

Cortical Cell Populations from Rabbit Kidney Isolated by Free-flow Electrophoresis: Characterization by Measurement of Hormone-sensitive Adenylate Cyclase

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ABSTRACT Free-flow electrophoresis allows the separation of different cell populations from a cell suspension isolated from rabbit kidney cortex after perfusion of the kidneys with a calcium-binder, followed by gentle mechanical treatment. After electrophoretic separation, analysis of the adenylate cyclase activities after stimulation by various hormones allows the precise determination of the origin of the cell populations with different electrophoretic mobilities. Adenylate cyclase from the slow-moving main cell population was only sensitive to parathyroid hormone. These cells had also high alkaline phosphatase content, further demonstrating their proximal origin. The various fast-moving cell populations had adenylate cyclase sensitive to isoproterenol and arginine vasopressin but were less sensitive to parathyroid hormone than the slow-moving cells. Their alkaline phosphatase content was also much lower. This indicates that these fast-moving cell populations originate from both the granulous segment of the distal tubule and from the collecting ducts. The adenylate cyclase activity and the cyclic AMP contents of isolated proximal cells maintained in culture medium were also investigated.

Recently, Heidrich and Dew have described a successful separation of cell populations from rabbit kidney cortex by free-flow electrophoresis (10). Single-cell suspensions were prepared after perfusion of kidneys with a Ca-binding medium (citrate). After electrophoretic separation, three types of cells (proximal, distal, and renin active cells) could be distinguished morphologically. In comparison with other proposed methods of separating the various types of cortical kidney cells (2, 15, 27, 29), this procedure has the advantage of avoiding any proteolytic treatment and/or gradient centrifugation for isolation and separation of cells. The technique could thus be useful in the study of drug metabolism, for example, and could provide information in addition to that already obtained using isolated microdissected tubules, particularly in the case of enzymatic and hormonal binding studies, which require large amounts of material. Such studies however, necessitate the isolation of well-defined cell populations whose location in the intact neph-

ron is precisely known. Except for ultrastructural differences concerning the presence or absence of microvilli, no reliable markers have been identified on these separated cells (10, 14). Morel's group (19), however, has recently demonstrated, using microdissected tubules from rabbit kidneys, that a fine functional segmentation corresponding to the different segments of the nephron can be established by adenylate cyclase measurements. In extensive studies they have shown precisely the different adenylate cyclase site sensitivities to various polypeptidic hormones (3, 5, 13, 18) and β -adrenergic agonist (4).

In the present study the different populations of isolated cells and the initial cortical cell suspension used for the experiments were analyzed using measurements of adenylate cyclase (AC), and alkaline phosphatase as a marker for microvilli (18). By analysis of the AC values, distinct populations of cells could be identified. AC activities and cyclic AMP contents (cAMP) were also measured in isolated cells kept in culture. The effects

of the Ca-binder medium and general problems connected with the isolation of specialized epithelial cells from an organ are also discussed.

MATERIALS AND METHODS

Preparation of Cell Suspension

Adult female New Zealand white rabbits were used in all experiments. The technique for the isolation of the cells from the cortical part of the kidney was that described earlier (10), with minor modifications. Four kidneys were normally perfused with Earle's balanced salt solution supplemented with 2.8 mM sodium citrate, 5 mM D-glucose and 0.005 vol % bovine serum albumin (BSA) for five min at a rate of 15 ml/min. In a separate series of experiments, the kidneys were perfused with either the same citrate solution for the same time, followed immediately by a standard Earle's medium perfusion containing 1.8 mM CaCl₂ for a further 5 min, or with the standard Earle's medium containing 1.8 mM CaCl₂, without citrate, for 10 min. At the end of the preparation, the cells were resuspended at a concentration of 25×10^6 cells/ml in electrophoresis medium (11 mM triethanolamine, 11 mM acetic acid, 5 mM D-glucose, 270 mM saccharose, 50 μ M CaCl₂, 0.5 mM MgCl₂, 1 mg/ml glutathione (reduced form), 0.005 vol % BSA, pH 7.4 (2 N KOH); osmolarity, 335 mOsm; conductivity, 6.5×10^2 μ mho).

Free-flow Electrophoresis

The electrophoresis was carried out in an FFV apparatus (Bender & Hobein, Munich, FRG) according to Hannig (8). The run was performed at 170 mA, 130 V/cm, 6°C; buffer flow 1.85 ml/fraction per hour; sample injection 1.8 ml/h. The cells were collected in tubes containing 2 ml of Earle's balanced salt solution. Under these conditions the cells were in the electric field for only 2 min and the entire cell suspension was separated after 2 h.

Protein Determination

Protein content was measured in a Technicon AutoAnalyzer System (ninhydrin reaction; Technicon Instruments Corp., Tarrytown, N.Y.) calibrated with albumin (9). To obtain a reliable value of protein per cell, six separate measurements of the mean protein content per 10^5 cells and per milliliter were performed for each separate fraction after the electrophoresis runs.

Alkaline Phosphatase Activity

Alkaline phosphatase activity was determined using disodium *p*-nitrophenyl phosphate as substrate (31) in each of the different fractions from the electrophoretic runs.

Permeabilization of the Isolated Cells for AC Assay

AC was assayed using a preparation of permeabilized broken cells. The same procedure was used for both the initial cortical cell suspension and the separated cell fractions. The cells were centrifuged at 200 g for 5 min and resuspended in a hypoosmotic medium (8 mM Tris/HCl, pH 7.5, 0.25 mM EDTA, 1 mM MgCl₂, 0.1 vol % BSA) at 4°C for 15 min. The volume of medium was chosen to give as nearly as possible an optimal concentration of 10×10^6 cells/ml. Cells were counted in a Neubauer chamber immediately after the addition of the hypoosmotic medium. To enhance membrane permeabilization, the samples were frozen rapidly in a dry ice-ethanol bath just before the AC assay, as described by Imbert et al. (12). After freezing, light microscopy showed that most of the cells were disrupted and that even the apparently intact cells were permeable to eosin and trypan blue. 5 μ l of this cell lysate was used for the AC assay.

