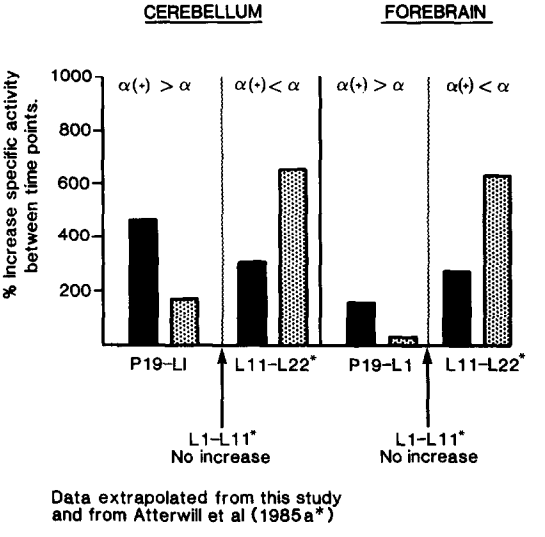

Fig. 2. Rate of development of Na^+,K^+-ATPase isozymes in rat brain regions peri and postnatally.

In P16 whole brain the α form activity (low ouabain affinity) was predominant (60% of total activity) and specific activities for both isoenzymes were low (Table 1). At P14.5 this was even more apparent (α = 73% total activity). Perinatally at P19 there were approximately equal proportions of the two forms in both brain regions (Table 1), whereas at Ll postnatally the α(+) form (high ouabain affinity) became predominant mirroring the profile in adult rat brain [3]. No differences in proportional activities were seen between cerebellum and forebrain at either P19 or Ll.

Experiments were also performed to examine the full ouabain-inhibition profile of Na^+,K^+-ATPase activity at the different stages using a ouabain concentration range of 10^{-8} to 5×10^{-3} M. It can be seen from Fig. 1 that in agreement with the above data Ll and P19 tissue preparations were more sensitive to ouabain inhibition than whole-brain at P16 which

Table 1. Developmental profile of Na^+,K^+ATPase isoenzyme activities in rat brain

			Total activities (μmol/hr/mg protein)						
			α(+)		α		OI		α(+):α ratio
Age	Brain region	N	Activity	%*	Activity	%*	Activity	%Δ	
P14.5	Whole brain	6	0.068 ± 0.02	27.4	0.180 ± 0.02	72.6	0.996 ± 0.04	80.1	0.378
P16	Whole brain	3	0.422 ± 0.02	42.8	0.565 ± 0.08	57.2	2.661 ± 0.35	72.9	0.747
P19	Cerebellum	3	0.453 ± 0.03	48.6	0.479 ± 0.09	51.4	1.408 ± 0.35	60.2	0.945
P19	Forebrain	3	0.775 ± 0.305	48.8	0.814 ± 0.22	51.2	2.230 ± 0.85	59.4	0.952
Ll	Cerebellum	4	2.556 ± 0.84	66.7	1.278 ± 0.4	33.3	2.478 ± 0.29	39.3	2.000
Ll	Forebrain	6	1.989 ± 0.42	64.6	1.088 ± 0.08	35.4	3.119 ± 0.18	50.3	1.828

* % Na^+,K^+-ATPase activity.
Δ% Total ATPase activity.
Activity expressed as x ± SEM.

Table 2. Na^+,K^+ isoenzyme activities in serum supplemented (S+) and serum free (S−) cultured whole brain reaggregates

		Total activities (μmol/hr/mg/protein)						
		α(+)		α		OI		α(+):α ratio
Media	N	Activity	%*	Activity	%*	Activity	%Δ	
S+	5	3.24 ± 0.39	62.8	1.92 ± 0.18	37.2	8.07 ± 1.67	61.0	1.69
S−	6	†1.59 ± 0.35	77.6	‡0.46 ± 0.05	22.4	‡2.81 ± 0.25	57.8	3.46

* % Na^+,K^+ATPase activity.
Δ % Total ATPase activity.
† *t*-test significant at 5% level.
‡ *t*-test significant at 1% level.
Activity expressed as x ± SEM.

tended towards a profile similar to that seen in cultured astrocytes and kidney [3]. Apparent K_i values for ouabain (Fig. 1) for the two isoenzymes did not vary during development and were similar to those obtained previously for late postnatal brain tissue [3].

In terms of the rate of the peri- and postnatal development of the specific activities of the two isoenzymes the perinatal rate of α(+) development was greater than that of α in both brain regions (see Fig. 2). Comparing this data with that obtained by Atterwill *et al.* [7] using Porton rats there appears to be a "latent" period immediately postnatally (see Fig. 2) and then the development of the α form appears to accelerate during the "critical growth period" (L11–L22).

These data also show that the proportion of ouabain-insensitive ATPase activity (OI = Mg^{2+}-ATPase activity) is much higher than Na^+,K^+-ATPase in more immature tissues (Table 1) and this proportion becomes smaller as the brain develops.

