Biochimica et Biophysica Acta 812 (1985) 453–459
Elsevier

BBA 72383

Na^+-dependent transport of α-aminoisobutyrate in isolated basolateral membrane vesicles from rat parotid glands

Taishin Takuma and Bruce J. Baum *

Clinical Investigations and Patient Care Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20205 (U S A)

(Received June 5th, 1984)
(Revised manuscript received September 12th, 1984)

Key words Basolateral membrane, Amino acid transport, Aminoisobutyrate; (Rat parotid)

Basolateral plasma membranes were prepared from rat parotid gland after centrifugation in a self-orienting Percoll gradient. K^+-dependent phosphatase ($(Na^+ + K^+)$-ATPase), a marker enzyme for basolateral membranes, was enriched 10-fold from tissue homogenates. Using this preparation, the transport of α-aminoisobutyrate was studied. The uptake of α-aminoisobutyrate was Na^+-dependent, osmotically sensitive, and temperature-dependent. In the presence of a Na^+ gradient between the extra- and intravesicular solutions, vesicles showed an 'overshoot' accumulation of α-aminoisobutyrate. Sodium-dependent α-aminoisobutyrate uptake was saturable, exhibiting an apparent K_m of 1.28 ± 0.35 mM and V_{max} of 780 ± 170 pmol/min per mg protein. α-Aminoisobutyrate transport was inhibited considerably by monensin, but incubating with ouabain was without effect. These results suggest that basolateral membrane vesicles, which possess an active amino acid transport system (system A), can be prepared from the rat parotid gland.

Introduction

The usefulness of isolated plasma membrane vesicles for transport studies has been amply demonstrated in various systems [1,2]. Using luminal (brush-border) or contraluminal (basolateral) plasma membrane vesicles, many absorption and reabsorption mechanisms of intestine and kidney have been investigated [1–5]. While the isolated plasma membrane would also appear to provide a potent tool for secretion studies, applications of this experimental technique to exocrine glands are very rare. For example, Milutinovic et al. [6–8] have isolated plasma membranes from cat pancreas for secretion and transport studies by centrifugation through a Ficoll-sucrose density gradient. More recently Mircheff et al. [9,10] have separated functional apical and basolateral membranes from the rat exorbital lacrimal gland by 'third-dimension separation procedures.'

Another often used model of exocrine secretion is the parotid salivary gland. Plasma membranes have been prepared from mouse [11], rabbit [12] and rat [13] glands by differential centrifugation and discontinuous sucrose gradient centrifugation. The methods utilized in these studies were in general complicated and time-consuming. Moreover, the luminal and basolateral membranes in these preparations were either not or insufficiently separated. Importantly, such preparations have not as yet been employed for functional studies.

Recently Scalera et al. [3] have introduced a simple and rapid method for isolation of basolateral membranes using self-orienting Percoll

* To whom all correspondence should be addressed.

Abbreviation: Hepes, 4-(2-(hydroxyethyl)-1-piperazineethanesulfonic acid

gradient centrifugation. This method has been utilized successfully to prepare membranes from intestine [3] and kidney [4,5] epithelial cells. In the present study, we have applied the Percoll method to rat parotid gland, and obtained basolateral membrane vesicles having an active amino acid transport system.

Materials and Methods

Materials. Animal used in these experiments were male, 250 g, Wistar strain rats purchased from Harlan-Sprague Dawley. Rats were fed ad libitum and killed between 9:30 and 10:30 a.m. α-Aminoiso[^{14}C]butyrate (56 mCi/mmol) was obtained from New England Nuclear. Unlabeled α-aminoisobutyrate, ouabain, monensin and phenylmethylsulfonyl fluoride (PMSF) were from Sigma. Percoll was purchased from Pharmacia. All other reagents utilized were the highest grade commercially available.

