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# $(Na^+ + K^+)$-ATPase ACTIVITY OF CRUDE HOMOGENATES OF RAT SKELETAL MUSCLE AS ESTIMATED FROM THEIR $K^+$-DEPENDENT 3-*O*-METHYLFLUORESCEIN PHOSPHATASE ACTIVITY

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**A highly sensitive fluorimetric assay using 3-*O*-methylfluorescein phosphate as substrate was used in the determination of $K^+$-dependent phosphatase activity in preparations of rat skeletal muscle. The gastrocnemius muscle was chosen because of mixed fibre composition. Crude, detergent treated homogenate was used so as to avoid loss of activity during purification. $K^+$-dependent phosphatase activities in the range 0.19–0.37 $\mu mol \cdot (g\ wet\ weight)^{-1} \cdot min^{-1}$ were obtained, the value decreasing with age and $K^+$-deficiency. Complete inhibition of the $K^+$-dependent phosphatase was obtained with $10^{-3}$ M ouabain. Using a KSCN-extracted muscle enzyme the intimate relation between $K^+$-dependent phosphatase activity and $(Na^+ + K^+)$-activated ATP hydrolysis could be demonstrated. A molecular activity of 620 $min^{-1}$ was estimated from simultaneous determination of $K^+$-dependent phosphatase activity and [$^3$H]ouabain binding capacity using the partially purified enzyme preparation. The corresponding enzyme concentration in the crude homogenates was calculated and corresponded well with the number of [$^3$H]ouabain binding sites measured in intact muscles or biopsies hereof.**

## Introduction

Our studies on the density of sodium pumps in intact skeletal muscle have repeatedly disclosed a much higher [$^3$H]ouabain binding capacity than would be expected from studies on the activity of enzyme purified from this tissue [1–3]. This discrepancy calls upon independent methods for the estimation of pump density. Unfortunately, the extremely low $(Na^+ + K^+)$-ATPase activity of muscle homogenates is overshadowed by unspecific ATPases making its measurement unreliable. The $K^+$-dependent phosphatase activity, which is associated with the sodium pump [4,5] seems more suitable for measurements by means of a highly sensitive fluorimetric assay for $K^+$-dependent phosphatase activity using 3-*O*-methylfluorescein phosphate [6]. This method allows the determination of the hydrolytic activity of the sodium pump in preparations with low activity such as present in crude homogenates [7] hereby avoiding complications due to isolation of the enzyme and ensuring that the total enzyme activity in the tissue is measured.

The muscle $(Na^+ + K^+)$-ATPase is of importance in muscular function under physiological and pathophysiological conditions. Thus, it is responsible for the rapid reuptake of the considerable amounts of $K^+$ lost during muscle activity [8]. Furthermore, it has been demonstrated that the number of sodium pumps in skeletal muscle estimated either from $(Na^+ + K^+)$-ATPase activity or from [$^3$H]ouabain binding capacity shows pronounced variation under a wide range of physiological and pathophysiological conditions such as age [9,10], $K^+$-depletion [11], hypo- and hyper-

thyroidism [12,13], denervation [14,15] and muscular dystrophy [16,17].

In the present study the $K^+$-dependent phosphatase activity has been measured by the fluorimetric assay in crude homogenates of skeletal muscle of $K^+$-depleted and normal rats of various age. This allowed comparison between results obtained using this method and measurement of [$^3$H]ouabain binding to intact muscles or biopsies hereof [18].

## Methods

*Animals.* All experiments were performed using fed male or female Wistar rats in the age range from 28 to 78 days. $K^+$-deficiency was induced by giving the animals Altromin $K^+$-deficient diet containing 0.75 mmol $K^+$ per kg and distilled water for 1 week. After 1 week on this diet determination of the total $K^+$-content in the gastrocnemius muscle showed this to be reduced by 50%.

