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Growth and Secretory Responses of Enriched Populations of Corticotropes*

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ABSTRACT. The purpose of this study was to learn whether enriched populations of corticotropes could be grown without the other pituitary cell types. Corticotrope populations were enriched to 80–90% by counterflow centrifugation in an elutriator with the Sanderson chamber. After initial separation into small, medium, and large fractions, the cells were stimulated for 3 h with 0.5 nM corticotropin-releasing hormone (CRH) and then re-eluted to remove the enlarged corticotropes. More ACTH (6- to 10-fold) was released in media with 10% fetal bovine serum (FBS) than was released in media with no serum. The effects of FBS could not be mimicked by 0.3% BSA. Corticotropes grew in serum-containing media as long as they were plated at a density of at least 2500 cells per well. The corticotropes expanded in size and assumed two major morphological subtypes. Both stored ACTH and β-endorphin. One subtype was flattened and pleomorphic. The other subtype was stellate with multiple processes. Cell counts showed a 2.5- to 3.8-fold increase in the number of labeled corticotropes during the first 21 days of culture. Then the numbers of cells declined rapidly. Basal secretion of ACTH rose 1.6-fold during the first week, plateaued after 14 days and then declined to <30% of first week levels. CRH stimulation produced dose-dependent increases in media ACTH. In 7 day cultures, both basal and stimulated levels of ACTH were similar to those in 7 day cultures of mixed pituitary cells (containing equivalent numbers of corticotropes). Stimulatory effects of CRH were evident for up to 42 days of culture. Arginine vasopressin enhanced CRH-mediated secretion in most cultures in the first week. Pretreatment with glucocorticoids (100 nM corticosterone) for 15 h blocked CRH-mediated secretion in all cultures. The studies showed that corticotropes do not need the other pituitary cell types for basic plating and basal and CRH-mediated secretory responses. Further tests of specific growth factors are needed to learn whether they will maintain function for longer periods. (*Endocrinology* **125:** 2540–2549, 1989).

IMPORTANT developments in our understanding of corticotrope function and growth have come from *in vitro* studies (1–11). The early studies showed that corticotropes could be detected in a mixed population for several weeks in culture (3, 6, 7). Furthermore, the cells secreted opiocortin peptides in response to secretagogues (1, 2, 6–13). Recent studies by May and Eipper (9) and Wand and Eipper (10) maintained corticotrope function for 21 days. They used serum-free medium supplemented with hormones and BSA.

All of these studies employed corticotropes in a mixed culture. However, corticotropes represent only 7–10% of all pituitary cells. Therefore, mixed cultures are not suitable for electrophysiological or biochemical studies of second messenger functions or ion channel activity (13, 14). One goal in this field has been to enrich or purify corticotropes.

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Recent studies in this laboratory have enriched this cell-type to 80–98% by centrifugal elutriation (15). The corticotropes are first separated into small, medium, and large fractions. Then, they are stimulated to enlarge by corticotropin releasing hormone (CRH) and re-eluted. The CRH-responsive corticotropes are thus separated into fractions enriched 8- to 9-fold.

There are no published studies of growth and function of enriched corticotropes. In fact, it is not known whether corticotropes can be cultured apart from other pituitary cell types. The cells may require factors from other pituitary cells to grow and function. Therefore, recent studies in this laboratory focused on their growth in culture. Storage and secretory responses were also tested. This report shows that corticotrope growth in isolation is possible if there is an adequate cell density in the monolayer. The report also describes responses to secretagogues, corticosterone, and morphological changes with time in culture.

Materials and Methods

Preparation of corticotrope-enriched fractions

Pituitary cells were separated by counterflow centrifugation as described in the previous study (15). Pituitaries from 9

groups of 6–8 male Sprague-Dawley rats (Timco, Houston, TX) were dispersed on the day of the experiment (15). The rats had been acclimated for 10 days. All experiments were done with pituitaries taken at 0730–0830 h. The dispersion produced 18–32 million cells (depending on the size of the rats).

The freshly dispersed cells were divided into two groups that were eluted identically. This was done to avoid overloading the Sanderson chamber which worked optimally with 10 million cells per load. Each group was loaded at 4 ml/min and then collected in 50 ml fractions at 20 ml/min (fraction 1); 30 ml/min (fraction 2); and 40 ml/ min as the centrifuge was stopped (fraction 3). All elutriations were done at 6–8 C (rotor temperature) with the elutriation buffer held at 8 C. During the loading and collection of fractions 1 and 2, the centrifuge was run at 1914–1924 rpm. Times were recorded during the collection of each fraction to monitor the accuracy of the flow rate. The contents of the chamber were collected and pooled with fraction 3.

After the two identical sets of fractions were obtained, the cells were spun at 900 rpm. Identical fractions were pooled and resuspended in 2 ml complete Eagle's minimal essential medium (MEM). The MEM included sodium bicarbonate, 0.3% BSA, HEPES (2.9 g/500 ml) and 10^{-4} M ascorbic acid. The pH was 7.3. Samples of cells (10 μl) were diluted 1:1 in 0.1% trypan blue and counted in a hemacytometer. Viability was 99–100% and yield was 50–107%. The higher yields were achieved by adequate conditioning of the tubing with Dulbeccos PBS + 1% BSA. The lower temperatures prevented cell clumping and sticking during the elutriation. Adequate alignment and cleaning of critical components in the elutriator rotor also improved cell yield.

After the first elutriation, areas and diameters of cells in each fraction were determined by image analysis (15). The cells were then exposed to 0.5 nM CRH for 3–4 h in a 37 C incubator under O_2, 5% CO_2 conditions. The CRH vehicle was MEM with 0.3%, BSA, 100 kallikrein inhibiting units of aprotinin (a protease ihbibitor), and 10^{-4} M ascorbic acid.

After 3–4 h in CRH, cells in each fraction were checked for viability and changes in area and diameter. Viability was 99–100% after CRH stimulation. A distribution analysis was used to demonstrate changes in cell size in the three fractions (15). When at least 10% of the cells in each fraction had enlarged, the fractions were re-eluted separately. Tests of media and incubation conditions showed no significant enlargement in any of the cell suspensions for up to 15 h unless CRH was added.

The fractions containing enlarged corticotropes were then re-eluted as follows: They were first monodispersed by passage through an 18 gauge needle (15). After loading at 10 ml/min, each fraction was re-eluted at its original flow rate. Then, the enlarged corticotropes were re-eluted in a separate fraction with flow rates 10 ml/min higher than the original flow rate. The contents of the chamber were pooled with each corticotrope-enriched fraction. Tests demonstrated 99–100% viability after re-elutriation. The final yield of corticotropes was 5–10% of the total number of pituitary cells loaded. For example, six to eight male rats produced 12–20 million cells depending on the size of the rat. Therefore, yields of 1–2 million corticotropes were obtained routinely. At least three fourths of the corticotropes originated from the small- and medium-sized fractions (collected at 20–30 ml/min).