AC Assay

The AC activity was measured essentially according to the method described by Morel et al. (19), except that the incubation was carried out in a final volume of 15 μ l in 1.5-ml Eppendorf plastic tubes. The final composition of the incubation medium was 100 mM Tris/HCl, pH 7.5, 1 mM cAMP, 0.25 mM [α -³²P]ATP (20 – 22×10^6 cpm per test), 20 mM creatine phosphate, 1 mg/ml creatine kinase, and various amounts of hormones and compounds as required. The reaction was initiated by the addition of 5 μ l of cell lysate (0.15 – 1.25×10^5 cells) and carried out for 30 min at 30°C. The reaction was stopped by the addition of 150 μ l of a solution containing 50 mM Tris/HCl, pH 7.5, 3.3 mM ATP, 5 mM cAMP, and

2×10^{-3} μ Cl [³H]cAMP for calculation of the cAMP recovery. After addition of 1 ml of water the cAMP formed was separated by passing the samples through Dowex and aluminum-oxide columns as described by Salomon et al. (24). The calculations were performed according to Morel et al. (19). Assays for the lysate from the initial cortical cell suspension were performed in duplicate. Only single assays were performed on the samples from separated cell fractions after free-flow electrophoresis, because less material was available, particularly in the fractions containing the fast-moving cells. The reaction was linear with time up to 30 min in both the cortical cell suspension and the separated fractions and was also proportional to the number of cells, up to a limit of 1.25×10^5 cells. All results were expressed in picomoles or femtomoles of cAMP generated per 30-min incubation time and per 10^5 cells or per milligram of protein. To further test the reliability of the test used here, we performed AC assays on isolated microdissected tubules prepared by the technique of Morel et al. (19). Under control and parathyroid hormone (PTH) (10 U/ml) stimulation, the results obtained with our test conditions (control: 10.8 ± 11 fmol/30 min per millimeter of tubular length, $n = 8$; PTH: 483.6 ± 51.9 , $n = 8$) were identical to those obtained by Chabardes et al. (3).

The hormones and compounds used in the AC measurements were synthetic 1–34 peptide from bovine PTH, 6,000 U/mg (Beckman Instruments, Microbic Division, Geneva, Switzerland); arginine-vasopressin (AVP) (Ferring Pharmaceuticals, Malmö, Sweden); L-isoproterenol D-bitartrate (ISO) (CIBA-Geigy, Basel, Switzerland); synthetic salmon calcitonin (SCT) (batch 20.0.51), (a gift from Sandoz AG, Basel, Switzerland); and sodium fluoride (NaF) (Merck, Darmstadt, FRG). The final concentrations of the different hormones and compounds used during the incubation period are given in the text, tables, and figures. In tables and figures the mean values \pm standard error are given. Student's *t* test was used for statistical analysis. The correlation coefficient was calculated by the least-squares method.

cAMP Assay

cAMP content in isolated cells was measured using the radioimmunoassay from New England Nuclear (Boston, Mass.) adapted from the procedures of Steiner et al. (28). Each incubation vessel contained 2×10^5 total cells ("viable" and "nonviable" as determined with eosin G dye) in 10 μ l of Weymouth MB 752/1 medium (Gibco), 80 μ l of Weymouth medium supplemented with 3 mM 3-isobutyl-1-methylxanthine (IBMX) as an inhibitor for cAMP-phosphodiesterase activity, and 10 μ l of PTH stock solution (to give 10 U/ml). Blanks did not contain hormone. After a 5-min preincubation without hormone and incubation for 10 min with PTH at 37°C, the reaction was stopped with 1 ml of ice-cold ethanol, and the samples were frozen for 60 min. The particulate material was spun down, and the supernatants were removed and dried at 40°C in a Speed Vac Concentrator (Savant Instruments, Inc., Hicksville, N. Y.). Each residue was dissolved in 0.05 M sodium buffer from the test kit, and 100 μ l of this solution was used for the radioimmunoassay, which was carried out after acetylation of the samples according to the instructions in the kit.

Proximal Cells in Culture

Pools from each of the main fractions from five separate electrophoresis runs (carried out in sterile conditions) were kept in culture for 3 d. The cells were maintained in plastic petri dishes in Dulbecco's modified Eagle's medium, supplemented with 100 μ g of streptomycin and 100 U of penicillin/ml, but without addition of serum. Cells were plated at 250,000 cells/ml and incubated at 37°C, 95% air, 5% CO₂. AC activities and cAMP contents were measured immediately after separation and after 1, 2, and 3 d of culture.

Electron Microscopy

Specimens for transmission electron microscopy were prepared using standard routine techniques including glutaraldehyde and osmium tetroxide fixation, embedding in Epon resin, and staining with uranyl acetate and lead citrate. Alkaline phosphatase was analyzed cytochemically using β -glycerophosphate as substrate.

RESULTS

Isolated Cortical Cell Suspension

Table I shows the AC activities measured on the total pool of cortical cells isolated after 5 min of citrate perfusion. The concentrations of hormones and compounds were identical to those used by Morel et al. (19), which give maximal AC stimulations. In the cell suspension prepared according to the

method described here, the stimulated AC activities were in every case significantly higher than the basal (control) activities measured in the absence of hormones. The absolute activities, however, were much lower than those found in microdissected tubules (see Discussion), and the highest AC activities were obtained with PTH and NaF stimulation. To test whether these low AC activities were related to some membrane modifications in the isolated cells caused by the depletion of cellular calcium induced by citrate perfusion, AC was measured in isolated cell suspensions prepared after citrate perfusion followed by a 1.8 mM calcium reloading or by a perfusion with Earle's solution containing the same amount of calcium (without citrate perfusion). The results of control, PTH, and NaF stimulations are shown in Fig. 1, compared with the previous values obtained after citrate perfusion alone. The highest values were obtained after reloading with calcium (control: 32.2 ± 5.1 pmol cAMP formed/30 min per milligram of protein; PTH: 143.6 ± 15.6 ; NaF: 136.2 ± 18.1 ; $n = 9$) but they were still lower than those reported for isolated microdissected tubules (3). Earle's Ca perfusion alone (without citrate) also gave high AC activities in the cell suspension. However, very few cells were isolated and the separation and the enzyme experiments described could not be carried out with that low number of cells. In addition, conditions in which kidneys were perfused or reloaded with calcium are not suitable for subsequent electrophoresis of the isolated cells since they promoted cell aggregation.