Preparation of basolateral membranes. The basolateral membrane vesicles were prepared by the method utilized for intestine [3] and kidney [4] with minor modifications. Parotid glands (approx. 2 g) from four rats were homogenized with 20 ml sucrose buffer comprising of 0.25 M sucrose/0.1 mM PMSF/10 mM Tris-HCl (pH 7.6) in a Polytron homogenizer (speed 5 for 15 s, twice) at 0°C. The homogenate was centrifuged at 2600 × *g* for 15 min in a Beckman JA 201 rotor, and the pellet was discarded. The soluble fraction was centrifuged at 23 500 × *g* for 20 min, and the resulting supernatant was discarded. No 'fluffy layer', such as observed with plasma membrane preparations of intestine and kidney, was obtained, so the whole 23 500 × *g* pellet was homogenized with 11.44 ml of the sucrose buffer in a Teflon-glass homogenizer (ten strokes, by hand) and was considered the crude plasma membrane preparation. Then, 1.56 ml Percoll (final concentration 12%) was added, mixed vigorously, and centrifuged at 41 700 × *g* for 30 min in a JA 20.1 rotor. After centrifugation, fractions (1.05-ml) were collected from the top by an Isco fractionator. Fraction numbers 3–6 were combined, diluted with loading buffer comprising of 100 mM KCl/100 mM mannitol/5 mM Hepes-Tris (pH 7.5) and centrifuged at 41 700 × *g* for 20 min. This same washing process was repeated two more times, before the pellet was finally suspended in a small volume of the loading buffer.

α-Aminoisobutyrate transport. Uptake of α-aminoiso[^{14}C]butyrate was measured by a rapid filtration technique through a Millipore filter (HA, 0.45 μm). Membrane vesicles (10 μl, 60–100 μg protein) were preincubated at 20°C for 1 min, mixed with 40 μl reaction mixture containing final concentrations of 178 μM α-aminoiso[^{14}C]-butyrate/80 mM NaCl/20 mM KCl/100 mM mannitol/5 mM Hepes-Tris (pH 7.5) and incubated at 20°C for various times. Reactions were stopped by adding 2 ml ice-cold buffer comprising 150 mM NaCl/1 mM Hepes-Tris (pH 7.5), passed over a Millipore filter and washed four times with 2 ml of the same buffer. Filters were extracted with Beckman Ready-Solv and radioactivity determined by liquid scintillation spectrometry. All determinations were carried out in triplicate and results are presented as the average ± S.E. of a typical experiment representing 2–4 separate experiments.

Enzyme assays. K^+-dependent phosphatase activity, a convenient and advantageous marker of ($Na^+ + K^+$)-ATPase in ouabain-resistant rat tissues [14], was measured as described by Arvan and Castle [13]. γ-Glutamyl transpeptidase (γ-GTPase) was measured by the method of Tate and Meister [15], alkaline phosphatase by the method of Bernt [16], cytochrome oxidase as described by Wharton and Tzagaloff [17]. Protein was determined by the method of Lowry et al. [18] using bovine serum albumin as a standard.

Results

Isolation of basolateral membrane vesicles

Fig. 1 shows the distribution of marker enzymes after an isopycnic centrifugation in 12% Percoll solution. This distribution pattern of marker enzymes was quite constant. Peak values of K^+-dependent phosphatase, a marker enzyme of basolateral membranes, were found in either fraction number 4 or 5. γ-GTPase, used as a marker enzyme of brush-border membranes in kidney and intestine, gave a bimodal distribution of activity; the main peak was located at fraction 9 or 10, and a small peak or shoulder was seen at fraction 4 or

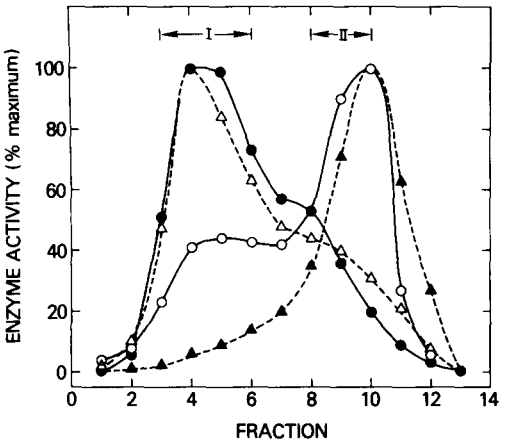

Fig. 1. Distribution of marker enzymes after centrifugation of crude plasma membrane fraction in a self-orienting Percoll gradient After differential centrifugation, the crude plasma membrane fraction was mixed with 12% Percoll and centrifuged at 41700×*g* for 30 min ●, K^+ −dependent phosphatase, ○, γ-glutamyl transpeptidase; △, alkaline phosphatase, ▲, cytochrome oxidase Fraction I (fraction numbers 3–6) was a basolateral membrane-enriched fraction. Fraction II (8–10) was presumed to be a luminal membrane-containing fraction.