*Enzyme preparation.* The gastrocnemius muscle is known to contain both type I and II fibres [19] and was therefore chosen as representative for the skeletal muscle system in general. After the animals had been killed by decapitation the muscle was dissected out. 1 g of tissue was minced in a garlic press leaving 2–3% behind, which probably represents connective tissue. The mince was homogenized in 9 ml buffer containing 30 mM histidine, 2 mM EDTA and 250 mM sucrose (pH 7.2) at 0°C with 10 strokes in a glass homogenizer with a tight fitting Teflon pestle operating at 1000 rpm. One hundred $\mu$l of this preparation was suspended in 900 $\mu$l of buffer containing 20 mM imidazole, 2 mM EDTA, 250 mM sucrose (pH 7.0) and 0–0.20% (w/v) sodium deoxycholate at 24°C for 0–60 min to demask latent ATPase activity.

In a few experiments the ($Na^+ + K^+$)-ATPase of muscle homogenate was partially purified by adding solid KSCN to a final concentration of 1 M to solubilize myosin and to suppress unspecific ATPase activity [20]. After stirring for 120 min at 24°C the preparation was centrifuged at 100 000 × *g* for 30 min, washed twice and resuspended to its original volumen in the histidine-sucrose buffer. The preparation was stored at −20°C until use and treated with detergent as described above. The final $K^+$ concentration in the enzyme preparation as determined by flame photometry was less than 0.5 mM, and protein concentration determined by the method of Lowry et al. [21] was around 3 mg/ml. Determination of [$^3$H]ouabain binding capacity was carried out in the presence of 3 mM $Mg^{2+}$, 120 mM $Na^+$ and 3 mM ATP at 37°C. After 120 min of incubation the preparation was washed twice by centrifugation at 0°C in the presence of $K^+$. $^3$H-activity was determined in the pellet and after correction for unspecific binding the [$^3$H]ouabain binding capacity was calculated.

Finally, partially purified ($Na^+ + K^+$)-ATPase from pig kidney outer medulla was prepared according to Jensen et al. [22] followed by treatment with SDS. Its [$^{48}$V]vanadate and [$^3$H]ouabain binding capacity was determined as described earlier [23].

*Assay of 3-O-methylfluorescein phosphatase activity.* The assay medium contained 19.5 $\mu$M 3-*O*-methylfluorescein phosphate, 4 mM $MgCl_2$, 1 mM EDTA, 80 mM Tris (pH 7.6) and 10–30 $\mu$l of the final homogenate corresponding to 100–300 $\mu$g wet wt. of tissue in a total volume of 2600 $\mu$l. $K^+$-dependent phosphatase activity was measured after addition of 2 M KCl to give a final $K^+$ concentration of 10 mM. To examine ouabain sensitivity this drug was added at a final concentration of $10^{-3}$ M and binding to the enzyme was allowed to take place at 37°C for 10 min before assaying enzyme activity. ATP activation in the presence of $Na^+$ was investigated with 1 mM $K^+$, 10 mM $Na^+$ and 5 $\mu$M ATP.

The fluorimetric assays were performed using a Perkin-Elmer MPF-44A spectrofluorimeter equipped with continuous stirring. The excitation wavelength was 475 nm, emission wavelength 515 nm and slitwidths 5 nm. The emission deflection was demonstrated to be proportional to the concentration of 3-*O*-methylfluorescein and a concentration of 0.1 $\mu$M in the assay medium was used as standard. A temperature of 37°C was ensured using continuous temperature recording. Based on the slope of the lines recording the fluorescence, the phosphatase activities were calculated and expressed as $\mu mol \cdot (g\ tissue)^{-1} \cdot min^{-1}$. Correction for spontaneous hydrolysis of 3-*O*-methylfluorescein phosphate was done and by substracting the value obtained in the absence from that obtained in the presence of $K^+$, the

$K^+$-dependent phosphatase activity was calculated.