Separated Isolated Cells after Free-flow Electrophoresis

CELL COUNTS AND PROTEIN CONTENT: Mean cell count and protein profiles of six separate experiments are shown in Fig. 2. As previously observed (10), no other definite peaks could be clearly resolved on either the cathodic or anodic sides of the main cell peak (fraction 30). The protein content profile was approximately parallel to the cell count profile. The highest protein content per cell was found in the main cell fraction (i.e., fraction 30; $15.7 \mu\text{g}/10^5$ cells) and in the five following fractions located on the anodic side of the main fraction. Thereafter the values decreased in the fractions located nearer the anode, i.e., the fast-moving cells. In every case, values were somewhat lower than the protein content per cell of the initial cortical cell suspension ($19.9 \mu\text{g}/10^5$ cells, $n = 32$).

ALKALINE PHOSPHATASE ACTIVITIES: Mean alkaline phosphatase activities of four experiments for each fraction are also given in Fig. 2. The highest activities were located in the region of the main cell peak but the maximum values were shifted to the left of this main cell peak to fraction 28. The

alkaline phosphatase activities were five to ten times lower in the fast-moving cells.

CONTROL AND NaF-STIMULATED AC ACTIVITIES: Fig. 3 shows the AC activities under basal (control) and NaF-stimulated (5×10^{-3} M) conditions. AC activities were expressed as femtomoles per 10^5 cells (and not per milligram of protein). The lowest control AC activity was found in the main cell peak (fraction 30). On the anodic side the control AC activities increased to three to four times the control activity of the main cell fraction. NaF induced a significant increase of AC activities in each of the fractions ($0.001 < P < 0.025$). The higher value of AC activity in the fast-moving cell fractions were maintained, with the maximum value in fraction 21.

PTH-STIMULATED AC ACTIVITIES: The main ratios of the PTH-stimulated (10 U/ml) to control AC activities (S/C ratios) from nine separate experiments are shown in the upper panel of Fig. 4. Three maxima could be identified. The first peak on the cathodic side had a maximum ratio value of 12.1 and was followed closely by main peak exactly superimposed on the main cell fraction. The S/C ratio in this fraction was 24.3. Thereafter ratios decreased to form a shoulder with a maximal ratio of 13.8 in fraction 26. No definitive peak could be observed in the fast-moving cells. These fractions showed a fourfold to sevenfold increase in AC activities under PTH stimulation. From electron microscopy observations (10), the main cell peak appears to be a rather homogeneous population of proximal cells. To confirm these observations, we measured dose response and sensitivity to PTH in this cell population. Fig. 5 shows the results obtained. The cAMP generated after

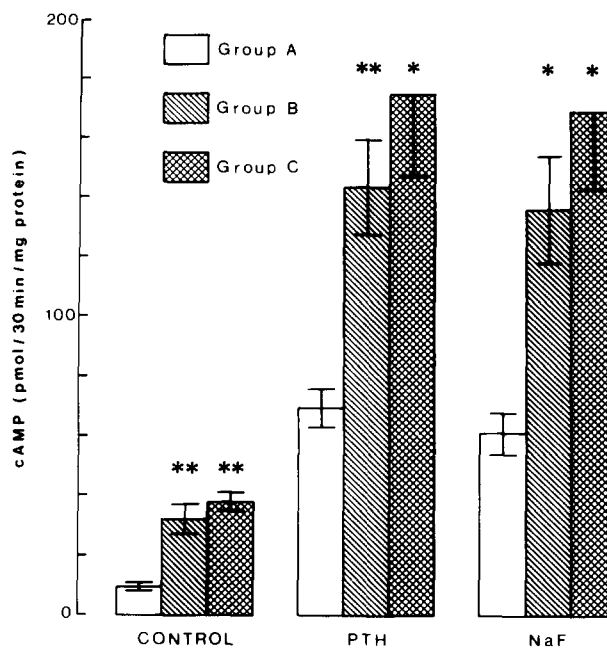


FIGURE 1 Effect of calcium loading on AC activities in cortical cell suspensions from rabbit kidney cortex. AC activities (expressed as pmol of cAMP formed per 30 min per mg of protein) in basal (control), PTH- (10 U/ml), and NaF- (5×10^{-3} M) stimulated conditions. The procedures for the kidney perfusion for isolation of cells were as follows: group A, citrate perfusion; group B, citrate perfusion followed by 1.8 mM CaCl_2 perfusion; group C, 1.8 mM CaCl_2 perfusion (without citrate). Values are the mean \pm standard error of nine experiments for each of the three groups. Each determination was performed in duplicate. Statistical significance (Student's *t* test) between group A and groups B and C are indicated above the bars (*, $P < 0.005$; **, $P < 0.001$).

TABLE I
Adenylate Cyclase Activities in Isolated Cortical Cell Suspension from Rabbit Kidney

	cAMP formed	S/C
Control	9.9 ± 1.0	—
PTH, 10 U/ml	69.7 ± 6.4	7.0
ISO, 10^{-6} M	18.5 ± 1.9	1.9
AVP, 10^{-6} M	18.1 ± 2.0	1.8
SCT, 100 ng/ml	16.9 ± 1.5	1.7
NaF, 5×10^{-3} M	61.6 ± 7.3	6.2

Values are means \pm standard error of nine different cortical cell preparations. Results are expressed in picomoles of cAMP formed/30 min per milligram of protein. S/C is the ratio of stimulated to control activities. In each case, the stimulated values differ significantly ($P < 0.005$) from the control values.