5. Alkaline phosphatase which usually is distributed in the same fraction as γ-GTPase, i.e., with the luminal membranes of kidney and intestine, in parotid gland showed a similar distribution profile to K^+-dependent phosphatase. This is similar to recent findings with the exorbital lacrimal gland [9,10]. The peak of cytochrome oxidase activity, used as a marker enzyme of mitochondria, appeared always 0.5–1 fraction later than did the main peak of γ-GTPase. According to this distribution pattern of marker enzymes, we have considered fraction I (fraction numbers 3–6) as a basolateral membrane-enriched fraction. Table I shows the distribution, relative specific activities and effective recoveries of marker enzymes during the purification process. The yield and enrichment factor of K^+-dependent phosphatase in fraction I were 9.6% and 10-fold, respectively, and those of γ-GTPase were 3.9% and 4.2-fold. The recoveries of all marker enzymes from the gradient were comparable to those shown previously with intestinal cells [3].

α-Aminoisobutyrate transport by basolateral membrane vesicles

To evaluate the suitability of the described method to obtain functional isolated basolateral membrane vesicles, the ability of these vesicles to actively transport α-aminoisobutyrate was examined. As shown in Fig. 2, the uptake of α-aminoiso[^{14}C]butyrate by vesicles was time- and Na^+-dependent. In the presence of a Na^+ gradient, vesicles showed an 'overshoot' accumulation of α-aminoisobutyrate. Vesicles reached equilibrium by 150 min when incubated with KCl, but not when incubated in mannitol alone. However, vesicles incubated either with KCl or mannitol showed no difference in uptake within the first 10 min.

To discriminate between the transport and binding of α-aminoisobutyrate, the effect of a diminution in intravesicular space on the extent of α-aminoisobutyrate uptake was examined by increasing the osmolarity of the bathing solution with mannitol (100–640 mM). As shown in Fig. 3, when results are extrapolated to an infinitely small intravesicular space, the line passes through zero. This suggests that α-aminoisobutyrate did not merely bind to membranes but was transported into the intravesicular space, since intravesicular space and α-aminoisobutyrate accumulation decreased uniformly.

The uptake of α-aminoisobutyrate into parotid basolateral membrane vesicles exhibited several other characteristics expected for a Na^+-dependent amino acid transport activity [1]. First, uptake was dependent on the concentration of vesicular protein present during incubation (Fig. 4). Second, the activity was proportional to the extravesicular NaCl concentration (Fig. 5). Third, Na^+-dependent transport was temperature-dependent; no significant α-aminoisobutyrate uptake was detectable at 0°C, while uptak e at 37°C was much greater than that at 20°C (data not shown).

Next, we examined the effect of α-aminoisobutyrate concentration on uptake at initial velocity (1 min). Na^+-dependent α-aminoisobutyrate transport was saturable (Fig. 6), further supporting the notion that this is a carrier-mediated process. On the other hand, Na^+-independent uptake increased linearly with α-aminoisobutyrate concentration, as would be expected with passive dif-

TABLE I

DISTRIBUTION OF PROTEIN AND ENZYME ACTIVITIES

Each fraction was prepared as described under Materials and Methods, except that fractions I, II and remaining fractions were washed and suspended in buffer comprising 100 mM NaCl/100 mM mannitol/5 mM Hepes-Tris (pH 7.5) Numbers in parentheses indicate relative specific activities compared to the homogenate fraction. Data are the average ± S E of three separate experiments. Effective recovery refers to total enzyme activity recovered from the Percoll gradient compared with the amount of activity placed on the gradient, as described by Scalera et al [3]