Comparison of Na^+,K^+*-ATPase molecular activities in serum-supplemented (S+) and serum-free (S−) cultured whole brain reaggregates (14 DIV).*

Reaggregate cultures were grown from P16 whole rat brain for 14 DIV in either a serum-supplemented medium (10% FCS:–S+) or a chemically-defined serum-free (S−) medium. The ratio of α+/α activities is *in vivo* at P16 is 0.75 (see Table 1 – starting activities approximately 0.4–0.6 μmols/hr/mg protein). In S+medium 14 DIV reaggregates displayed adult proportions of the two isoenzyme activities (Table 2) with α(+) activity developing rapidly in culture (as *in vivo*) to give an α(+)/α ratio of 1.69. The specific activities of the two forms were approximately equivalent to *in vivo* brains values by 14 DIV.

In contrast to serum-supplemented cultures, the development of both Na^+,K^+-ATPase forms was retarded by the absence of serum factors, but compared to α(+) the α form activity (low ouabain affinity) was retarded much more.

DISCUSSION

The two new findings from this study are that, firstly, no perinatal differences in either the proportions or rates of development of the two Na^+,K^+-ATPase isoenzymes were found in rat cerebellum and forebrain to account for the previously demonstrated differential sensitivity of the α isoenzyme (low ouabain affinity) to neonatally induced hypothyroidism [7]. Secondly, it was found that serum-deprivation of reaggregated whole brain cells compared to those supplemented with serum factors causes a similarly retarded development of the Na^+,K^+-ATPase isoenzyme *in vitro*.

Our data agree with previous studies where it has been shown that prenatally until around 14 days of gestation in the rat the α form of Na^+,K^+-ATPase predominates [5, 8]. It has been suggested that the early prenatal α form is probably neuronal since few glia are present and both immature and mature neurones can express this isoenzyme [3, 8] even though astroglia and oligodendrocytes appear to express predominantly the α form throughout the CNS. It was found previously that neonatal thyroid hormone deficiency may lead to a retarded development of a specific cerebellar cell population containing predominantly this Na^+,K^+-ATPase isoenzyme with low ouabain affinity [7]. This cell population may have been either astrocytes, oligodendrocytes or a specific neuronal type. In this context, oligodendrocytes and certain neurones have been shown to be independently regulated by T_3 *in vitro* whereas cultured astrocytes are insensitive to the hormone [9, 14].

Interestingly, certain correlations were found with respect to the Na^+,K^+-ATPase isoenzymes between the *in vivo* findings from hypothyroidism and serum-deprivation of whole brain reaggregate cultures where the development of the α form was selectively retarded in both cases. However, in contrast to the cerebellum *in vivo* this was the Na^+,K^+-ATPase form present predominantly in the rat brain at P16 from which the serum-deprived reaggregates were cultivated and may thus have predisposed this form to serum-factor deficiency, T_3 may have been this crucial missing factor essential for the isoenzyme development, since its replacement in serum-deprived cells restored total Na^+,K^+-ATPase activity [9].

Morphologically, preliminary experiments have indicated that serum-deprivation also leads to the

absence of the "glia limitans" in whole-brain reaggregates [7]. It is equally possible, therefore, that this also contributed to the observed reduction of α isoenzyme activity.

As well as controlling the development of Na^+,K^+-ATPase activity in cultured neural cells acute addition of serum factors other than T_3 increase Na^+,K^+-ATPase activity in a variety of cell types (including neural cells) due to activation of the Na^+/H^+ antiporter (see Refs 15 and 16). It would be interesting, therefore, to see if a specific isoenzyme was acutely and selectively activated in reaggregate cultures under such conditions since it has recently been demonstrated that the two isoenzymes can show differences in cooperativity towards Na^+ [1] or Na^+-dependent activation of their phosphorylation.

It is still impossible, from the present data, to link the developing Na^+,K^+-ATPase isoenzymes with specific neural cell types in rat brain. It is possible that T_3 is in fact controlling the α isoenzyme in all neural cell types. Useful additional information on this front may come from the use of specific neurotoxins *in vitro*. Preliminary data from our laboratory have in fact shown that exposure of reaggregates to low concentrations of ECMA (ethylcholine mustard aziridium ion: a cholinergic neurotoxin *in vivo* [17, 18] can produce specific loss of cholinergic function *in vitro* [18, 19] together with a preferential loss of α isoenzyme activity (60% reduction versus 30% reduction in $\alpha(+)$ activity: Atterwill *et al.*, unpublished data). Coincidentally, serum-deprivation itself also leads to marked reductions in cholinergic activity in the rat brain reaggregate cultures [20].

We can conclude from the present work, therefore, that the predominant effect of *in vivo* hypothyroidism on the cerebellar but not the forebrain α isoenzyme is not due to regional, perinatal differences in the proportions of the two forms of Na^+,K^+-ATPase. Furthermore, the comparable *in vitro* deficit of the α isoenzyme of Na^+,K^+-ATPase produced by serum deprivation of brain reaggregate cultures *in vitro* provides a useful model with which to study direct drug and hormone effects on the specific isoenzymes in culture. Until now the only models available were those where the $\alpha(+)$ isoenzyme Na^+,K^+-ATPase is specifically inhibited using chemical agents.

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