*Chemicals.* All chemicals were of analytical grade. $K^+$-deficient fodder was from Altromin Co. Lage, F.R.G. 3-*O*-methylfluorescein and 3-*O*-methylfluorescein phosphate were from Sigma Chemicals, St. Louis, MO, U.S.A. and ouabain from Merck Co., Darmstadt, F.R.G. [$^3$H]Ouabain was from the New England Nuclear Corporation, Boston, MA, U.S.A. and [$^{48}$V]vanadate from Amersham International, Amersham, U.K.

## Results

*Experiments with partially purified ($Na^+ + K^+$)-ATPase from pig kidney and KSCN-extracted rat muscle homogenate*

In order to characterize the basic properties of the $K^+$-dependent phosphatase activity of muscle homogenate, experiments were performed on partially purified enzyme preparations. First, the $K^+$-dependent 3-*O*-methylfluorescein phosphatase activity of the conventional ($Na^+ + K^+$)-ATPase preparation from pig kidney was determined. It could be demonstrated to obey simple Michaelis-Menten kinetics with respect to 3-*O*-methylfluorescein phosphate. Based on [$^{48}$V]vanadate and

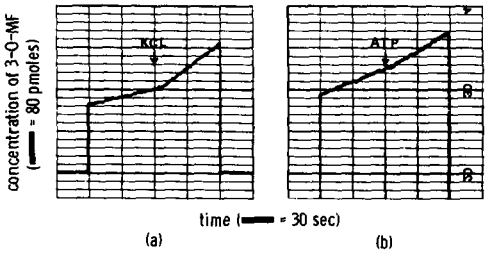


Fig. 1. The effect of $K^+$ (a) and ATP (b) on 3-*O*-methylfluorescein phosphatase activity of partially purified muscle homogenates prepared from the gastrocnemius muscle of 28-day-old rats. After extraction of the homogenate with KSCN and subsequent detergent treatment the 3-*O*-methylfluorescein phosphatase activity was determined using 10 $\mu$l of enzyme corresponding to 30 $\mu$g protein or 500 $\mu$g of tissue and 19.5 $\mu$M 3-*O*-methylfluorescein phosphate as substrate in a final volume of 2600 $\mu$l. In (a) the medium contained 4 mM $MgCl_2$, 1 mM EDTA and 80 mM Tris (pH 7.6). 10 mM KCl was added as indicated. In (b) the medium contained 4 mM $MgCl_2$, 1 mM EDTA, 80 mM Tris (pH 7.6), 1 mM KCl and 10 mM NaCl. 5 $\mu$M ATP was added as indicated. 3-O-MF, 3-*O*-methylfluorescein.

[$^3$H]ouabain binding capacities of 1.26 and 1.30 nmol · (mg protein)$^{-1}$, respectively, a molecular activity of 440 min$^{-1}$ could be calculated for this preparation.

Hereafter, the $K^+$-dependent 3-*O*-methylfluorescein phosphatase activity of the KSCN-extracted homogenate from muscle was determined. It could also be demonstrated to obey Michaelis-Menten kinetics with respect to 3-*O*-methylfluorescein phosphate and a substrate concentration of 19.5 $\mu$M appeared to be close to optimum. Since this preparation is devoid of unspecific phosphatase, the activity before the addition of $K^+$ represented spontaneous hydrolysis of 3-*O*-methylfluorescein phosphate. As can be seen from Fig. 1a the addition of 10 mM $K^+$ caused a marked increase in the phosphatase activity. This activity could be completely inhibited by $10^{-3}$ M ouabain. Furthermore, $Na^+$ is known to be inhibitory to the $K^+$-dependent phosphatase and the inhibition can be overcome by ATP [24]. From Fig. 1b it can be seen that 10 mM $Na^+$ in the presence of 1 mM $K^+$ reduced the phosphatase activity to around 30% of the activity in the presence of $K^+$. The addition of 5 $\mu$M. ATP partially relieved this inhibition, increasing the activity to around 70% of the $K^+$-dependent phosphatase activity. Taken together, the above-mentioned observations verify the intimate connection between $K^+$-dependent phosphatase activity and overall ($Na^+ + K^+$)-activated ATP hydrolysis in homogenates of muscle. Based upon the [$^3$H]ouabain binding capacity of the KSCN-extracted muscle homogenate, a molecular activity of 620 min$^{-1}$ could be determined, i.e. in reasonable agreement with the molecular activity determined for the kidney enzyme.