Fraction	Protein	K^+-dependent phosphatase	γ-Glutamyl transpeptidase	Alkaline phosphatase	Cytochrome oxidase
Homogenate	100%	100% (1)	100% (1)	100% (1)	100% (1)
Pellet (2600 × g)	32.2 ±0.3	46.3 ±3 3 (1 44 ± 0 11)	49.7 ±5 7 (1 55 ± 0 19)	51.9 ±6 6 (1 61 ± 0 22)	65 3 ±1.0 (2.04 ±0 03)
Supernatant (23500 × g)	55.4 ±0 6	32.6 ±1.4 (0 59 ± 0 03)	32 1 ±1.4 (0 58 ± 0 03)	40 4 ±3.7 (0 72 ± 0 07)	6 05 ±0 74 (0 108 ± 0 013)
Crude membrane	6.99 ± 1.39	23 3 ±2 6 (3.99 ± 1 16)	19 4 ±1 7 (3.38 ± 1 03)	11 3 ±1 3 (1.95 ± 0 59)	15 3 ±2 2 (1 77 ±0.30)
Fraction I (tubes 3–6)	0 95 ± 0 11	9 63 ± 0 83 (10 2 ±0.55)	3 93 ± 0 35 (4 19 ± 0 34)	3 40 ± 0 62 (3 52 ± 0.43)	0 65 ±0 04 (0 69 ±0 14)
Fraction II (Tubes 8–10)	1 86 ± 0 45	1 73 ± 0 15 (1 20 ± 0 37)	8 33 ± 0 98 (5 55 ± 1 47)	2 20 ± 0 33 (1 33 ± 0 21)	4 95 ±0 04 (2 08 ±0 09)
Remaining fractions	0 94 ± 0.14	1.83 ± 0 39 (2 23 ± 0 77)	2.77 ± 0.12 (3 08 ± 0 31)	1 67 ± 0 17 (1 92 ± 0 42)	2.25 ±0 46 (2 06 ±0 18)
Effective recovery	55.7 ±3.6	57.2 ±2.0	78 2 ±5.9	66 0 ±4 1	52 6 ±4 2

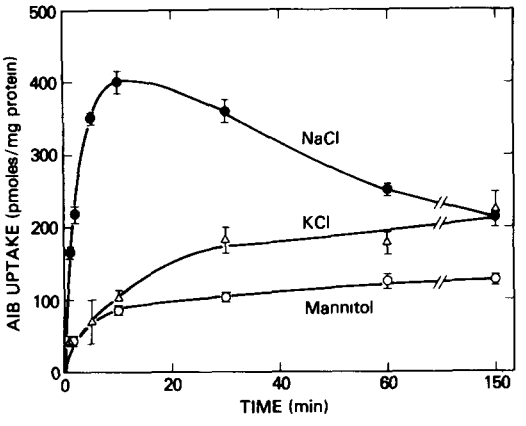

Fig. 2. Time-course of α-aminoiso[^{14}C]butyrate (AIB) uptake by isolated basolateral membrane vesicles. Membrane vesicles were incubated with 178 μM α-aminoiso[^{14}C]butyrate in the presence or absence of NaCl at 20°C for indicated times NaCl: 80 mM NaCl/20 mM KCl/100 mM mannitol; KCl: 100 mM KCl/100 mM Mannitol; Mannitol 20 mM KCl/260 mM mannitol.

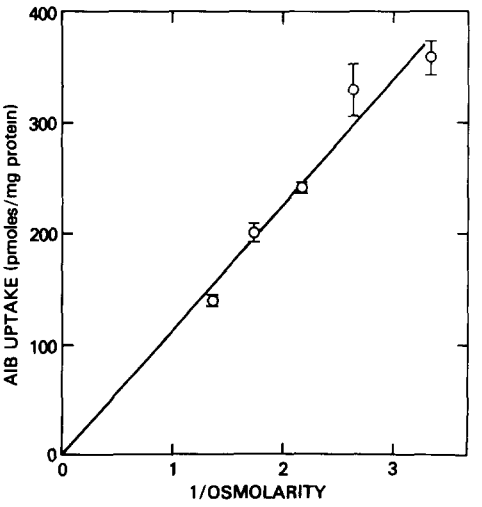

Fig. 3. Effect of extravesicular osmolarity on α-aminoisobutyrate (AIB) uptake by basolateral membrane vesicles Vesicles were incubated with increasing concentrations of mannitol (100–640 mM) in the presence of 178 μM α-aminoiso[^{14}C]butyrate, 80 mM NaCl and 20 mM KCl at 20°C for 5 min. The line shown was calculated by a least-squares regression analysis, $r = 0.938$