After re-elutriation, corticotrope-enriched fractions from original fractions 1 and 2 were pooled to form the small and medium sized subset. This was done because previous studies had shown the two populations were identical in storage characteristics and responses to CRH (15). Corticotropes from fraction 3 were the largest subset. In later experiments all small, medium, and large corticotropes were pooled.

Growth of enriched corticotropes

Corticotropes were plated on glass coverslips (Arthur H. Thomas, Philadelphia, PA) (15) by adding 1,500–12,500 cells to the center in a 15–20 μl drop of MEM + 0.3% BSA. Most (80–90%) settled in 15 min. The cells adhered to the coverslip rapidly (1 h). Then, 300–400 μl MEM + 10% fetal bovine serum (FBS) and 0.3% BSA were added. The cells were grown for 1–42 days in 400–500 μl MEM (containing 0.3% BSA and 10% FBS). Media were changed every 3–4 days.

Tests of FBS, BSA, and CRH

In separate sets of experiments, tests of the requirements for BSA, FBS, and CRH were done. Cells were plated in three types of media: MEM + 0.3% BSA + 10% FBS; MEM + 0.3% BSA; and MEM alone. The MEM in all three groups contained 1–2.5 μg/ml insulin, 10–100 ng/ml transferrin, and 30 nM sodium selenite. In some of the cultures, 0.5 nM CRH was added with each medium change (every 3 days). The media were collected with each change and assayed by RIA for ACTH.

The above experiments established a requirement for BSA and FBS for the growth of enriched corticotropes. However, CRH also clearly enhanced ACTH secretory activity. Parallel electrophysiological studies required that the cells be maintained in a basal state (Silberberg, S., T. Lacerda, G. V. Childs, and A. M. Brown, in progress). Therefore, chronic administration of CRH was not done. Thus, the long-term growth tests included only weekly 4 h exposures to 0.5 nM CRH. Tests of secretory activity reported in this study were done 6–7 days after this 4 h CRH exposure.

Tests of ACTH secretion

Tests of secretory responses were begun after 3 days of culture to allow the cells to flatten and adhere to the coverslip more tightly. Cells were washed in MEM to remove all traces of FBS. Cultures were divided into two groups. One group was pretreated for 15 h with 100 nM corticosterone. The second group was given MEM vehicle only. As in previous reports, the corticosterone stock was 30 mM in 95% ethanol. It was diluted to 100 nM with MEM. The ethanol concentration was minimal. After 15 h at 37 C, the cells were exposed to 500 μl 0.5 nM CRH for 4 h with or without 10 nM arginine vasopressin (AVP). Peptides were diluted in MEM + BSA, aprotinin, and ascorbic acid as described in the previous section on elutriation. Media were collected after the 4 h exposure to the neuropeptides and frozen for ACTH RIA. To test degradation of ACTH by cells or media components, 10 pg to 100 ng/ml ACTH (III World

TABLE 1. Effect of serum and BSA on ACTH secretion from cultures of pooled corticotropes (3 days; 2500 cells per well)

Serum supplements[a]	(pg/ml ACTH/3 days)	
	−CRH	+CRH (0.5 nM)
+ BSA + FBS	804 ± 223	9225 ± 620
+ BSA	299 ± 11	814 ± 82
None	233 ± 5	270 ± 84

Values are averages of three wells ± SE. FBS, fetal bovine serum, 10%.

[a] All media were MEM containing 2.5 μg/ml insulin, 100 ng/ml transferrin; and 30 nM sodium selenite.

Standard) was added to 3 or 7 day cultures of corticotropes for 4 h. Media were removed and assayed by RIA for ACTH.

RIAs for all tests of secretion were done by Dr. T. J. Collins with the ACTH RIA kit generously supplied by the Hormone Distribution Program, NIADDK. Interexperimental and intraexperimental variations were less than 10%. Each group was the average of three wells. Each experiment was repeated three times. Analysis of variance (ANOVA) followed by Duncan's multiple range test was used to test significant differences between means ($P < 0.05$ was considered significant).

Comparative studies of mixed pituitary cultures

Mixed cultures of anterior pituitary cells were also exposed to 0.1–5 nM CRH (diluted in MEM) to compare their responses to those of enriched corticotropes. These mixed cultures were plated to contain an equivalent number of corticotropes. Since corticotropes represent 7–10% of a mixed population (14–17), equivalent cell numbers ranged from 40,000–65,000 cells per well. Previous studies (17) had shown that percentages of corticotropes are not changed in mixed cultures for up to 7 days. Therefore we tested mixed cultures 3 and 7 days after dissociation.

Cytochemical labeling

After all treatments, the enriched corticotropes were fixed and immunolabeled for ACTH and β-endorphin (END) as described previously (15). Antisera dilutions were 1:5,000 anti-β-END and 1:30,000 anti-$^{17-39}$ACTH. Some groups of 7 day cultures were exposed to biotinylated CRH (Bio-CRH) for 5 min and labeled with avidin peroxidase (16, 18). The biotinylated CRH was produced by Drs. J. Morel and G. Aguilera (NIH) and characterized as described in the previous study (18). The biotin was attached to the N-terminal serine. It was equipotent when compared with unlabeled CRH (18).

Controls have been described in previous studies (14–19). Briefly they involve omission of the primary antibody or the biotinylated CRH. They were applied to corticotropes during the first week. Competition of soluble antigen for antibody binding sites (15) or CRH for bio-CRH sites (16, 18) was also done. Controls have also been run with immunoblots testing cross-reactivity of anti-$^{17-39}$ ACTH or anti-β-END with opiocortin antigens (19). These antisera reacted only with their homologous antigens. Anti-ACTH sera did not react with other antigens tested including: 16 K fragment of POMC, β-lipotropin, or met-enkephalin. Anti-β-END showed a weak reaction only with β-lipotropin. Counts of percentages of corticotropes were performed on three wells per experimental group. Each experiment was run at least three times. ANOVA followed by Duncan's multiple range tests determined the significance of differences between control and stimulated populations (5% level; Lionheart Statistics).

Analysis of cell number and surface area

An analysis of cell number and surface area was performed on corticotropes identified by labels for ACTH and β-END.

