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Calbindin D-28k and parvalbumin expression in mitotic cells of rat primary cortical cultures

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CALBINDIN D-28k and parvalbumin expression was studied immunocytochemically in monolayer mixed cortical cultures from rats aged 16 (E16), 17 (E17), and 19 (E19) days of gestation using monoclonal antibodies and the avidin–biotin immunoperoxidase method. Whereas *in vivo* both calcium-binding proteins are exclusively expressed in postmitotic neuronal populations, they were demonstrated in mitotic cultured cells at the three fetal ages and the three maturation times considered (6, 12, and 15 days). A subpopulation of immunostained mitotic cells was identified as astroglial cells after a double labelling study using an antibody against glial fibrillary acidic protein. These results suggest that conditions *in vitro* may induce the expression of calbindin D-28k and parvalbumin in mitotic cells.

Key words: Calcium-binding proteins; *In vitro*; Mitosis

Introduction

Calbindin D-28k (CaBP D–28k) and parvalbumin (PV) are two calcium-binding proteins which belong to the 'EF-hand' family.[1] The physiological function of these calcium-binding proteins remains unknown, although it has been postulated that they constitute an intracellular buffering mechanism to adjust calcium physiological levels.[1,2] CaBP D-28k and PV are present, among other locations, in specific neuronal populations widely distributed throughout the brain. In *in vivo* conditions, both proteins are used as specific neuronal markers.[2–5]

During brain development, CaBP D-28k and PV are expressed at very different stages and only in postmitotic populations. CaBP D-28k appears in a given brain region almost invariably 1–2 days after the last mitosis, when neuronal migration and the extension of neuronal processes begin.[6] Thus, it has been postulated that CaBP D-28k is involved in the development of calcium-dependent processes during neuronal migration.[6] PV, by contrast, is expressed when morphogenetic migration is concluded, and neuronal differentiation is complete: 5–10 days after the last mitosis in rat cerebral cortex and hippocampus.[7]

It has been reported that CaBP D-28k and PV show a relatively common expression pattern *in vivo* and *in vitro*, including the exclusive expression of both proteins in neuronal populations.[8–13] We studied the presence of these two calcium-binding proteins in rat mixed nervous cultures, and found expression of both CaBP D-28k and PV in cultured mitotic cells.

Materials and Methods

Primary dispersed cell cultures of fetal rat cerebral cortex were prepared according to previous reports.[14,15] Timed pregnant Wistar rats were raised in our laboratory. On days 16, 17, and 19 of fetal life, embryos were removed from the ketamine-anesthetized (Ketolar, 50 mg kg^{-1} body weight) mothers. Cortical regions of the fetal brain were dissected out in sterile 0.1 M phosphate buffer, pH 7.3 (PB). After rinsing three times with Hank's balanced salt solution, the tissues were mechanically dispersed. The cellular dispersion was resuspended before plating in 10% fetal calf serum (Gibco), 5% horse serum (Gibco), 2% L-glutamine (Gibco) and 2% penicillin/streptomycin (Lab-Clinics) supplemented MEM (Flow). Then, 1.5 ml of the cell suspension were plated in 35-mm plastic Petri dishes at a final concentration of 5×10^6 cells per dish.

When the maturation time was complete, the medium was discarded and the culture dishes were carefully washed for 1 min in PB. Cells were then fixed for 30 min in a solution containing 4% paraformaldehyde and 15% saturated picric acid in PB. After fixation, cultures were washed with PB (3 × 5 min). Endogenous peroxidase activity was eliminated by incubating the cultures in a solution of 3% hydrogen peroxide in methanol. Plates were then processed for CaBP D-28k- or PV-immunocytochemistry. The dishes were sequentially incubated in: (a) 0.1% Triton X-100 in PB (2 h at 4°C); (b) primary antibody (anti-CaBP D-28k or anti-PV) diluted 1:2000 in PB (48 h at 4°C); (c) biotinylated anti-mouse immunoglobulin

(Vector Labs, Burlingame, CA) diluted 1:200 in PB (1 h at room temperature); and (d) avidin–peroxidase complex (Vector Labs) diluted 1:250 in PB (90 min at room temperature). Peroxidase was visualized with 0.05% 3,3′-diaminobenzidine (Sigma) and 0.003% hydrogen peroxide in 0.2 M Tris-HCl buffer, pH 7.6.