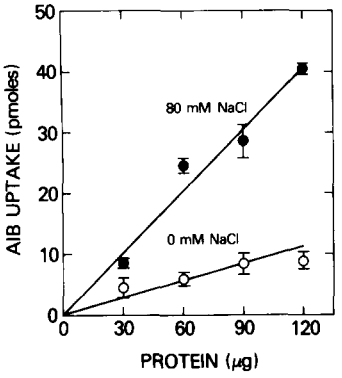

Fig. 4 Effect of vesicular protein concentration on α-aminoisobutyrate (AIB) uptake by basolateral membrane vesicles. Different amounts of vesicles were incubated in the presence or absence of 80 mM NaCl at 20°C for 5 min

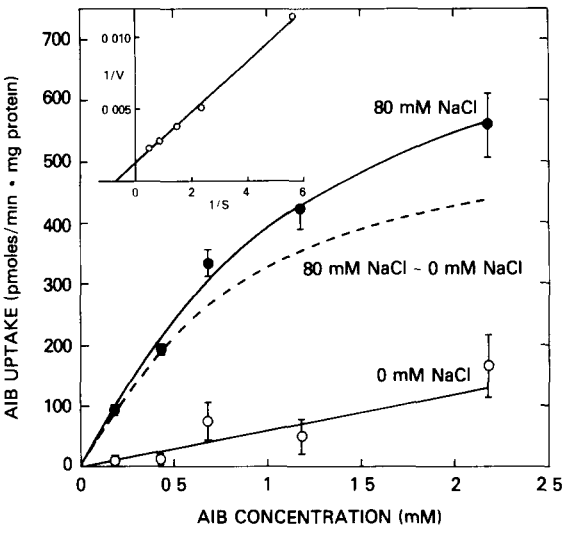

Fig. 6 Effect of α-aminoisobutyrate (AIB) concentration on α-aminoisobutyrate uptake by basolateral membrane vesicles Vesicles were incubated with increasing concentrations of α-aminoisobutyrate (178 μM–4.178 mM) in the presence or absence of 80 mM NaCl at 20°C for 1 min.

fusion. Double-reciprocal analysis of four separate experiments showed that the apparent K_m of Na^+-dependent α-aminoisobutyrate uptake was 1.28 ± 0.35 mM (mean ± S.E.) and V_{max} was 780 ± 170 pmol/min per mg protein.

Finally, we studied the effect of monensin and ouabain on Na^+-dependent α-aminoisobutyrate transport in these vesicles (Table II). Monensin, a sodium ionophore, markedly inhibited α-aminoisobutyrate uptake at 1 and 5 min. However, ouabain, an inhibitor of $(Na^+ + K^+)$ATPase, was without any effect on this process. After 120 min, all incubations reached an equilibrium level. Similar results were obtained after preincubation with vesicles and these reagents for 20 min at 20°C (not shown).

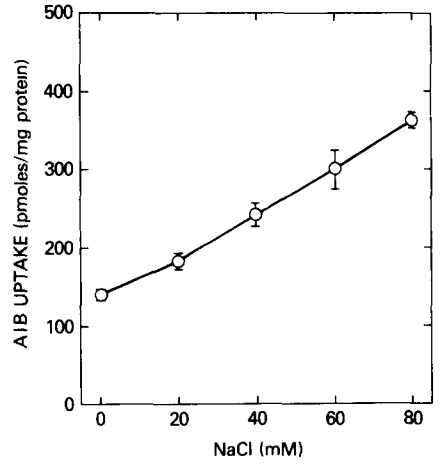

Fig. 5. Effect of extravesicular NaCl concentration on α-aminoisobutyrate (AIB) uptake by basolateral membrane vesicles Vesicles were incubated with increasing concentrations of NaCl (0–80 mM) at 20°C for 1 min