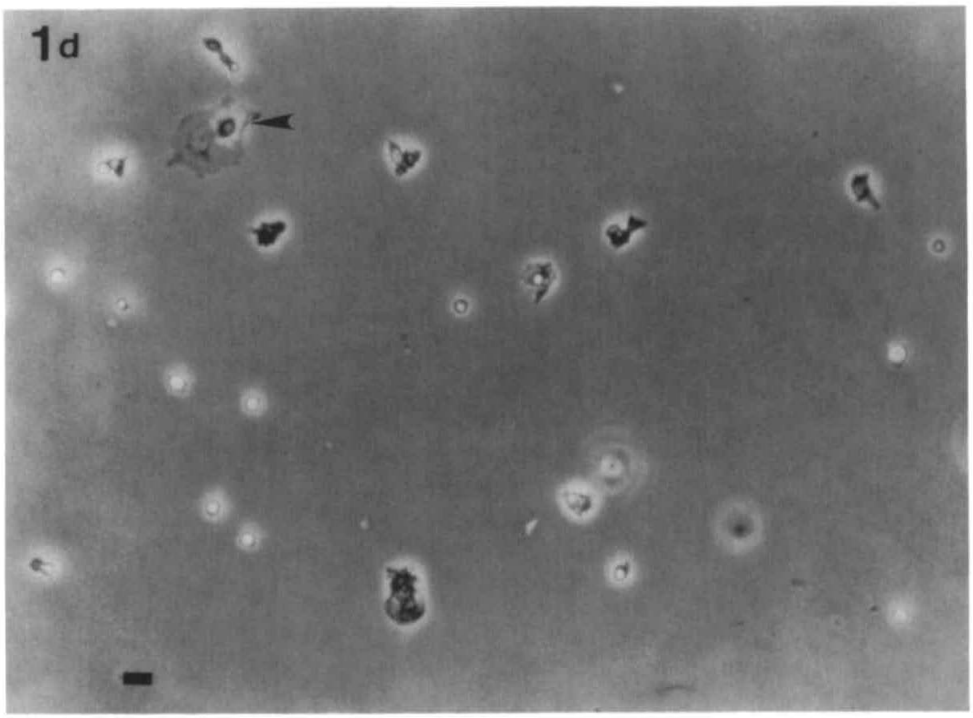


FIG. 1. Phase contrast view of field of corticotropes 1 day after dispersion and elutriation. Cultures were labeled for ACTH and β-END. The phase-bright glow indicates the presence of intense label. Cells that are more diffusely labeled are dark in this micrograph. The enrichment is over 90%. Note that the cells exhibit differences in shape. Most are round or ovoid. Magnification, ×154; *bar*, 20 μm.

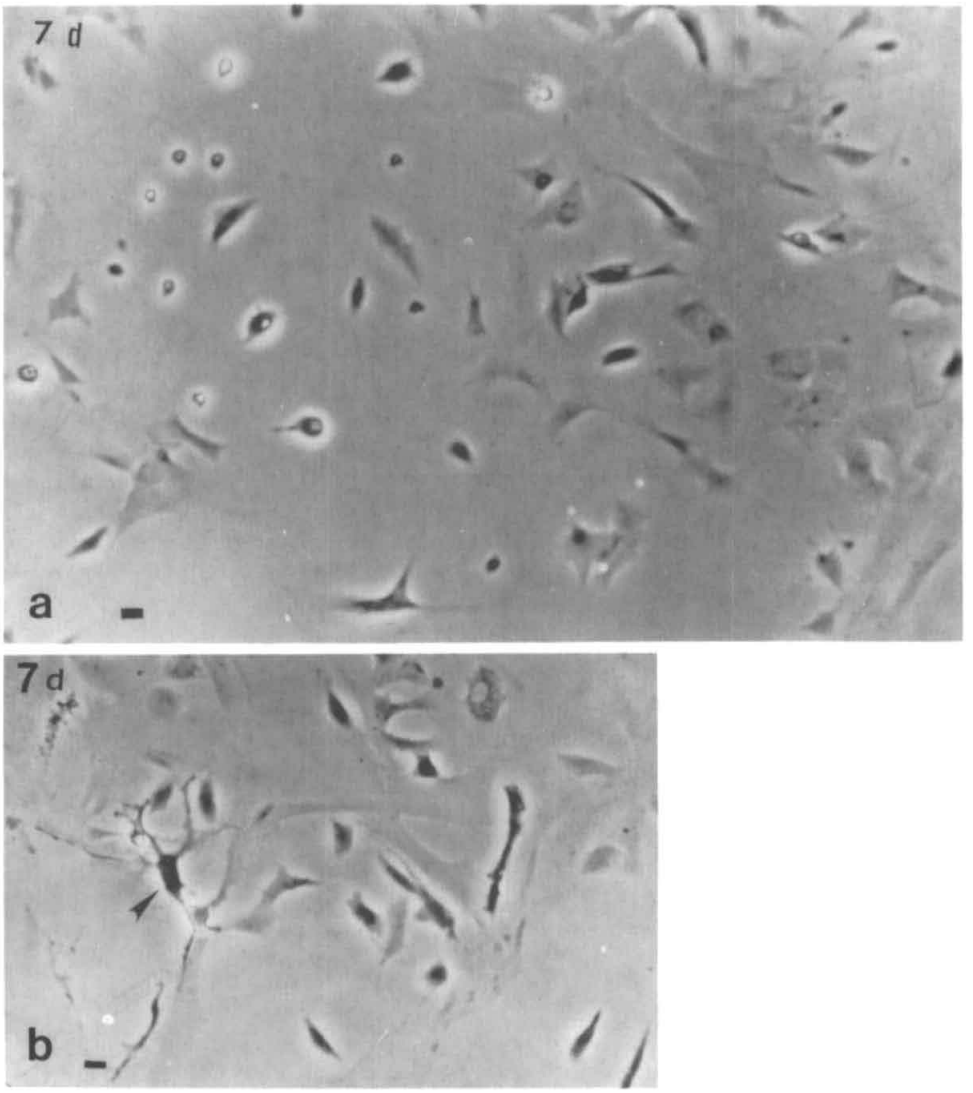


FIG. 2. Phase contrast views of the same culture after 7 days in serum-containing media (10% FBS). Some areas contain scattered cells that are labeled (dark or phase-bright) for both ACTH and β-END. Flattened fibroblasts are seen in some regions. They provide a cellular substrate for the corticotropes. The field in panel b shows a developing stellate subtype (*arrow*) that is extending long slender processes. It is labeled intensely for both hormones. Magnification, × 154; *bar*, 20 μm.