Five plates from each group (E16, E17, E19; 6, 12 and 15 maturation days) were double-immunolabelled to study the presence of CaBP D-28k-PV colocalization. Other six plates from each group were double-immunolabelled for CaBP D-28k and glial fibrillary acidic protein (GFAP) (three plates from each group) and PV and GFAP (three plates from each group) in order to determine the possible expression of CaBP D-28k or PV in cultured astroglial cells. For the CaBP D-28k–PV double-immunolabelling, the plates were incubated after PV-immunocytochemistry in: (a) polyclonal anti-CaBP D-28k serum diluted 1:2000 in PB (48 h at 4°C); and (b) Texas Red-labelled anti-rabbit serum diluted 1:50 in PB (1 h at room temperature). For the CaBP D-28k-GFAP and PV-GFAP double-immunolabelling, the plates were incubated after CaBP D-28k- or PV-immunocytochemistry in: (a) polyclonal anti-GFAP serum diluted 1:1000 in PB (48 h at 4°C); and (b) Texas Red-labelled anti-rabbit serum diluted 1:50 in PB (1 h at room temperature). After each step, the dishes were carefully washed in PB. Finally, the cultures were counterstained with Mayer's haematoxylin for 20 s, washed in PB (3 × 5 min) and mounted in Aquatex (Merck).

The primary antibodies used have been fully characterized (anti-PV,[16] monoclonal anti-CaBP D-28k,[17] polyclonal anti-CaBP D-28k,[18] anti-GFAP[19]) and widely used in both *in vivo* and *in vitro* studies.[2,5,9,13] The specificity of the immunostaining was controlled by omitting the first or second antibody in the incubation procedure. No residual immunoreactivity was found.

From each group (E16, E17, E19; 6, 12 and 15 maturation days), five culture dishes were processed and analysed for CaBP D-28k- and five for PV-immunocytochemistry. In each dish and for each protein, 100 randomly selected mitoses were typified as CaBP D-28k- or PV-labelled or unlabelled. The number of total stained cells was counted in 40 randomly selected fields (41.400 μm^2 each) per dish. Percentages of labelled mitotic cells to total mitoses were calculated for both calcium-binding proteins in each dish. The mean and s.e.m. for each group were calculated using the corrected average for every individual dish. The results were statistically analysed using ANOVA. Values of $p < 0.01$ for Fisher PLSD and Scheffé-F tests jointly were considered statistically significant.

Results

Cells attached to the surface of the culture dishes within a few hours. The number of cells per dish increased in the cultures from 6 to 12 days and decreased from 12 to 15 days. This culture evolution happened simultaneously with a gradual development of the monolayer: after 6 days cells formed a continuous monolayer with a few cell clusters. After 2 weeks, the monolayer became discontinuous and the number of cell aggregates increased.

At each of the three fetal ages (E16, E17 and E19) and throughout all maturation times (6, 12 and 15 days), CaBP D-28k- and PV-immunoreactivity appeared in only a few cells, scattered in the monolayer. In contrast to *in vivo* conditions, subpopulations of the CaBP D-28k- and PV-immunoreactive cultured cells were GFAP-immunopositive. In addition, the CaBP D-28k–PV double-labelling study demonstrated the presence of a few cultured cells showing coexpression of CaBP D-28k and PV, in agreement with the quantitative observations (Table 1).

Some of these CaBP D-28k- or PV-immunolabelled cells were mitotic cells (Fig. 1 a,c,e,f). Mitotic cells were observed in all dishes, at all maturation times and for all fetal ages. The number of dividing cells was low in relation to the total number of cells (< 1%) and they appeared scattered in the monolayer without any particular distribution. Approximately half (31–68%) of the mitotic cells were labelled for either CaBP D-28k or PV (Table 1). Immunostained mitotic cells appeared in all mitotic phases. Comparing CaBP D-28k- and PV-immunopositive mitotic cells, the percentage of labelled to total mitoses was higher for PV than for CaBP D-28k at each fetal age (E16, E17 and E19) and at all maturation times (6, 12 and 15 days), and the differences were statistically significant ($p < 0.01$) in all cases. Finally, although all double-labelled dishes demonstrated the presence of PV- (Fig. 1 c, d), CaBP D-28k-, and GFAP-single-immunolabelled mitotic cells, colocalization of CaBP D-28k and GFAP (Fig. 1 a,b), PV and GFAP, or CaBP D-28k and PV (Fig. 1 e,f) was also observed in mitotic cells.

The morphology of mitotic cells labelled for either CaBP D-28k or PV was similar. Both CaBP D-28k- and PV-labelled mitotic populations were small (9–12 μm) round cells without visible processes (Fig. 1c), or fusiform cells medium in size (16–19 μm) with one or two processes (Fig. 1 e), or stellate or polygonal cells, medium or large in size (21–24 μm), with short and simple processes (Fig. 1a). Labelled mitotic cells showed diaminobenzidine reaction product uniformly distributed in the cytoplasm, and the chromosomes were not immunolabelled. The staining was generally

Table 1. Percentages (mean ± s.e.m.) of CaBP D-28k- and PV-positive mitotic cells relative to total mitotic cells