PTH (10 U/ml) stimulation was proportional to the number of cells (left panel). The fact that the regression line passes near the origin assures the reproducibility of the method and the homogeneity of cells in the main cell fractions of all the performed experiments ($y = 0.69x + 0.04$; $r = 0.030$; $n = 36$). The right-hand panel shows that the PTH concentration inducing threshold response was between 0.01 and 0.1 U/ml with a maximal stimulation at 10 U/ml. The half-maximal stimulation was obtained with 0.5 U/ml.

ISO-, AVP-, AND SCT-STIMULATED AC ACTIVITIES: The ratio of ISO- and AVP-stimulated AC activities to control activities (S/C ratios) are shown in Fig. 4 (lower panel). For ISO (10^{-6} M) the highest activities were found in the fast-moving cells, with a maximum in fraction 19 (S/C ratio of 6.5); the S/C ratios decreased on the cathodic side, forming a small

peak in the fractions 33–36. The AVP (10^{-6} M) S/C ratios presented a profile similar to that for ISO. However, the S/C ratios were lower than after ISO stimulation. No definite peak could be observed after SCT stimulation (100 ng/ml). Here, again, the highest ratios were found in the same fractions as after ISO and AVP treatment.

ABSOLUTE AC ACTIVITIES OF THE MAIN CELL POPULATIONS: Table II summarizes protein content, alkaline phosphatase, and AC activities under the different conditions of stimulation in the different cell populations as characterized by the S/C peak ratios. PTH and NaF induce a highly significant rise of AC activities as compared with control values in all of the fractions. No significant differences could be observed in fractions 35 and 30 after ISO or AVP, except in fraction 35 for the latter ($P < 0.025$). However, they both induced signif-

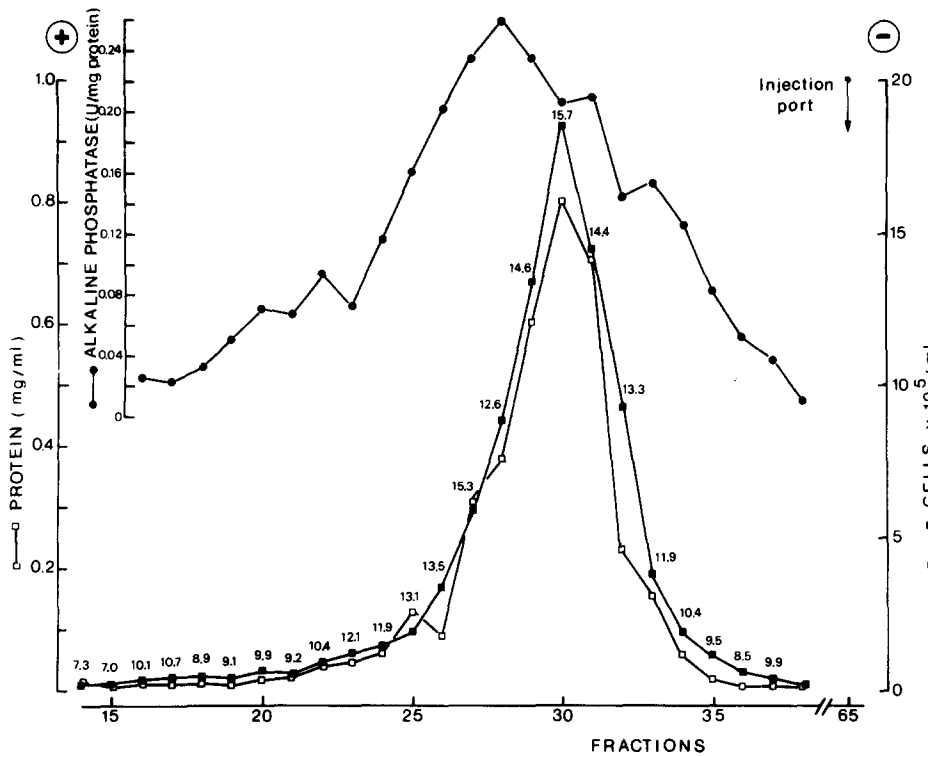


FIGURE 2 Cell count, protein content, and alkaline phosphatase activities in the different fractions separated by free-flow electrophoresis. The two bottom curves represent the mean values of six separate electrophoretic runs. (■) The cell number per ml; (□) the protein content expressed in mg per ml. The numbers above the squares give the protein content in μg per 10^5 cells. The curve at the top of the figure represents the mean values of four separate runs for alkaline phosphatase activities (expressed as U/mg of protein).

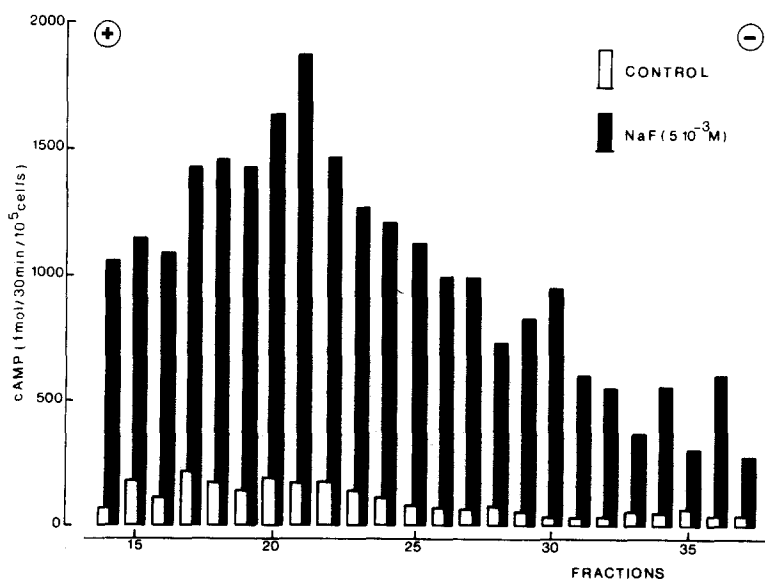


FIGURE 3 Control and NaF-stimulated AC activities in the cell fractions separated by free-flow electrophoresis. Each bar represents the mean value of nine separate electrophoresis runs. AC activities are expressed as fmol of cAMP formed per 30 min per 10^5 cells. Open bars refer to the control values. Black bars refer to the NaF-stimulated AC activities.

icant stimulation in all of the other fractions ($0.05 < P < 0.001$). SCT did not induce a specific increase in any fractions.