This was done to provide an estimate of morphometric changes in the population. These changes were then compared with changes in basal ACTH secretion in individual populations.

The protocol was similar to that described in a previous publication (16). First, a calibrated grid was placed over the field (Cue 3 image analysis system). The approximate area covered by the entire culture was measured at low magnifications with this grid. The plating density (cells/mm^2) was determined by counting the number of cells in each of six grid spaces and dividing the number by the area of the grid spaces. The six values were then averaged. The cell number was calculated as the average plating density (no. of cells/mm^2) × the total area of the culture.

The cell surface area was determined by counting the number of points in the calibrated grid that fell over a corticotrope. This gave a volume fraction reading which was converted to average cell surface area as in previous reports (16). The formula was: volume fraction × total grid area = cell surface area/grid. Four to six grid spaces were analyzed and then averages were made of data from three wells.

Finally, an estimation of total cell surface area was obtained by first dividing the total area covered by the cells by the grid area. This value was then multiplied by the average cell surface area per grid. These morphometric analyses were performed on three coverslips per culture × three separate populations of corticotropes. ANOVA followed by Duncan's multiple range tests determined significant differences.

Results

Growth characteristics of corticotropes

Separated corticotropes could be grown in an enriched population for 42 days if initial plating densities were at least 2500 cells per well tray. The contaminating fibroblasts provided a cellular substrate for the corticotropes. These results agree with a number of studies that demonstrated the importance of plating density in the survival of other types of primary cultures. Adequate cell numbers promote cell-cell contacts and increase concentrations of growth factors needed for attachment and plating (reviewed in Ref. 20).

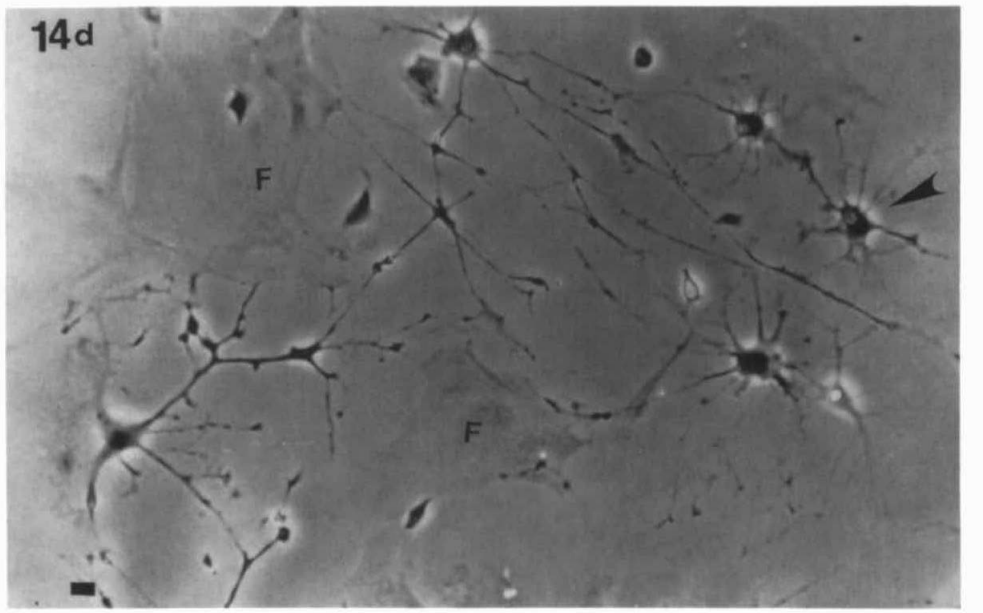


FIG. 3. Phase contrast view of cultured corticotropes after 14 days of culture. Two major morphological subtypes are evident. One is flattened and stores the opiocortin hormones in diffuse patches (F). The other is stellate (*arrowhead*). The region around the nucleus is intensely labeled. Numerous processes extend to nearby cells or interdigitate with one another. Magnification, ×154; *bar*, 20 μm.

When ACTH secretion was tested after growth in the three test media for 3–14 days, MEM with FBS promoted the highest basal secretory activity. MEM with BSA produced a 50% increase in basal secretion of ACTH over that produced by serum- or BSA-deficient MEM. The increment was not as high as that produced by MEM with FBS. An example from a set of 3 day cultures is shown in Table 1.

ACTH release was not increased over basal levels if corticotropes were grown in media containing CRH, but not BSA and FBS. In contrast, there was a 3- to 5-fold increase in ACTH released by parallel cultures grown in media containing CRH and BSA. Even more striking 6- to 10-fold increases were seen in cultures grown in media containing CRH, BSA, and FBS. These differences are shown in the example in Table 1. The same responses were evident in 3, 6, 9, 12, and 14 day cultures. The data indicated that media without FBS were considered too stringent for the initial studies of enriched corticotrope populations. Therefore, the initial studies were done with MEM containing BSA and FBS.

Cell content assays were not done for this pilot study because of the limited number of cells available for assays and immunocytochemistry. Ongoing studies testing the effect of different growth media and substrata on morphology, ACTH content, and ACTH secretion will be reported in a subsequent paper (Childs, G. V., G. Unabia, and J. Bottenstein, studies in progress).