	CaBP D-28k			PV		
	E16	E17	E19	E16	E17	E19
6 day	42 ± 4	45 ± 3	32 ± 3	63 ± 4	66 ± 2	50 ± 3
12 day	43 ± 5	47 ± 5	34 ± 4	60 ± 3	68 ± 6	51 ± 3
15 day	35 ± 3	43 ± 4	31 ± 4	60 ± 5	64 ± 2	50 ± 4

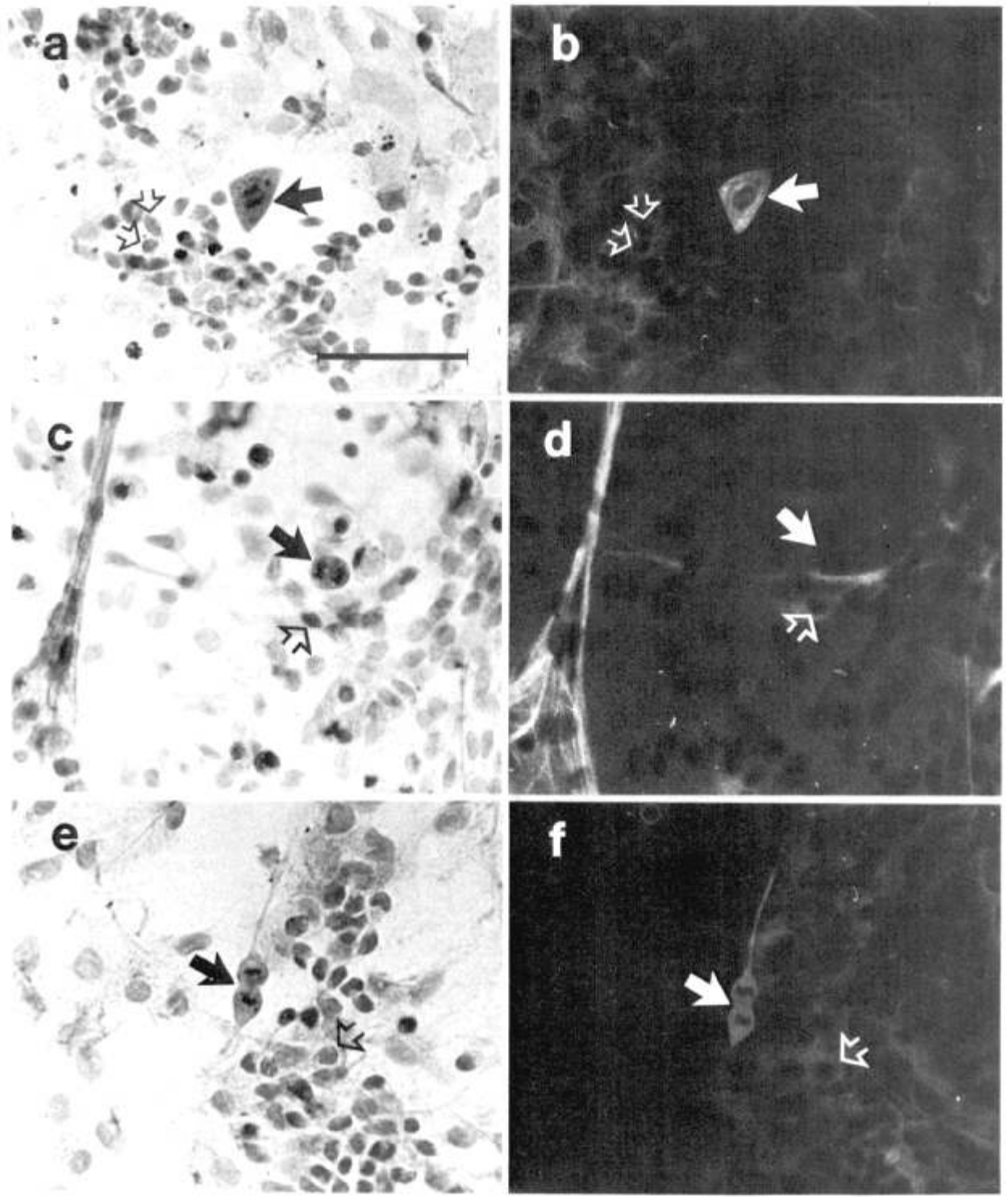

FIG. 1. Mitotic cells in mixed nervous cultures after CaBP D-28k-, PV-, or GFAP-immunostaining. Cell nuclei are counterstained with Mayer's haematoxylin. Left panels show bright-field photomicrographs of mitotic cells labelled with CaBP D-28k (**a**) and PV antibodies (**c** and **e**) using DAB as chromogen. Right panels show the same sections illuminated to reveal immunofluorescence of GFAP (**b** and **d**) and CaBP D-28k (**f**). Some cell nuclei are used as reference in the double immunostaining (open arrows). Scale bar for all figures = 50 μm. (a) CaBP D-28k-immunopositive mitotic cell (solid arrow). (b) Same cell as in (a) showing GFAP-immunostaining (solid arrow). (c) PV-immunoreactive mitotic cell (solid arrow). (d) The same field as in (c) showing that the PV-positive cell is GFAP-immunonegative (solid arrow). (e) PV-immunopositive mitotic cell (solid arrow). (f) Same cell as in (e) showing CaBP D-28k-immunostaining (solid arrow).