TABLE II

EFFECT OF MONENSIN AND OUABAIN ON α-AMINOISOBUTYRATE UPTAKE INTO PAROTID BASOLATERAL VESICLES

Data represent the average ± S.E. of four determinations in a representative experiment in which all treatments were performed simultaneously. Complete refers to transport studies in which vesicles were incubated in 80 mM NaCl/20 mM KCl/100 mM mannitol/5 mM Hepes-Tris (pH 7 5) at 20°C. Where NaCl was left out, it was replaced by KCl.

Treatment	α-Aminoisobutyrate uptake (pmol/mg protein)		
	1 min	5 min	120 min
Complete	381 ± 30	632 ± 26	467 ± 57
− NaCl	138 ± 12	197 ± 25	467 ± 18
+ Monensin (50 μM)	250 ± 5	300 ± 23	475 ± 12
+ Ouabain (1 mM)	403 ± 52	590 ± 40	497 ± 21

Discussion

The present study has shown that a basolateral plasma membrane-enriched fraction was readily isolated from rat parotid gland by self-orienting Percoll gradient centrifugation. The specific activity of K^+-dependent phosphatase, a marker enzyme of basolateral membranes, was enriched in our preparation 10-fold, similar to the enrichment factor seen for this enzyme in intestine [3] and kidney [4,5]. Although the isolation procedure used in the present study was almost identical with the procedures applied to intestine and kidney, the distribution of alkaline phosphatase activity was completely different. In kidney and intestine, alkaline phosphatase moved together with γ-GTPase, and was often used as a marker enzyme for brush-border membranes. In the exocrine exorbital lacrimal gland, however, alkaline phosphatase and K^+-dependent phosphatase are distributed in a very similar density region [9,10]. Similarly, in the parotid gland, we observed that alkaline phosphatase codistributed with K^+-dependent phosphatase. In a recent report, Arvan and Castle [13] prepared plasma membranes, by different methods, from rat parotid gland. Interestingly, the resulting membranes displayed a very low enrichment of alkaline phosphatase activity. The reason for this lack of ageement between the two studies is not clear, although Arvan and Castle [13] did utilize very young animals (100–125 g) of a different strain, which were starved overnight.

From the data available we cannot conclude that alkaline phosphatase is a constituent of basolateral membrane in parotid gland. However, alkaline phosphatase in the parotid gland does distribute quite differently from the enzyme in the kidney and intestine.

As noted above, γ-GTPase is often considered a marker of epithelial cell luminal membranes. This has also been suggested to be true for rat parotid cells [13]. Two distinct peaks of γ-GTPase activity were seen here with the Percoll gradient; the major peak was distinct from K^+-dependent phosphatase activity and is considered here to represent a parotid luminal membrane-enriched fraction. A lesser peak of activity consistently codistributed with the basolateral membrane fraction (K^+-dependent phosphatase-enriched). Heterogeneity of membrane fractions containing γ-GTPase has been previously reported for both the kidney [19] and the parotid gland [13], although a reasonable explanation for these differences is not yet apparent.

To evaluate the suitability of isolated basolateral plasma membrane vesicles for use with in vitro functional studies, we examined the ability of vesicles to transport α-aminoisobutyrate (system A). We chose this transport activity because α-aminoisobutyrate transport has been studied extensively by Keryer and Rossignol [20] using rat parotid gland minces. Thus, we are able to compare our data obtained with membrane vesicles to earlier results obtained with intact cells.

As shown above, α-aminoisobutyrate uptake was Na^+-dependent, osmotically sensitive and temperature-dependent, suggesting that α-aminoisobutyrate did not bind to the membrane but was transported into membrane vesicles. In the presence of a NaCl gradient, α-aminoisobutyrate transport into vesicles showed an overshoot accumulation. This implies that α-aminoisobutyrate was transported beyond its equilibrium concentration (uphill transport) energized by the Na^+ gradient. These characteristics of α-aminoisobutyrate transport have been observed also in intact parotid cells [20]. The K_m for Na^+-dependent α-aminoisobutyrate transport in basolateral membrane vesicles (1.28 ± 0.35 mM) was lower than that found in parotid minces (4.89 mM). The reason for this may be partly due to differences in the accessibility of α-aminoisobutyrate to plasma membranes in the two systems.