Morphological Comparison between Slow- and Fast-moving Cells

As was already shown using scanning electron microscopy (10), the main cell peak contains only cells with long microvilli. These cells possess alkaline phosphatase activity that can be localized cytochemically on the brush-border microvilli (Fig. 6). On the other hand, the fast-moving cells in fractions 15–24

possessed almost no microvilli, in contrast to the cells from the main peak (fraction 30), as is illustrated in Fig. 7a and b. However, the fast-moving cells without microvilli are still contaminated with cells possessing microvilli, and this contamination varies from preparation to preparation.

AC Activities of Proximal Cells (Fraction 30) and cAMP Formation in Culture

AC activities were measured on proximal cells 1–3 d after their electrophoretic separation, to discover whether activities were maintained or enhanced after a few days in culture medium. During this period most of the cells excluded trypan blue and eosin dye. After 2 d of culture, the AC activity was still 83% of the initial PTH-stimulated activity. On the 3rd d of culture, the basal (control) activity rose, whereas the PTH-stimulated AC activity was reduced to 66% of the initial value. During this time the cells tend to aggregate without forming monolayers and no cell proliferation was observed. Also, cAMP contents were measured in the isolated cells kept in culture. Fig. 8 shows that under PTH stimulation cAMP concentrations rise as the cells recover in culture from the isolation procedure. On day 3, however, PTH-stimulated cAMP formation begins to break down.

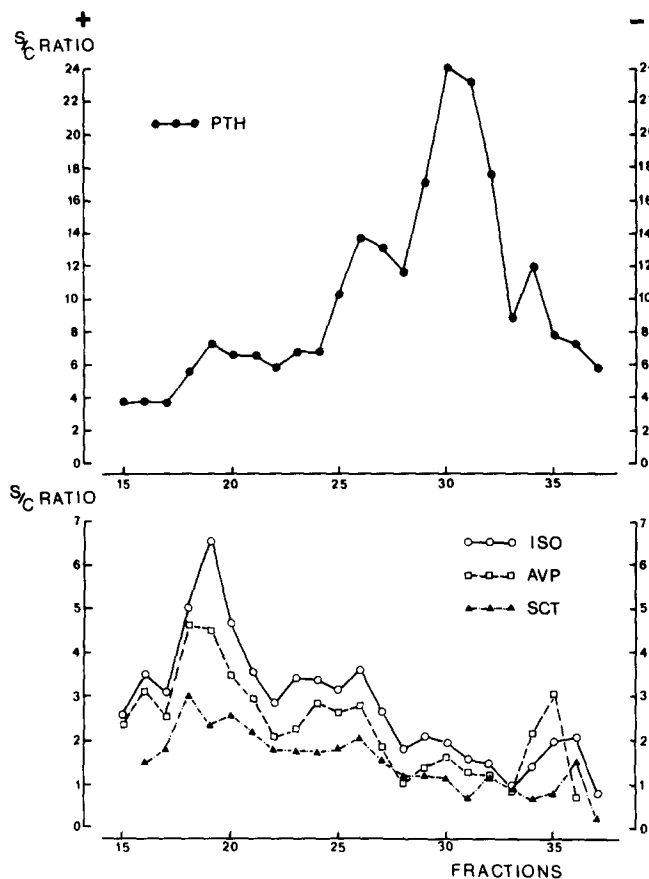


FIGURE 4 Ratios of PTH-, ISO-, AVP-, and SCT-stimulated AC activities to control AC activity in the cell fractions separated by free-flow electrophoresis. Each point represents the mean S/C AC ratio of nine separate electrophoretic runs. Upper panel: (●) the PTH (10 U/ml) S/C ratios. Lower panel: (○) the ISO (10^{-6} M) S/C ratios, (□) the AVP (10^{-6} M) S/C ratios, and (▲) the SCT (100 ng/ml) S/C ratios.

DISCUSSION

Previous observations by Heidrich and Dew (10) indicated that relatively homogeneous cell populations from rabbit kidney cortex could be separated by means of free-flow electrophoresis. The cell populations were distinguished morphologically. These results are confirmed by the present results from AC measurements on such separated cell populations. The experiments demonstrate that different types of tubular cells from cortex could be isolated by combining a mild calcium-binding perfusion, mechanical disruption (without any additional enzymatic treatment), and electrophoretic separation. The starting cortical cell suspension for the electrophoretic separations contains the different types of tubular cells. This can be seen from Table I, in which a significant increase in AC activities after stimulation by each of the various hormones is demonstrated. The S/C ratios and absolute activities were comparable to those obtained using the different polypeptide hormones and β -adrenergic agonist on crude homogenate of rat kidney cortex (1, 6, 7, 16, 17, 20). The question arises, however, why such isolated cells present much lower AC activities than those reported by microdissected tubules by Morel and co-workers (19). It could be that, during the cell isolation, membrane modifications occur, either as a result of the breaking of cell