Morphological changes in serum-containing medium

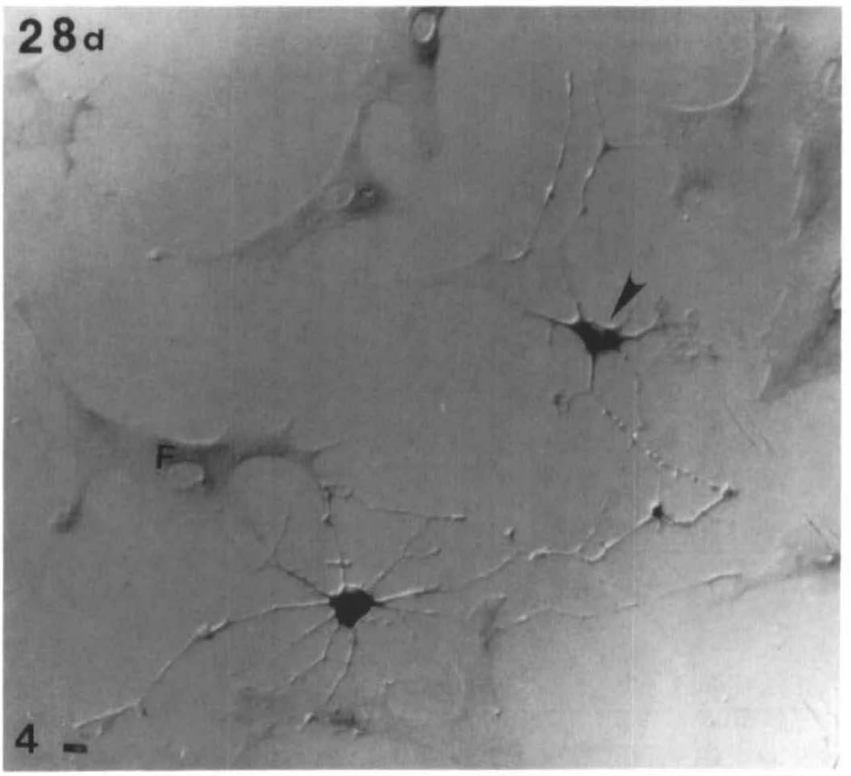
The small-medium or large subtypes did not show obvious differences in morphology with time in culture. Therefore, the following discussion applies to all populations. During the first week of culture, some corticotropes sent small processes to adjacent cells. Others remained small and round or ovoid with one or more blunt processes (Figs. 1 and 2). Double labels for ACTH or END showed that most corticotropes were labeled for both hormones. In 7–14 day cultures, fibroblast outgrowth was evident. The fibroblasts were not prominent in later cultures (21–42 days). Figure 2 illustrates a population after 7 days of culture in which these cell types are represented.

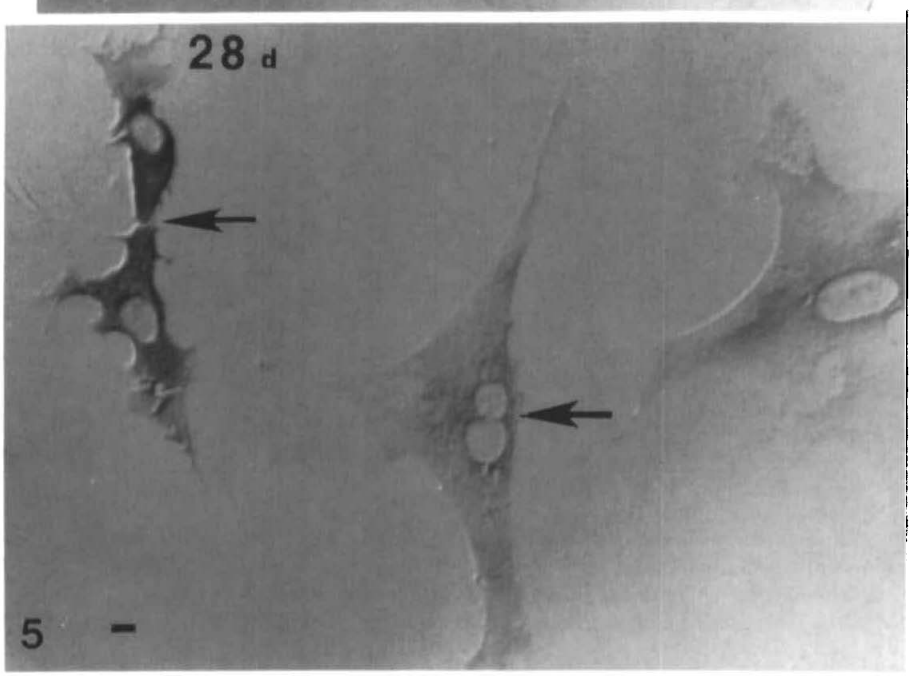
After 1 week of culture, a few cells extended longer processes (Fig. 2b). In 14–28 day cultures, colonies of stellate corticotropes developed (Figs. 3, 4, and 6). They extended beaded processes. Some of the processes showed extensive branching. The β-END label was most intense in the region around the nucleus. Label for ACTH was densely distributed in the same region and also in the cellular processes.

The second cell type evident in 14–35 day cultures was pleomorphic with several blunt processes (Figs. 4 and 5). Such cells were also found in clusters (Fig. 5) or interspersed with the stellate cells (Fig. 4). The label for ACTH and END was a diffuse mixture of gray and amber in the cytoplasm. Occasional flattened cells exhibited signs of mitoses, including cytokinesis and two nuclei (Fig. 5).

Controls to test ACTH degradation

Controls were performed to test degradation of ACTH by 3 or 7 day cultures of corticotropes. RIAs showed that exogenous ACTH survived the 4 h incubation (37 C) in the presence of enriched corticotropes. In 3 day cultures treated with 1, 10, and 100 ng/ml ACTH, 1.2 ± 04 10 ± 2 and 88 ± 18 ng/ml were recovered, respectively (average of 3 wells ± SD). In parallel tests of 7 day cultures, 1.1 ± 0.5, 11.5 ± 2, and 112 ± 12 ng/ml ACTH were recovered 4 h after adding 1, 10, or 100 ng/ml, respectively. As will





FIGS. 4 and 5. Nomarski optics were used to photograph the stellate and flattened subsets seen in the 28 day cultures. Label is very intense in the region around the nucleus in stellate cells (*arrowhead*). The flattened cells contain diffuse patches of label. In Fig. 5, a cluster of flattened corticotropes is evident. Two of the cells may have divided (*arrows*). The cells on the *left* resemble two daughter cells. The cell on the *right* contains two nuclei. The label is a mixture of gray (ACTH) and orange (β-END) and varies in intensity. Magnification, ×154; *bar*, 20 μm.

be shown below, a 4 h test of basal secretion adds 150–200 pg/ml ACTH from the cultures themselves. Tests of degradation also showed that even 100 pg/ml exogenous ACTH could be detected above the normal basal levels.

Basal secretory responses

The first part of this section will show averaged data from three to four experiments. The second part will illustrate secretion from two of the individual cultures to show the variability. Basal levels of ACTH in 3 day cultures of small-medium corticotropes were 147 ± 26 pg/ml (averages ± SE of 3 experiments; 3,500–4,000 cells per well) in 4 h tests. This was identical to basal levels from parallel 3 day cultures of mixed pituitary cells (139 ± 2 pg/ml; 45,000 cells per well). It was also not significantly different from levels secreted by large corticotropes (164 ± 65 pg/ml) (2,500–3,000 cells per well).