However, the $K^+$-dependent phosphatase activity of KSCN-extracted muscle homogenate appeared to be only a fraction, roughly 1/3, of the activity of the crude homogenate. Therefore, the crude homogenate was used in the following experiments for the quantification of the total enzyme activity, but the molecular activity determined on the KSCN-extracted preparation was used for the calculation of the corresponding total enzyme concentration.

*Experiments with crude muscle homogenates*

Because of vesicle formation by plasma mem-

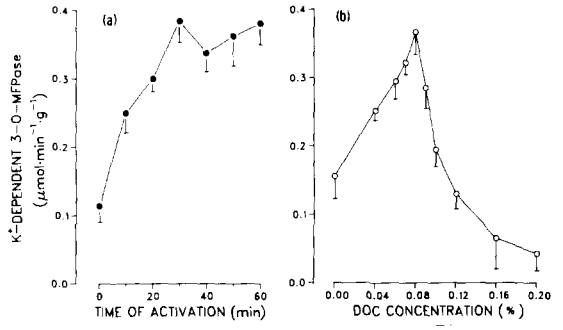


Fig. 2. The effect of deoxycholate (DOC) treatment of crude muscle homogenate on $K^+$-dependent 3-*O*-methylfluorescein phosphatase (3-O-MFPase) activity. The homogenate was prepared from gastrocnemius of 28-day-old rats. Ten $\mu$l of enzyme corresponding to 100 $\mu$g of tissue was used for the assay. The medium contained 4 mM $MgCl_2$, 1 mM EDTA and 80 mM Tris (pH 7.6) and as substrate was used 19.5 $\mu$M 3-*O*-methylfluorescein phosphate in a final volume of 2600 $\mu$l The $K^+$-dependent phosphatase was calculated as the difference between the 3-*O*-methylfluorescein phosphatase activity in the presence and the absence of 10 mM $K^+$. In (a) a deoxycholate concentration of 0.08% was used and the activity was measured after 0–60 min of activation. In (b) a deoxycholate concentration of 0–0.20% was used and the activity was measured after 30 min of activation. Each point represents the mean of three assays with bars denoting S.E.

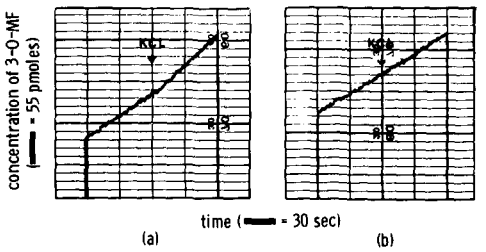


Fig. 3. The effect of $K^+$ (a) and ouabain plus $K^+$ (b) on 3-*O*-methylfluorescein phosphatase activity of crude muscle homogenates prepared from the gastrocnemius of 28-day-old rats. After activation with 0.08% deoxycholate for 30 min the 3-*O*-methylfluorescein phosphatase activity was determined using 10 $\mu$l of enzyme corresponding to 100 $\mu$g of tissue in a final volume of 2600 $\mu$l. In (a) the medium contained 4 mM $MgCl_2$, 1 mM EDTA and 80 mM Tris (pH 7.6) and as substrate was used 19.5 $\mu$l 3-*O*-methylfluorescein phosphate. 10 mM KCl was added as indicated. In (b) the experimental conditions were as in (a) except that 1 mM ouabain was added to the medium 10 min before the assay. 3-O-MF, 3-*O*-methylfluorescein.