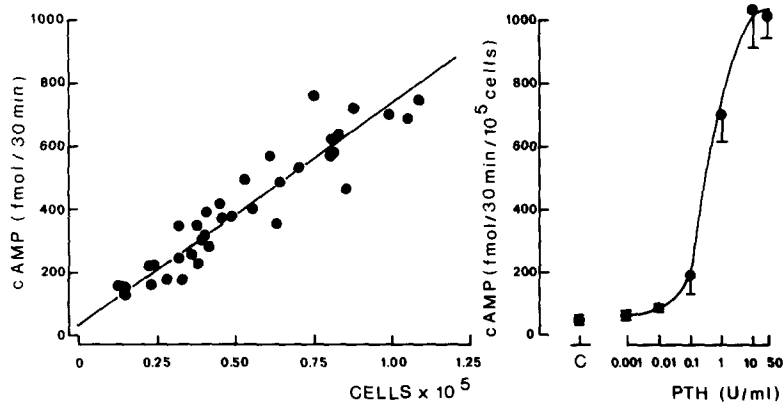


FIGURE 5 PTH-sensitive AC activities in the main cell fraction (fraction 30). Left panel: cAMP formation (expressed as fmol of cAMP generated per 30 min) as a function of the number of cells in the presence of PTH (10 U/ml). Each point represents the individual values of the four fractions surrounding the mean cell peak (fractions 28–31) in nine separate experiments. Right panel: AC sensitivity to PTH (log scale) in the main cell fraction. Each point represents the mean value \pm SE of the AC activity (expressed as fmol of cAMP formed per 30 min per 10^5 cells) of five separate experiments. C refers to the activity measured in the absence of PTH (control).

TABLE II
Protein Content and Alkaline Phosphatase and AC Activities
of the Different Main Cell Populations after Free-flow Separation

Frac- tion	Protein (6)	Alkaline Phospha- tase (4)	AC activities (9), fmol cAMP formed/30 min/10 ⁵ cells										
			Control	PTH	S/C	ISO	S/C	AVP	S/C	SCT	S/C	NaF	S/C
35	9.5	0.084 (± 0.036)	65.8 (± 26.8)	510.6 (± 170.0)	7.7	130.3 (± 46.5)	2.0	199.5 (± 24.5)	3.0	52.9 (± 34.1)	0.8	311.2 (± 45.7)	4.7
30	15.7	0.208 (± 0.016)	37.7 (± 12.3)	917.7 (± 103.0)	24.3	73.2 (± 12.3)	1.9	60.5 (± 12.6)	1.6	47.8 (± 12.4)	1.3	954.8 (± 158.4)	25.3
26	13.5	0.210 (± 0.022)	69.8 (± 12.3)	966.3 (± 377.1)	13.8	251.9 (± 54.9)	3.6	192.3 (± 37.7)	2.8	171.6 (± 43.6)	2.5	982.6 (± 133.5)	14.1
19	9.1	0.056 (± 0.005)	143.4 (± 29.8)	1056.5 (± 212.6)	7.4	937.6 (± 198.0)	6.5	248.2 (± 172.6)	4.5	330.6 (± 70.0)	2.3	1433.7 (± 230.7)	10.0
16	10.1	0.026 (± 0.007)	193.5 (± 52.4)	751.6 (± 244.4)	3.9	682.5 (± 156.5)	3.5	612.3 (± 140.5)	3.2	278.0 (± 108.2)	1.4	1195.7 (± 377.0)	6.2

Values are the means ± standard error. Protein are given as micrograms per 10⁶ cells. Alkaline phosphatase are expressed as units per milligram of protein. S/C represents the stimulated AC over control ratio. The final concentrations of hormones and compounds were as follows: PTH (10 U/ml), ISO (10⁻⁶ M), AVP (10⁻⁸ M), SCT (100 ng/ml), NaF (5 × 10⁻³ M). Parentheses indicate the number of electrophoresis runs performed for each result.

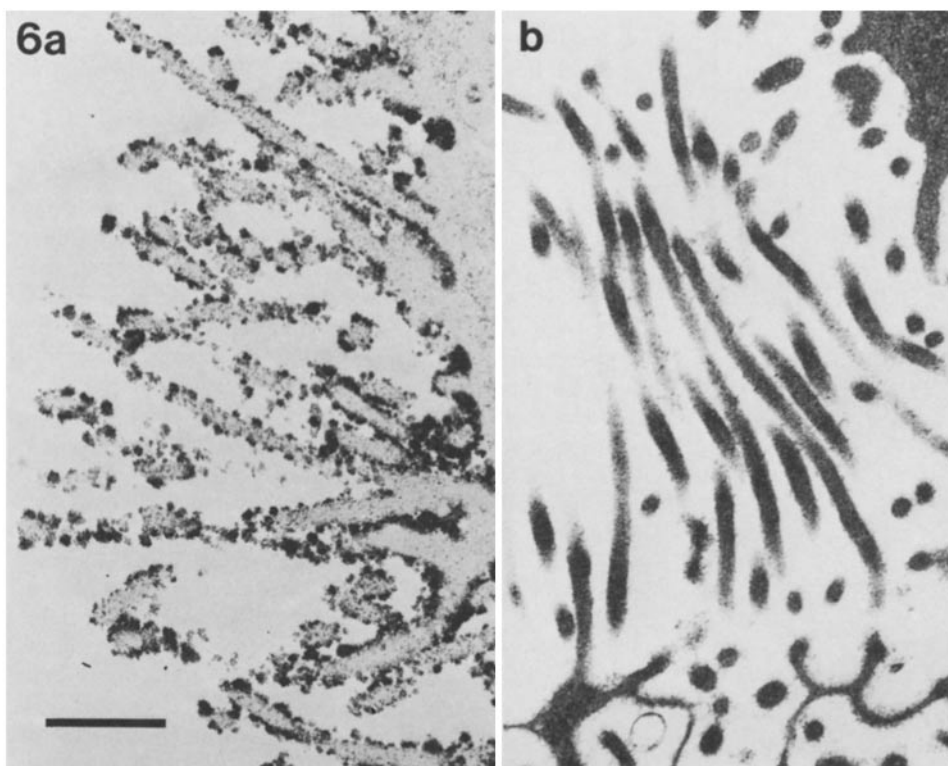


FIGURE 6 Cytochemical demonstration of alkaline phosphatase activity on the microvilli of isolated and separated proximal tubule cells (fraction 30). A modified Gomori lead salt technique with sodium β -glycerophosphate as substrate was used for a. Even alkaline phosphatase is a very soluble enzyme and is easily mobilized from tissue; high activities of this enzyme are still present on the isolated cells. (b) The control without substrate. Bar, 0.5 μ m.