During the first 14 days of culture, average basal ACTH secretion by four groups of small-medium corti-

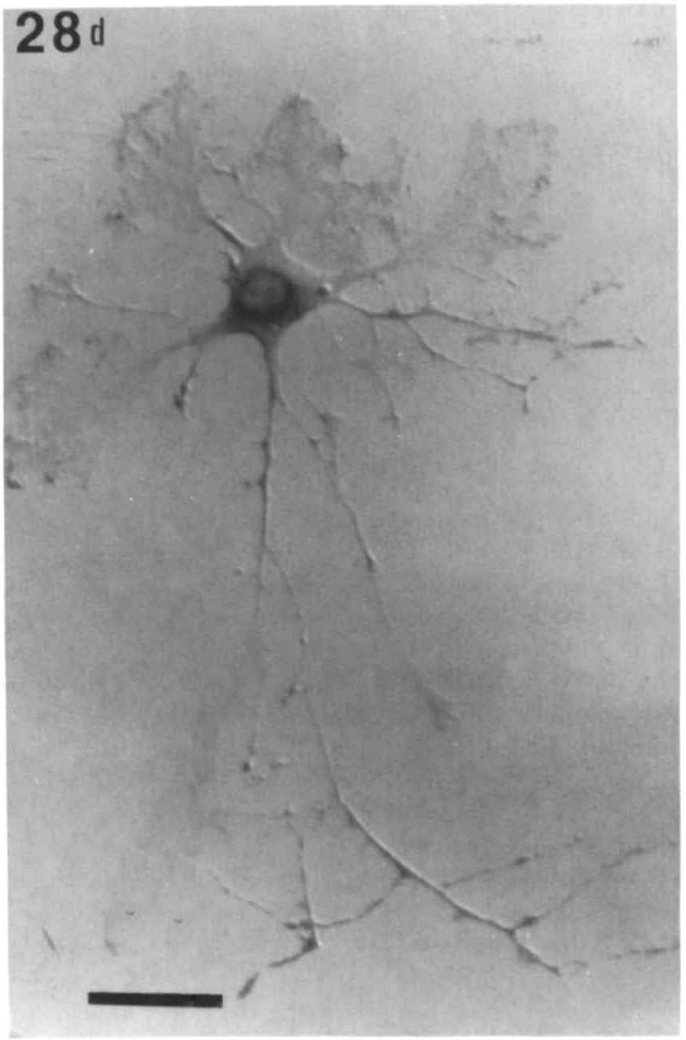


FIG. 6. Higher magnification of a stellate subtype grown for 28 days. The label is a mixture of gray (ACTH) and orange (β-END) in the region around the nucleus. The photograph was taken with Nomarski Optics. Magnification, ×773; *bar*, 20 μm.

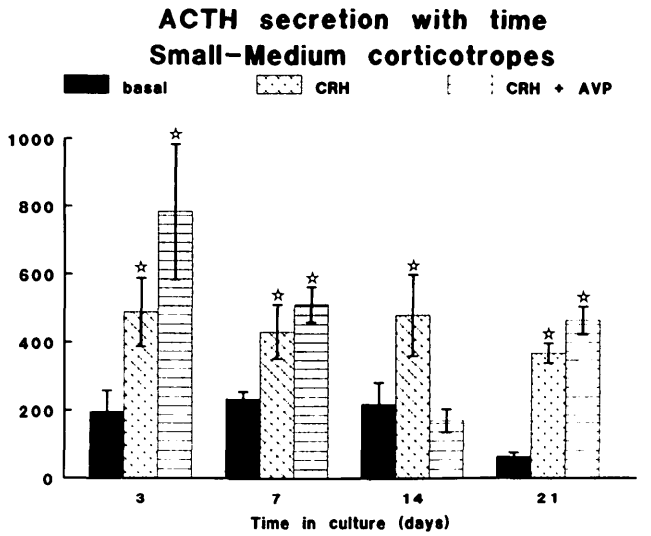


FIG. 7. ACTH secretion from the July 11, 1988 collection of small/medium corticotropes showing maintenance of first week basal levels for 14 days. These corticotropes were plated initially at 3,000–4,000 cells per well and their numbers during the first 3 weeks of culture rose to over 11,000 cells per well (see text). This rise does not correlate with a rise in secretion, however. *, Significantly different from basal. CRH, 0.5 nM; AVP, 10 nM.

TABLE 2. Dose-response tests on enriched small-medium corticotropes

	3 day cultures	14 day cultures
Basal	106 ± 27	40 ± 16
0.1 nM CRH	162 ± 11[a]	230 ± 23[a]
0.5 nM CRH	284 ± 13[a]	364 ± 117[a]
1.0 nM CRH	n.d.	618 ± 108[a]
5.0 nM CRH	697 ± 41[a]	n.d.

Values are average of three samples ± SE; 3,000–4,000 cells per well. n.d., Not done. BSA, bovine serum albumin, 0.3%.

[a] Different from basal levels ($P < 0.01$).

cotropes increased significantly to 231 ± 4 pg/ml (average of four experiments ± SE). There was no increase in secretion in any of the four cultures of large corticotropes beyond that seen at 3 days. There was no further increase in average levels in the 21 day cultures of small-medium or large corticotropes. In 28–42 day cultures, basal ACTH release declined to a range of 61–87 pg/ml.

In 7 day cultures of pooled corticotropes, (4,500 cells per well), average basal secretion was 194 ± 30 (after 4 h). This level was higher than basal levels secreted by equivalent numbers of corticotropes in 7 day mixed pituitary cultures (107 ± 20 or 139 ± 2 pg/ml). It was not different from levels secreted by either small-medium or large subsets. In 14 day cultures, pooled corticotropes secreted higher average basal levels (287 ± 35 pg/ml). These levels were maintained at 325 ± 40 pg/ml at 21 days. Basal ACTH levels then declined to 60 ± 20 pg/ml in 28–35 day cultures.

Thus, there was an average 1.6-fold increase in basal secretion during the first 7–14 days. Did this increase reflect an expansion in cell size or cell numbers? Image analysis was done to correlate changes in ACTH secretion with changes in corticotrope numbers or surface area of labeled corticotropes.

In the population of small-medium corticotropes collected August 17, 1988, there was a 3.3-fold increase in the number of labeled corticotropes per well during the 28 day culture period. Cell numbers then declined to first week levels. Total corticotrope cell surface area also increased 64-fold by 28 days. The secretory data from this population of corticotropes showed similar basal levels when 7 and 28 day cultures were compared. However, CRH stimulation produced a 5-fold enhancement in the 28 day cultures compared to a 2-fold enhancement in the 7 day cultures. This enhancement correlated with the increase in cell numbers and surface area.