branes during homogenization, unmasking of the enzyme activity by making them leaky to substrate and ligands was necessary. Deoxycholate was chosen as detergent and experiments were performed in order to define the optimal activation with respect to time and concentration. As can be seen from Fig. 2(a) the $K^+$-dependent phosphatase activity in the presence of 0.08% deoxycholate reached a maximum after 30 min and remained almost constant for up to 60 min of activation. This is in good agreement with results obtained using purified renal ($Na^+ + K^+$)-ATPase [25]. Fig. 2b shows the $K^+$-dependent phosphatase activity after an activation period of 30 min as a function of deoxycholate concentration. Maximum activity was obtained at 0.08% deoxycholate and this was followed by a rapid decline of activity. The unspecific phosphate activity did not change with the deoxycholate concentration. Based upon these experiments a standard activation procedure with 0.08% deoxycholate for 30 min was used in the following experiments.

Fig. 3 shows the increase of 3-*O*-methylfluorescein using crude homogenate as a function of time. As can be seen from Fig. 3a there was a considerable phosphatase activity, around 2/3 of the total activity, before addition of $K^+$. This represents spontaneous hydrolysis and unspecific $Mg^{2+}$-activated phosphatase activity of the crude homogenate. The addition of $K^+$ caused an increase in the phosphatase activity of around 1/3 of the total activity. A similar pattern is seen using crude homogenates of human red cells [26]. It can be seen from Fig. 3b, that the addition of $10^{-3}$ M ouabain totally abolished $K^+$-activation of the phosphatase activity. When ouabain was added simultaneously with or after the addition of $K^+$ no or only a minor inhibition could be demonstrated, indicating that the inhibition of ouabain is slow and antagonized by $K^+$.

In order to assess the reproducibility of the method, the $K^+$-dependent phosphatase activity was determined in a series of crude homogenates of gastrocnemius muscles from $K^+$-depleted and normal rats of various ages. This would allow comparison with results obtained using intact muscles or biopsies hereof. From Table I it can be seen that the $K^+$-dependent phosphatase activity in muscle homogenates of 35-day-old female rats was 0.329 $\mu$mol · $min^{-1}$ · (g wet wt.)$^{-1}$. At 78 days of life the $K^+$-dependent phosphatase activity in

TABLE I

THE EFFECT OF AGE AND $K^+$-DEPLETION ON 3-*O*-METHYLFLUORESCEIN PHOSPHATASE ACTIVITY IN CRUDE MUSCLE HOMOGENATES OF GASTROCNEMIUS

Rats of the indicated age and treatment were used. Experimental conditions as in Fig. 3a. The results are given as mean values ± S.E. with the number of assays in parentheses. In each group at least four different animals were used. The *P* values were calculated using the *t*-test for groups of non-paired observations. The estimated number of sodium pumps was calculated based on a molecular activity of 620 $min^{-1}$.

| Age and treatment | 3-*O*-methylfluorescein phosphatase activity ($\mu mol \cdot min^{-1} \cdot g^{-1}$) | | | | Estimated number of sodium pumps ($pmol \cdot g^{-1}$) |
|---|---|---|---|---|---|
| | Basal | Total | $K^+$-dependent | | |
| Female, 35 days normal | 0.569 ± 0.013 (12) | 0.898 + 0.022 (12) | 0.329 ± 0.012 (12) | | 530 |
| Female, 35 days $K^+$-depleted 1 week | 0.487 ± 0.011 (16) | 0.692 ± 0.014 (16) | 0.205 ± 0.007 (16) | $P < 0.001$ | 330 |
| Female, 78 days normal | 0.319 ± 0.018 (10) | 0.511 ± 0.023 (10) | 0.192 ± 0.012 (10) | $P < 0.001$ | 310 |

muscle homogenates of female rats was decreased by 42% and the unspecific phosphatase activity by 44%. Furthermore, 1 week on $K^+$-deficient fodder caused a decrease of 38% in the $K^+$-dependent phosphatase activity in muscle homogenate of 35-day-old female rats, whereas the unspecific phosphatase activity only decreased by 14%. Based on a molecular activity of 620 $min^{-1}$ determined on KSCN-extracted muscle homogenate, the corresponding enzyme concentration could be calculated (Table I).

