A RAPID METHOD FOR THE PREPARATION OF SARCOLEMMAL VESICLES FROM RAT AORTA, AND THE STIMULATION OF CALCIUM UPTAKE INTO THE VESICLES BY CYCLIC AMP-DEPENDENT PROTEIN KINASE

Keith J. BROCKBANK and Paul J. ENGLAND

Department of Biochemistry, University of Bristol Medical School, University Walk, Bristol BS8 1TD, England

Received 15 October 1980

1. Introduction

In vascular smooth muscle, extracellular Ca^{2+} is required for the maximal development of tension in response to hormonal or electrical stimulation [1–3]. During subsequent relaxation, Ca^{2+} must be transported back across the sarcolemma by an energy-dependent process [4]. This relaxation can be stimulated by hormones which elevate intracellular cyclic nucleotide concentrations [5,6]. The known action of cyclic nucleotides in eukaryotes is to activate protein kinases leading to phosphorylation of substrate proteins [7]. We have therefore investigated the phosphorylation of sarcolemmal vesicles from rat aorta, and the effect of this on Ca^{2+} transport.

A number of methods of preparation of sarcolemmal vesicles from a variety of tissues have been reported. These include enzymatic disaggregation and/or osmotic shock [8,9], and various methods of homogenisation [10,11], in each case followed by sucrose density gradient centrifugation. With preparations of sarcolemmal vesicles from smooth muscle using these methods, contamination by sarcoplasmic reticulum has been reported [11]. The effect of phosphorylation of sarcolemmal vesicles from smooth muscle by cyclic AMP-dependent protein kinase has also been studied. Using crude membrane fractions from trachea [12], it was reported that on incubation with [γ-^{32}P]ATP and cyclic AMP-dependent protein kinase phosphorylation of sarcolemmal proteins did not occur, and there was no change in oxalate-dependent Ca^{2+} uptake. In contrast, using a more purified preparation from rat aorta [13], a protein of M_r 44 000 was phosphorylated under similar conditions, and an increase in Ca^{2+} uptake was reported.

We now report a new, rapid method for the preparation of purified sarcolemmal vesicles free of mitochondrial and cytoplasmic contamination using Percoll. The preparation also appears to have a low level of contamination by sarcoplasmic reticulum. On incubation with [γ-^{32}P]ATP and cyclic AMP-dependent protein kinase, proteins of app. M_r 11 000, 21 000, 44 000 and 110 000 are phosphorylated. The phosphorylation also causes a 2–3-fold stimulation of oxalate-independent Ca^{2+}-uptake which is inhibited by ouabain.

2. Methods

2.1. *Preparation of sarcolemmal vesicles*

Thoracic aortae were removed from freshly-killed Wistar rats (200–250 g), stripped of adhering tissue, and homogenised in 0.25 M sucrose, 50 mM imidazole, 14 mM 2-mercaptoethanol, 1 mM EDTA, 10 mM $MgCl_2$ (pH 7.4) (5 ml/g tissue) using a Polytron homogeniser (setting 5 for 20 s). All procedures were done at 4°C. The homogenate was filtered through glass wool, which was then washed with an equal volume of homogenisation medium. The pooled filtrate was centrifuged at 50 000 × *g* for 45 min, and the pellet resuspended in 1 ml Percoll medium/g original tissue [Percoll medium: 0.17 ml Percoll (Pharmacia Ltd)/ml in 0.3 M KCl, 0.25 M sucrose, 50 mM imidazole, 14 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 1 mM EDTA (pH 7.4)]. The initial density of the Percoll in the medium was 1.05. This was thoroughly mixed with a further 8 vol. Percoll medium, and centrifuged at 10 000 × *g* for 15 min [14]. Fractions of 1 ml were taken from the bottom of the tube.

2.2. *Measurement of calcium uptake and protein phosphorylation*

Ca^{2+} uptake into sarcolemmal vesicles was measured by filtration on Millipore membranes using $^{45}CaCl_2$ in the absence of EGTA, essentially as in [15]. The concentration of vesicles in the uptake medium was 30 μg protein/ml. Oxalate was omitted from certain incubations as indicated in section 3.