limited to the cell body, but some cells demonstrated labelled processes. The immunostaining intensity was variable: some cells were weakly stained whereas others showed strong immunolabelling. The intensity of the immunostaining was independent of the fetal age and the maturation period, and it had no evident relationship to the morphology, size or mitotic stage of the labelled cells.

Analysing each fetal age (E16, E17 and E19), the proportions of CaBP D-28k- or PV-positive to total

mitotic cells were relatively constant (no statistically significant differences; $p > 0.01$) at the three maturation times considered (6, 12 and 15 days) with the exception of CaBP D-28k-immunolabelled cultures from E17, where there was a statistically significant ($p < 0.01$) decrease between 12 and 15 days. Analysing each maturation time an increase in the percentage of positive to total mitotic cells from E16 to E17, and a decrease from E17 to E19 were observed. However, whereas the increase between E16 and E17 was not statistically significant ($p > 0.01$), the decrease from E17 to E19 was significant ($p < 0.01$) at the three maturation periods (Table 1).

Discussion

Mixed nervous cell cultures were used in the present study since the presence of non-neuronal cells in the culture influences its evolution, promotes the maturation of neurones, and provides a more natural environment for the cultured cells.[10,20] Our results on the morphology and evolution of the cultures are coincident with those from previous reports using mixed nervous cultures.[9,10,21]

In vivo, antisera against both calcium-binding proteins labelled neurones exclusively.[2,5] *In vitro*, expression of CaBP D-28k in astroglial cells in neurone-rich cultures was not found previously.[11] Nevertheless, the present data demonstrate that subpopulations of CaBP D-28k- and PV-immunopositive cells were GFAP-immunoreactive. Thus, it can be assumed that both calcium-binding proteins cannot be used in cultures as specific neuronal markers and culture conditions may induce the expression of CaBP D-28k or PV in astroglial cells.

Whereas in the cerebral cortex of the adult rat, CaBP D-28k and PV have a different and mostly complementary distribution,[2] a small cell population in our cultures was positive for both calcium-binding proteins. Although this could be another difference between *in vivo* and *in vitro* conditions, the nature of these cells and their correspondence with the cells in the mature nervous system remain to be elucidated. One possibility would be that these colocalizing cells correspond to astroglial cells or to Cajal-Retzius cells. Cajal-Retzius neurones are transiently present in the cerebral cortex during development. In the monkey, these cells colocalize CaBP D-28k and PV.[22] In rodents, however, Cajal-Retzius cells transiently express CaBP D-28k and constantly express calretinin, but not PV.[23]

CaBP D-28k- and PV-positive mitotic cells were found in all culture dishes studied, whereas *in vivo* both proteins are exclusively expressed in postmitotic cells.[6,7] The total population of labelled cells increased from E16 to E17 at all three maturation times (data not shown), but the percentages of labelled mitotic cells to total mitoses remained constant at both fetal ages for both proteins and at all three maturation times (no statistically significant differences; $p > 0.01$; see Table 1). From E17 to E19, the percentage of labelled mitoses decreased ($p < 0.01$) although the percentage of total labelled cells increased ($p < 0.01$).

The presence of PV in previously negative cells *in vitro*, using a rat cDNA as a nuclear episome, increased the length of G1 and mitosis.[24] These data suggest that portions of the cell cycle are sensitive to calcium-buffering, and this control could be mediated through EF-hand calcium-binding proteins. In addition, it has been demonstrated that other EF-hand calcium-binding proteins such as calretinin and calmodulin play important roles in cell cycle regulation. Calretinin takes part in the dynamic phenomena involved in the separation of the chromosomes,[25] whereas intracellular calmodulin levels determine the G1/S, G2/M and metaphase/anaphase boundary progressions.[24] Nevertheless, a role in the mitotic process has not been previously attributed to CaBP D-28k or PV. The present results indicate that the *in vitro* artificial conditions may induce the expression of CaBP D-28k and PV in mitotic cells.

Conclusion

The presence of mitotic cells, some of them GFAP-immunopositive, expressing CaBP D-28k and PV in mixed nervous cultures is a distinctive difference between *in vivo* and *in vitro* developmental conditions. This *in vitro* expression may indicate the need for a better calcium-buffering system in dividing cultured cells to control calcium intracellular levels and to avoid calcium-mediated neuronal death.

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