## Discussion

Determination of $(Na^+ + K^+)$-ATPase activity of muscle homogenates is unreliable by conventional end-point $P_i$ measurements due to the large contamination with unspecific ATPases (for a discussion, see Ref. 27). A continuous, sensitive recording of phosphatase activity utilizing a fluorigenic substrate, 3-*O*-methylfluorescein phosphate, has been described (Hill et al. [6]. The advantages of a kinetic versus an endpoint assay include the ability to use the same sample as its own reference for the determination of unspecific and total phosphatase activity. The sensitivity of the fluorimetric assay implies that only small amounts of tissue homogenates need to be employed and the ratio of $K^+$-dependent phosphatase/total phosphatase activity seems more favourable using this assay. Furthermore, graded ouabain inhibition in human red cells has shown that the $K^+$-dependent phosphatase activity associated with the $(Na^+ + K^+)$-ATPase is proportional to the overall hydrolysis of ATP [26].

Measurements of the hydrolytic activity or the number of sodium pumps in skeletal muscle have usually been performed using $(Na^+ + K^+)$-ATPase preparations or membrane fractions isolated by differential centrifugation. Due to difficulties in ensuring complete recovery of the $(Na^+ + K^+)$-ATPase activity as well as exposure of all available sites or ligands, this approach yields little or no information about the total enzyme activity per g tissue. In the present study the above-mentioned kinetic method for the determination of $K^+$-dependent phosphatase activity using 3-*O*-methylfluorescein phosphate as substrate was used on crude homogenates of skeletal muscle. Using the molecular activity obtained from partially purified muscle enzyme it became possible to quantitate the total number of sodium pumps.

The anticipated association between $K^+$-dependent phosphatase activity and overall $(Na^+ + K^+)$-activated ATP hydrolysis was demonstrated using the KSCN-extracted muscle enzyme. Thus, complete ouabain inhibition of the $K^+$-dependent phosphatase activity as well as relieve of $Na^+$ inhibition by ATP could be clearly demonstrated. However, a considerable loss of $K^+$-dependent phosphatase activity was the consequence of this partial purification of the enzyme by isolation of a

particulate fraction of the homogenate. Huang et al. [28] noticed the same using heart muscle. Contrary to their observation our recordings on crude homogenates were linear and reproducible. Crude homogenates were thus preferred for the KSCN-extracted homogenate for quantitation of enzyme activity. Still, for convenience the molecular activity, i.e. the relationship between ouabain binding capacity and phosphatase activity was determined on the KSCN-extracted muscle homogenate. This molecular activity appeared reasonably similar to that determined on purified pig kidney $(Na^+ + K^+)$-ATPase.

The concentration of the sodium pumps in the crude muscle homogenate was calculated on the basis of $K^+$-dependent phosphatase activity and molecular activity and compared to the number of [$^3$H]ouabain binding sites determined in intact muscles or biopsies hereof. It has been demonstrated, that the number of [$^3$H]ouabain binding sites in intact soleus muscle of female rats decreased from around 430 pmoles · (g wet wt.)$^{-1}$ at 35 days to around 240 pmol · (g wet wt.)$^{-1}$ after 85 days of life [9]. In the present study the estimated number of sodium pumps in the gastrocnemius muscle decreased from around 530 pmol · (g wet wt.)$^{-1}$ at 35 days to around 330 pmol · (g wet wt.)$^{-1}$ at 78 days of life. The soleus muscle predominantly contains type II fibres whereas gastrocnemius contains both type I and II fibres [19]. Furthermore, it has been shown that the number of [$^3$H]ouabain binding sites is 20% higher in type I than in type II fibres [29]. Taking into consideration the difference in fibre composition between soleus and gastrocnemius, there seems to be reasonable agreement between the methods with respect to changes with age. Furthermore, a decrease in $K^+$-dependent phosphatase activity associated with $K^+$-depletion by 38% is in close accordance with the results obtained using intact soleus muscles [15]. Thus, there seems to be a good agreement between the number of sodium pumps in skeletal muscle estimated by [$^3$H]ouabain binding and $K^+$-dependent phosphatase activity.