Monensin, a Na^+ ionophore, markedly inhibited α-aminoisobutyrate transport in these vesicles, presumably by abolishing the Na^+ gradient. This was also the situation observed in intact cells [20]. Ouabain, an inhibitor of $(Na^+ + K^+)$-ATPase which inhibited α-aminoisobutyrate uptake in intact cells, was without effect on transport in membrane vesicles. Preincubation with 1 mM ouabain for 20 min also resulted in no change from controls. It is not surprising that $(Na^+ + K^+)$-ATPase is not involved in α-aminoisobutyrate transport in this vesicle system, since incubations are performed without ATP.

In conclusion, these results suggest that basolateral plasma membrane vesicles prepared by a

Percoll gradient method, from rat parotid glands, have an active amino acid transport system like intact parotid cells and plasma membrane vesicles from cat pancreas [8]. Such a preparation should provide a potent tool for studying transport and secretion mechanisms of salivary glands.

Acknowledgements

We would like to thank Drs. Kenneth Spring, Joseph Handler, James Turner and Bertram Sacktor for many helpful discussions and suggestions, and Drs. Linda Cheng and C. Tony Liang for help in the development of the methods used here.

References

1 Murer, H and Kinne, R. (1980) J Membrane Biol. 55, 81–95
2 Sachs, G.R., Jackson, J and Rabon, E.C. (1980) Am. J. Physiol. 238, G151–G164
3 Scalera, V, Storelli, C., Storelli-Joss, C., Haase, W. and Murer, H. (1980) Biochem. J. 187, 177–181
4 Sactor, B, Rosenbloom, I.L., Liang, C T. and Cheng, L (1981) J. Membrane Biol. 60, 63–71
5 Inui, K., Okano, T, Takano, M., Kitazawa, S and Hori, R. (1981) Biochim. Biophys. Acta 637, 150–154
6 Milutinovic, S., Sachs, G., Hasse, W and Schulz, I. (1977) J. Membrane Biol 36, 253–279
7 Milutinovic, S., Argent, B.E., Schulz, I. and Sachs, G. (1977) J. Membrane Biol 36, 281–295
8 Tryakowski, T, Milutinovic, S. and Schulz, I. (1978) J Membrane Biol. 38, 333–346
9 Mircheff, A.K., Conteas, C.N., Lu, C C., Santiago, N.A., Gray, G M. and Lipson, L G (1983) Am. J. Physiol. 245, G133–G142
10 Mircheff, A K, Lu, C.C. and Conteas, C.N. (1983) Am J. Physiol. 245, G661–G667
11 Durham, J P, Gialanti, N. and Revis, N W (1975) Biochim. Biophys. Acta 394, 388–405
12 Williams, M.A., Pratten, M.K, Turner, J W. and Cape, G H. (1979) Histochem. J. 11, 19–50
13 Arvan, P. and Castle, J.D. (1982) J Cell Biol 95, 8–19
14 Schwartz, A, Lindermagen, G.E and Allen, J.G. (1975) Pharm Rev. 27, 3–85
15 Tate, S.S. and Meister, A. (1974) J Biol Chem. 249, 7593–7602
16 Bernt, E. (1974) in Methods of Enzymatic Analysis, Vol. 2 (Bergmeyer, H.U., ed.), pp 868–870, Academic Press, New York
17 Wharton, D.C and Tzagaloff, A. (1967) Methods Enzymol 10, 245–250
18 Lowry, O.H., Rosebrough, N.J, Farr, A.L. and Randall, R.J. (1951) J. Biol Chem. 193, 265–275
19 Mamelok, R.D., Groth, D.F. and Prusiner, S B. (1980) Biochemistry 19, 2367–2373
20 Keryer, G and Rossignol, B. (1980) Am. J. Physiol 239, G183–G189