Phosphorylation of sarcolemmal vesicles was carried out by incubation with added cyclic AMP-dependent protein kinase. For the investigation of the effect of phosphorylation on Ca^{2+} uptake, vesicles (~2 mg protein/ml) were preincubated for up to 10 min at 37°C with 0.1 mg/ml partially-purified beef heart protein kinase [16], 40 μM cyclic AMP, 20 mM ATP, 0.8 mM theophylline, 21.6 mM $MgCl_2$, 1.6 mM NaF, 0.4 mM EGTA, 1.7 mM 2-mercaptoethanol, 20 mM Tris-phosphate (pH 7.0). For the investigation of ^{32}P-incorporation into the vesicles, the conditions above were used but with 0.1 mM [γ-^{32}P]ATP (spec. radioact. 20–25 Bq./pmol) and 1.7 mM $MgCl_2$. ^{32}P-incorporation was maximal after 1 min incubation. The reaction was stopped by the addition of trichloroacetic acid (final conc. 0.5 M), and the precipitated protein redissolved in buffer and subjected to SDS–polyacrylamide gel electrophoresis as in [17]. Protein was detected by staining with Coomassie brilliant blue, and protein-bound ^{32}P measured by densitometric scanning after radioautography of the gel.

2.3. *Methods of enzyme assays*

Ouabain-sensitive K^+-stimulated phosphatase was measured with *p*-nitrophenyl phosphate as substrate [18]. Ca^{2+}/Mg^{2+}-ATPase and Na^+/K^+-ATPase were measured as in [18]. 5′-Nucleotidase was assayed with 5′-AMP as substrate [19]. Alkaline phosphatase was assayed at pH 9.0 with *p*-nitrophenyl phosphate as substrate [20]. Succinate dehydrogenase was measured with dichlorophenylindophenyl as electron acceptor [21]. Lactate dehydrogenase was assayed with pyruvate as substrate [22]. Protein was measured [23] with bovine serum albumin as standard.

3. Results and discussion

3.1. *Characterisation of sarcolemmal vesicles*

Fig.1 shows the profiles of protein, Ca^{2+} uptake (in the presence of oxalate), 5′-nucleotidase and succinate dehydrogenase in fractions from the Percoll density gradient centrifugation. There were two major protein-containing fractions, a pellet fraction and one near the top of gradient. The pellet was rich in succinate dehydrogenase, but had no detectable 5′-nucleotidase. The upper fraction contained all the 5′-nucleotidase in the gradient, and very low levels of succinate dehydrogenase. The upper fraction therefore appeared to be microsomal in origin, while the pellet was at least partly derived from mitochondria. Both fractions exhibited Ca^{2+} uptake, although the microsomal fraction was more active.

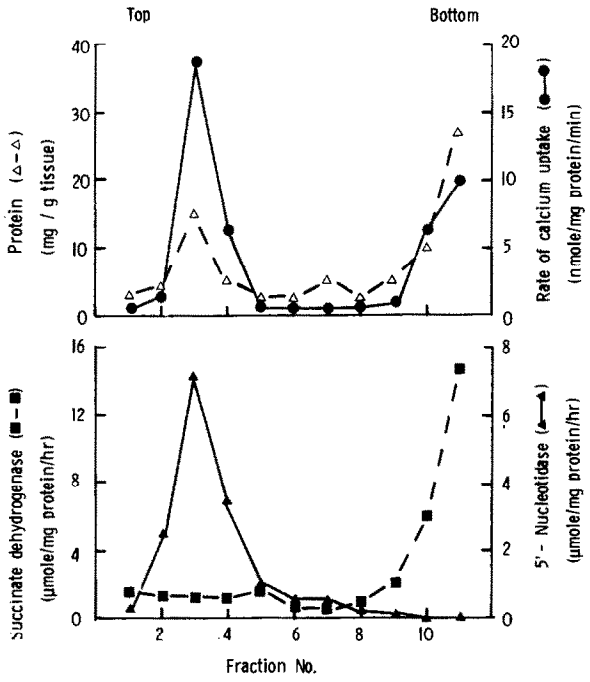

Fig.1. Distribution of enzyme activities after Percoll density gradient centrifugation. Material from 0.5 g rat aorta was used for the centrifugation, and 1 ml fractions were taken for assay of enzyme activities. Protein (△), Ca^{2+} uptake (●), 5′-nucleotidase (▲) and succinate dehydrogenase (■) were assayed as in section 2.