A simple and reliable method for the determination of the number of sodium pumps in muscle homogenates was needed. We believe that the method presented here offers adequate reproducibility for the quantitative analysis of the number of sodium pumps in skeletal muscle under various conditions, and it may be an attractive alternative to measurements of [$^3$H]ouabain binding to intact muscle cells or biopsies hereof.

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## References

1 Sulakhe, P.V., Fedelesova, M., McNamara, D.B. and Dhalla, N.S. (1971) Biochem. Biophys. Res. Commun. 42, 793–800
2 Andrew, C.G. and Appel, S.H. (1973) J. Biol. Chem. 248, 5156–5163
3 Seiler, S. and Fleischer, S. (1982) J. Biol. Chem. 257, 13862–13871
4 Albers, R.W. and Koval, G.J. (1966) J. Biol. Chem. 241, 1896–1898
5 Askari, A. and Koyal, D. (1968) Biochem. Biophys. Res. Commun. 32, 227–232
6 Hill, H.D., Summer, G.K. and Waters, M.D. (1968) Anal. Biochem. 24, 9–17
7 Huang, W. and Askari, A. (1975) Anal. Biochem. 66, 265–271
8 Sreter, F.A. (1963) Am. J. Physiol. 205, 1295–1298
9 Kjeldsen, K., Nørgaard, A. and Clausen, T. (1982) Biochim. Biophys. Acta 686, 253–256
10 Vigne, P., Frelin, C. and Lazdunski, M. (1982) J. Biol. Chem. 257, 5380–5384
11 Nørgaard, A., Kjeldsen, K. and Clausen, T. (1981) Nature 293, 739–741
12 Asano, Y., Liberman, U.A. and Edelman, I.S. (1976) J. Clin. Invest 57, 368–379
13 Biron, R., Burger, A., Chinet, A., Clausen, T. and Dubois-Ferriere, R. (1979) J. Physiol. 297, 47–60
14 Clausen, T., Sellin, L.C. and Thesleff, S. (1981) Acta Physiol. Scand. 111, 373–375
15 Clausen, T., Kjeldsen, K. and Nørgaard, A. (1983) J. Physiol., 345, 123–134
16 Desaiah, D., Mishra, S.K. and Hobson, M. (1981) J. Neurol. Sci. 51, 457–464
17 Desnuelle, C., Lombet, A., Serratrice, G. and Lazdunski, M. (1982) J. Clin. Invest. 69, 358–367
18 Nørgaard, A., Kjeldsen, K., Hansen, O. and Clausen, T. (1983) Biochim. Biophys. Res. Commun. 111, 319–325
19 Close, R.I. (1972) Physiol. Rev. 52, 129–197
20 Klodos, I., Ottolenghi, P. and Boldyrev, A. (1975) Anal. Biochem. 67, 397–403
21 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275

22 Jensen, J., Nørby, J.G. and Ottolenghi, P. (1984) J. Physiol., 346, 219–241
23 Hansen, O. (1982) Biochim. Biophys. Acta 692, 187–195
24 Koyal, D., Rao, S.N. and Askari, A. (1971) Biochim. Biophys. Acta 225, 11–19
25 Jørgensen, P.L. and Skou, J.C. (1971) Biochim. Biophys. Acta 233, 366–380
26 Huang, W. and Askari, A. (1976) Arch. Biochim. Biophys. 175, 185–189
27 Corrie, W.S. and Bonting, S.L. (1966) Biochim. Biophys. Acta 120, 91–96
28 Huang, W., Rhee, H.M., Chiu, T.H. and Askari, A. (1979) J. Pharmacol. Exp. Ther. 211, 571–582
29 Clausen, T., Hansen, O., Kjeldsen, K. and Nørgaard, A. (1982) J. Physiol. 333, 367–381