Not all cultures showed this correlation, however. Se-

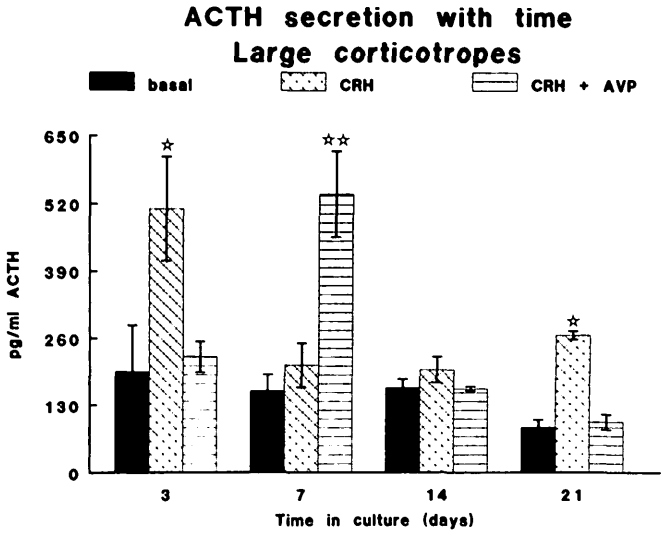


FIG. 8. ACTH secretion from the July 11, 1988 collection of large corticotropes (taken from the same group illustrated in Fig. 7). Basal secretion is maintained for only 14 days after which it declines. Responses to 0.5 nM CRH are lower after 7 days which reflects the lower plating density (2,000 -2,500 cells per well). Only 7 day cultures showed responses to 0.5 nM CRH + 10 nM AVP. *, Significantly different from basal; **, significantly different from basal and CRH-treated.

cretory responses of another group of small-medium cultures collected July 11, 1988 are illustrated in Fig. 7. There was a 3.8-fold increase in corticotrope numbers when 14–21 day cultures were compared with 3 day cultures. An initial 2-fold increase was seen during the first week of culture. Total cell surface area increased only 5.9-fold from 3–7 days and 10-fold by 14–21 days. These cultures maintain basal levels of ACTH for 14 days. However, CRH-stimulated levels are not higher than those of 3 day cultures. There is also a gradual decline in basal ACTH secretion after 14 days in spite of the maintenance of high cell numbers and surface area in the 21 day cultures (Fig. 7).

Stimulated secretory responses

Even the oldest cultures responded to CRH. Table 2 shows representative dose-response tests in 3 and 14 day cultures of small-medium corticotropes (3000–4500 cells per well). In both cultures, there is a progressive increase in ACTH levels to nearly 7× basal levels in the presence of 1–5.0 nM CRH. ED_{50} values were not obtained because of the limited numbers of wells available for these experiments. The dose range did not include a plateau point.

The averaged results of weekly tests of CRH-mediated secretion showed a 2.6 ± 0.5 fold increase in ACTH release from 7 day small-medium corticotropes (average ± SE of 4 experiments). If 10 nM AVP was added with the CRH, the average increment in ACTH release was 4.3 ± 1 fold basal values. This was significantly higher than the increase produced by CRH alone. Figure 7 illustrates data from a representative culture.

There was variability in the responses of large corticotropes. When the results from all four experiments were averaged, the increment in ACTH secretion after CRH exposure was 4.2 ± 2 fold basal. AVP stimulated an increase of 8.6 ± 4 fold basal ACTH release (average ±SE). This increment was not significantly larger than that of the small corticotropes because of the interexperiment variation. A representative experiment is illustrated in Fig. 8.

Analysis of data from 21–28 day cultures showed that CRH stimulated an average 4-fold increment in ACTH levels. After 28 days, the increment in CRH-mediated secretion averaged 2- to 3-fold. However, basal levels are declining during this same period. Thus, the CRH-mediated release in 28–42 day cultures produced levels that were slightly above basal levels measured at 7 days.

Mixed pituitary cell cultures were also tested after 7 days of culture. Cultures of 45,000 mixed pituitary cells (estimated 4,500 corticotropes) secreted 139 ± 2 pg/ml ACTH basally, and 1,417 ± 107 pg/ml and 2,122 ± 73 pg/ml ACTH after 0.5 and 5 nM CRH, respectively. These levels are similar to basal (194 ± 30 pg/ml) and 0.5 nM CRH-stimulated levels (1,615 ± 208 pg/ml) levels secreted by parallel cultures of 7 day enriched pooled corticotropes.

Loss of responses to AVP

In 90% of 3–7 day cultures, AVP enhanced CRH-mediated responses. However, enhanced responses were variable at 14 days and absent by 21 days. Furthermore, in 10% of all tests, the combination of CRH and AVP did not augment ACTH release above basal levels. This contrasted with the augmentation seen with CRH alone [see Fig. 7 (14 days) and Fig. 8 (3 days) for examples].

Effect of corticosterone inhibition on basal and stimulated ACTH release

Pretreatment with 100 nM corticosterone for 15 h significantly inhibited secretion stimulated by CRH and AVP in all cultures tested (7–35 days). ACTH levels in corticosterone-pretreated cultures were only slightly above, or not different from levels in cultures treated with vehicle only.

Discussion

This study of enriched corticotropes was begun initially to learn whether the ACTH cells would survive and secrete without other pituitary cell types. The initial experiments showed that survival in relative isolation was possible. To our knowledge, this is the first report describing primary cultures of enriched corticotropes.

An initial plating density of at least 2500 cells per well (in 24 well trays) was required. After survival was established, tests were conducted to learn whether secretory responses were maintained in media with and without serum or BSA. The results demonstrated a requirement for components in serum to maintain basal and CRH-mediated secretory levels of ACTH. Furthermore, in agreement with May and Eipper (9), chronic treatment with CRH enhanced media levels of ACTH in cells grown in serum-free, BSA-, and hormone-supplemented media. However, a greater enhancement was seen in these isolated corticotropes if the media also contained FBS. Thus, additional, unknown factors may be needed to promote differentiated function of these enriched corticotropes. Even when serum was added, there was variability from culture to culture in survival and functional responses after 14 days.

Parallel electrophysiological studies of these corticotrope cultures required that they be maintained in a basal (unstimulated) state. Therefore, daily exposure to CRH was not used in initial attempts to maintain the cultures. Trials showed that the percentage of CRH-bound cells was reduced by 50% after 7 days in culture. The percentages then recovered to 87% after 4 h in CRH (Childs, G. V., and G. Unabia, studies in progress). The results of these experiments led us to give 0.5 nM CRH for 4 h every 7 days as a minimal stimulation. The tests of basal and stimulated ACTH secretion described in these studies were conducted 6–7 days after these exposures to CRH.