contacts or as a result of the removal of Ca⁺⁺ ions by citrate, which results in poor stimulation of the very sensitive adenylate cyclase enzyme. To test the second possibility, we prepared cells without calcium chelator during the kidney perfusion and with a physiological amount of Ca⁺⁺, or reloaded the kidneys with Ca⁺⁺ after citrate perfusion. Under such conditions (Fig. 1), a significant rise in the AC activities was observed but in no case did they reach the values found for microdissected tubules, which are ~30 times higher. Thus the low stimulated-AC activities seem mostly to be due to modifications in the membrane structures after disruption of the cell contacts, e.g., the junctions. It has been shown in experiments on urinary bladder epithelial cells (21) and in MDCK cells (23, 25) that, during isolation of cells, redistribution of functional groups can occur. Such redistribution of membrane components might well take place in the kidney cells described here during their isolation.

Since the AC receptors are known to be located in the basolateral regions (26), such phenomena could explain the low AC activities. However, it is more difficult to draw conclusion as to the precise molecular mechanism responsible for these low activities. There is no further loss of AC activity in the electric field, as was proven in experiments with and without electric current in the separation chamber. Such a loss resulting from redistribution of membrane components might be expected since, as shown by Poo et al. (22), an electric field can move and redistribute functional molecules within a membrane.

Despite these problems, which arise from the preparation procedures, AC activity could be measured in each of the separated fractions and expressed in femtomoles per 10⁵ cells. In this way the results can be compared with those reported for microdissected tubules where AC activities are expressed per millimeter of tubular length (12). Analysis of the S/C ratios

allows certain conclusions to be drawn about the origins of the different fractions separated by free-flow electrophoresis. The main cell fraction (fraction 30, Table II) shows a 24-fold increase in AC under PTH stimulation, which is the highest stimulated ratio observed. In comparison with the other cell fractions the main cell fraction shows the highest protein content per cell, the lowest AC control activities, relatively low NaF stimulation, and no significant increase of AC activities with AVP, SCT, and ISO. These characteristics are consistent with those observed for microdissected proximal convoluted tubules (3), indicating that the fraction consists of a homogeneous population of proximal cells. This conclusion is sup-

ported by the morphological observations of Heidrich and Dew (10), who observed by scanning electron microscopy a homogeneous cell population with well-individualized long microvilli (see also Fig. 7 *a*). Furthermore this fraction has a high alkaline phosphatase content, a marker for microvilli, which can also be observed electron microscopically after cytochemical reactions on the separated cells.

On the anodic side of this main peak are several fractions of fast-moving cells with lower alkaline phosphatase activities. The separation profile of the isolated cells is a function of the different surface charges and can be compared with the separation of luminal (microvilli) and basolateral membranes by