The corticotropes expressed responsiveness to CRH and glucocorticoids at all periods tested. In contrast, they responded to the synergistic effects of AVP only for 7 days. Responses to AVP were variable in 14 day cultures or absent in 21–42 day cultures. These data confirm those of Wand and Eipper (10) who studied AVP responses in mixed cultures. In addition, Wand and Eipper showed no significant increases in total ACTH production in the presence of AVP alone for a 14 day test period (10). Finally, it is unclear why a few (10%) tests showed lower secretory activity in the presence of CRH and AVP. They secreted well when CRH was added alone.

The initial increase in basal secretory activity seen in most cultures correlated partially with the increase in cell number and surface area. However, while the cultures increased 2- to 3-fold in cell number, basal secretory levels increased only 1.6-fold. This suggested that factors needed to maintain normal secretory function were missing in the serum-supplemented MEM.

Losses in cell numbers evident after 21 days may reflect a limited lifespan of differentiated corticotropes. However, it also may reflect deficiencies in the medium and substrates. The work by May and Eipper (9) and Wand and Eipper (10) demonstrated the value of chronic treatment with CRH in maintaining corticotrope cell number and function for 21 days. This was coupled with the use of nutrient-enriched growth medium (Hams F_{12} and DME). This approach may be even more critical for isolated subsets of corticotropes. Important growth factors produced by other pituitary cell types may also be needed to maintain growth and function (20).

All subsets of corticotropes stored ACTH and END. However, they exhibited surprising changes in morphology. For the first week, the stellate, ovoid, or round shapes were like those of the freshly dispersed corticotrope population. Some of the corticotropes began to extend processes that became more complex in older cultures.

The other subset was more flattened and pleomorphic. These corticotropes also projected a few thin processes. Occasionally these cells resembled an intermediate stage between the flattened and stellate subsets. The flatter cells sometimes contained two nuclei or evidence of cytokinesis indicating that they were dividing. The increases in cell numbers also suggested the FBS containing media supported cell proliferation. Corticotropes have been shown to increase 2- to 3-fold after chronic exposure to CRH, or adrenalectomy (14, 16, 21). However, studies with [^{3}H]thymidine (12) are required to confirm mitosis *in vitro* in the corticotrope population.

In conclusion, this study has demonstrated growth and functioning of enriched corticotropes for 14–21 days in serum-containing media without the other pituitary cell types. Their secretory responses during the first week are similar to those of their counterparts in a mixed pituitary cell population. This is indirect evidence that they do not depend upon factors from other pituitary cell types for initial plating and ACTH secretion. Further work with defined media and substrata are needed to maintain differentiated functions for longer periods.

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References

1. Fleischer N, Rawls WE 1970 ACTH synthesis and release in pituitary monolayer culture: effect of dexamethasone. Am J Physiol 219:445
2. Sayers G, Portanova R 1974 Secretion of ACTH by isolated pituitary cells: kinetics of stimulation by CRF and inhibition by corticosterone. Endocrinology 4:1723
3. Bacsy E, Tougard C, Tixier-Vidal A, Martin J, Stark E 1976 Corticotroph cells in primary cultures of rat adenohypophysis: a light and electron microscopic immunocytochemical study. Histochemistry 50:161

4. Vale W, Rivier C 1977 Substances modulating the secretion of ACTH by cultured anterior pituitary cells. Fed Proc 36:2094
5. Vale, W, Rivier C, Yang L, Minick S, Guillemin R 1978 Effects of purified hypothalamic corticotropin-releasing factor and other substances on the secretion of adrenocorticotropin and β-endorphin like immunoreactivities *in vitro*. Endocrinology 103:1910
6. Rappay G 1980 Auto-and heterosynthesis in pituitary corticotropes in culture. Acta Histochem [Suppl] 22:253
7. Rappay G, Makara GB 1981 A quantitative approach to trace the corticotropes in culture after adrenalectomy. Histochem 73:131
8. Vale W, Vaughn J, Smith M, Yamamoto G, Rivier J, Rivier C 1983 Effects of synthetic ovine corticotropin-releasing factor, glucocorticoids, catecholamines, neurohypophysial peptides, and other substances on cultured corticotropic cells. Endocrinology 113:1121
9. May V, Eipper BA 1986 Long term culture of primary rat pituitary adrenocorticotropin/endorphin-producing cells in serum-free medium. Endocrinology 118: 1284
10. Wand GS, Eipper BA 1987 Effect of chronic secretagogue exposure on pro-adrenocorticotropin/endorphin production and secretion in primary cultures of rat anterior pituitary. Endocrinology 120:953
11. Watanabe T, Orth DN 1988 Effects of several *in vitro* systems on the potencies of putative adrenocorticotropin secretagogues on rat anterior pituitary cells. Endocrinology 122:229
12. McNicol AM, Kubba MAG, McTeague E 1987 The mitogenic effects of corticotropin-releasing factor on the anterior pituitary gland of the rat. J Endocrinol 118:237
13. Marchetti C, Childs GV, Brown AM 1987 Membrane currents of identified isolated rat corticotropes and gonadotropes. Am J Physiol 252:E340
14. Childs GV 1987 Cytochemical studies of the regulation of ACTH secretion Ann NY Acad Sci 512:248
15. Childs GV, Lloyd JM, Rougeau D, Unabia G 1988 Enrichment of corticotropes by counterflow centrifugation. Endocrinology 123:2885
16. Westlund KN, Aguilera G, Childs GV 1985 Quantification of morphological changes in pituitary corticotropes produced by *in vivo* corticotropin-releasing factor stimulation and adrenalectomy. Endocrinology 116:439
17. Westlund KN, Wynn PC, Chmielowiec S, Collins TJ, Childs GV 1984 Characterization of a potent biotin-conjugated CRF analog and the response of anterior pituitary corticotropes. Peptides 5:627
18. Childs GV, Marchetti C, Brown AM 1987 Involvement of sodium channels and two types of calcium channels in the regulation of ACTH release. Endocrinology 120:2059
19. Childs GV, Westlund KN, Unabia G, Characterization of anterior pituitary target cells for arginine vasopressin: including cells that store adrenocorticotropin, thyrotropin-β, and both hormones. Endocrinology, 125:554
20. Bottenstein J 1983 Growth requiredments of neural cells *in vitro*. In: Fedoroff S, Hertz L (eds) Advances in Cell Neurobiology, ed 4. Academic Press, New York, p 333
21. Gertz BJ, Conteras LN, McComb DJ, Kovacs HI, Tyrell JB, Dallman MF 1987 Chronic administration of corticotropin-relaasing factor increases pituitary corticotroph number. Endocrinology 120:381