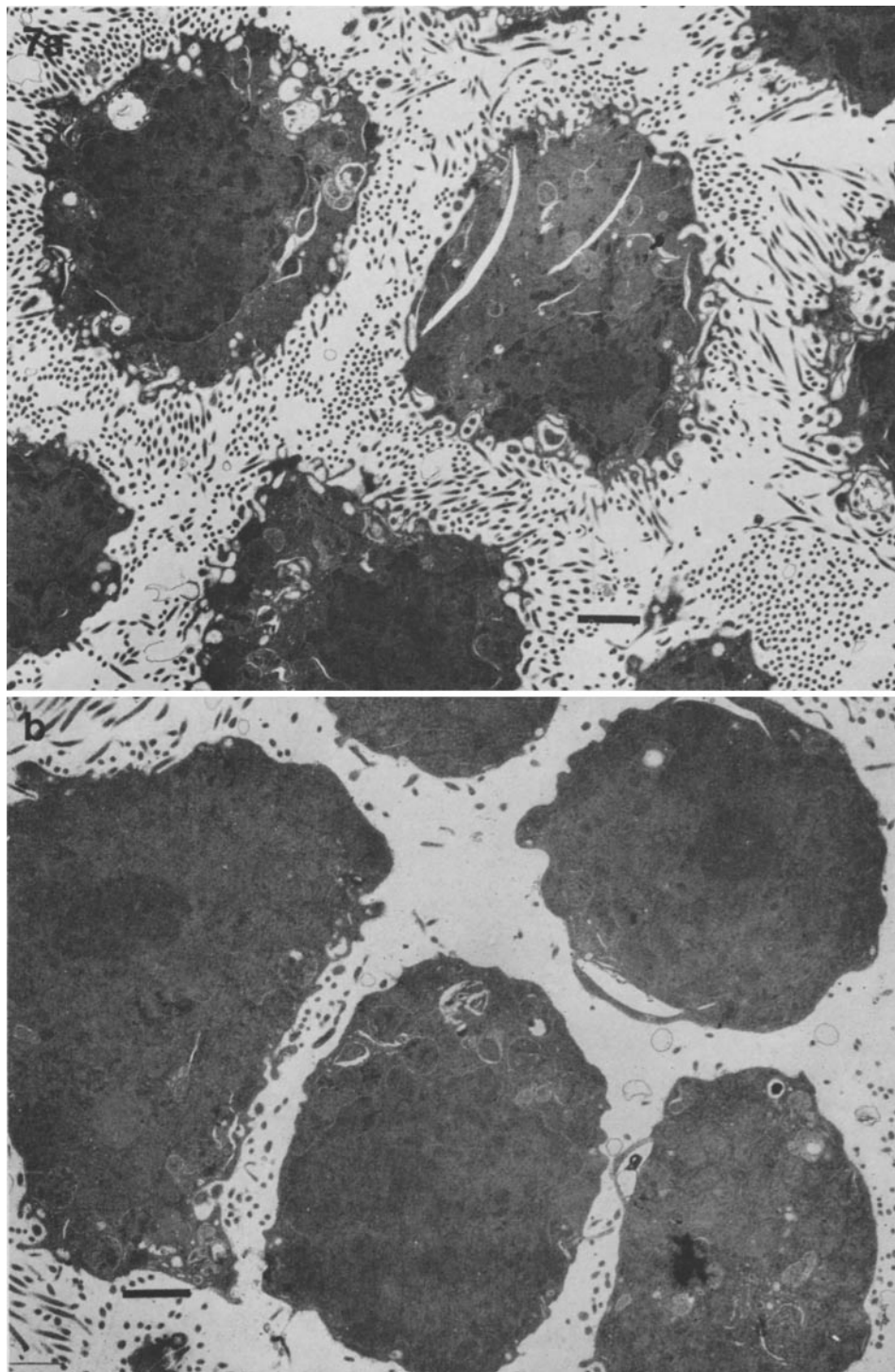


FIGURE 7 Morphological comparison between electrophoretically slow- and fast-moving cells. Thin sections from fraction 30 (*a*) and from fractions 15-24 (*b*) of the electrophoresis run show a distinct difference. The slow-moving cells possess long microvilli (proximal tubule cells), whereas the fast-moving cells have no or only short microvilli. The latter cell population is not homogeneous but rather is contaminated with cells from the proximal tubule. Bars, 1 μ m.

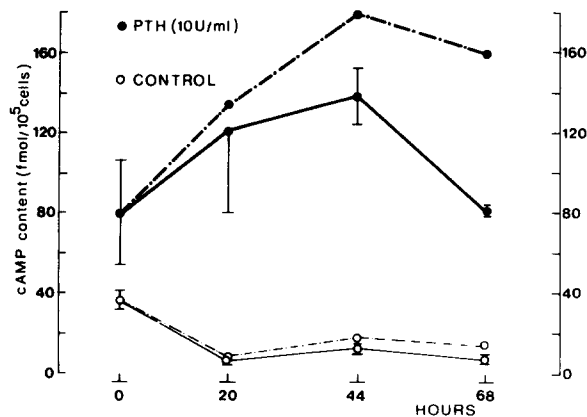


FIGURE 8 cAMP production in isolated proximal cells in culture. Each point represents the mean values \pm standard error of four experiments. cAMP contents in isolated cells were measured under control conditions (O) and PTH (10 U/ml) stimulation (●) at 0, 20, 44, and 68 h of culture. Broken lines represent the cAMP values theoretically expected if all cells in the test sample were "viable," i.e., eosin G dye negative.

the same electrophoresis technique (20). In the latter experiments it was shown that the microvilli, as a result of their low-charged glycocalyxes, have much slower electrophoretic mobility than basolateral membranes, which are deflected more towards the anode. Similarly, in the separation of kidney cells, the slow-moving cells are those with long microvilli and high alkaline phosphatase activity. Thus the fast-moving cells without alkaline phosphatase should represent the "distal" cells. The measurements of stimulated AC activities confirm this conclusion and enable the recognition of different cell types. Fractions 25–28 were sensitive to PTH and could also be stimulated by ISO. However, these cells also have high alkaline phosphatase activities. This fraction is probably composed mainly of proximal cells with small proportion of "distal" cells responsible for the AVP and ISO sensitivities. This is certainly the result of an overlap between the main proximal cell population and the faster-moving cells. The fastest-moving cells (fractions 15–24), which possess no or only short microvilli, appear to contain cells derived from the granulous segments of the distal and collecting tubules. They show a sixfold to sevenfold stimulation by PTH and ISO and a threefold to fourfold stimulation for AVP. This corresponds to the S/C ratios observed for these hormones on microdissected distal and collecting segments (3, 4, 13). As can be seen from the electron micrographs of this fraction, the cells are contaminated with proximal tubule cells. This probably accounts for the high standard error values of the control and stimulated AC activities in fractions 19 and 16 shown in Table II. In none of the fractions did AVP induce a stimulation higher than that obtained with ISO. However, in the fastest fractions (15–17), AVP stimulation reached the same level as ISO stimulation, indicating that some of the cells in these fractions are derived from the cortical collecting segments. A clear stimulation of AC with SCT, a marker for cells from the cortical thick ascending limb (18), was not observed in any fraction. The small peak around fractions 34–36 (Fig. 4) defined by PTH, ISO, and AVP S/C ratios contained smaller cells whose origin and nature are still unknown.

In the previous investigation, the quality and vitality of the isolated cells were tested in culture by uridine incorporation and dye exclusion (10). We performed similar experiments on the isolated proximal cell population (fraction 30) after free-

flow electrophoresis. Under our culture conditions, 83% of the initial PTH-stimulated activity was still present after 48 h. In addition, cAMP content measurements in basal conditions and under PTH stimulation were carried out on the isolated cells up to 68 h as the cells recovered in culture from the isolation procedure. The presence of AC and the ability of the isolated cells to form cAMP from its precursors still present in the cells for at least 2 d in culture could offer some possibilities for long-term metabolic studies. Since isolation of tubular cells appears to be responsible for a loss in the activities of certain membrane enzymes, for example AC, the question arises whether or not such a technique of cell separation could be harmful to the cells and could also lead to a loss of intracellular enzymes. Preliminary results indicate, for example, that the intracellular enzyme phosphoenolpyruvate carboxykinase is found with similar activity in isolated proximal cells and in microdissected proximal tubules (30).

For conclusion, the present work shows that separation by free-flow electrophoresis is a successful method for obtaining distinct cell populations from rabbit kidney cortex. These populations can be characterized by their AC activities. Despite the problems involved, the possibility of obtaining rapidly a large number of cells gives this method distinct advantages for many kinds of studies on tubular kidney cells.

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