The microsomal fraction was further characterised by measuring the activities of several enzymes of known intracellular localisation [18]. Table 1 shows these activities in the initial homogenate, in the pellet obtained after the first centrifugation, and in the microsomal fraction. The microsomal fraction was enriched with 5′-nucleotidase, K^+-stimulated phosphatase and Na^+/K^+-ATPase, all three of which are localised in the sarcolemma. There was no detectable

Table 1
Enzyme specific activities in fractions during the preparation of sarcolemmal membranes

	Yield (mg protein)	5'-Nucleotidase	K^+-stimulated phosphatase	Na^+/K^+-ATPase	Ca^{2+}/Mg^{2+}-ATPase	Alkaline phosphatase	Lactate dehydrogenase	Succinate dehydrogenase
Homogenate	139 ±25	0.95 ±0.06	3.70 ±0.64	0.12 ±0.02	0.030 ±0.006	15.1 ±1.9	13.7 ±1.1	7.31 ±1.17
50 000 × *g* pellet	37 ±6	2.17 ±0.10	6.44 ±0.61	0.55 ±0.07	0.55 ±0.05	0.77 ±0.48	0.43 ±0.01	15.5 ±1.3
Sarcolemmal membranes	1.5 ±0.1	7.15 ±0.63	10.4 ±0.9	0.83 ±0.07	0.018 ±0.005	Not detectable	Not detectable	1.47 ±0.06

All enzyme specific activities in μmol product formed . mg protein^{-1} . h^{-1}. Figures are presented as mean ± SEM, $n \geq 3$

alkaline phosphatase or lactate dehydrogenase, indicating the fraction had no cytoplasmic contamination. Similarly, the low activity of succinate dehydrogenase indicated that mitochondrial contamination was also very low. The yield of microsomal protein (1.5 mg/g tissue) was comparable to that reported from smooth muscle using other methods of preparation [11].

The above results indicate that the microsomal fraction was at least partly sarcolemmal in origin. Experiments were also performed to test for the presence of vesicles originating from the sarcoplasmic reticulum. It has been reported [11] that Ca^{2+} uptake into sarcoplasmic reticulum vesicles is dependent on the presence of oxalate, whereas the uptake into sarcolemmal vesicles is not. Ca^{2+} uptake into the microsomal fraction was therefore measured in the presence and absence of oxalate. Both the basal rate of Ca^{2+} uptake, and the stimulated rate after phosphorylation (see section 3.2) were the same in the absence and presence of oxalate (fig.2). This indicates that the microsomal fraction had little or no contamination by sarcoplasmic reticulum. In addition, the effect of ouabain on the Ca^{2+} uptake (see section 3.2) is indicative of the sarcolemmal origin of the vesicles.

This method of preparation of membranes is much more rapid (90 min) than those in [8–11], in that only two centrifugations are required. The method is simple to perform and also eliminates the requirement for a preformed density gradient [14]. Furthermore, the yield is comparable to other methods, and gives a good separation of sarcolemmal membranes from other cell components.

3.2. *Phosphorylation of sarcolemmal vesicles*

Fig.2 shows that the vesicles as prepared by the above method had a low rate of Ca^{2+} uptake, which was independent of the presence of oxalate and was not inhibited by 10^{-8} M ouabain. After preincubation with cyclic AMP-dependent protein kinase and ATP there was a 2–3-fold stimulation of Ca^{2+} uptake which was also independent of oxalate (fig.2). However, this stimulation of uptake was abolished by 10^{-8} M ouabain. There was also a 2–3-fold increase in Na^+/K^+-ATPase activity after preincubation which was independent of the presence of Ca^{2+}.

These results suggest a dual mechanism of Ca^{2+} uptake into sarcolemmal vesicles. In the basal condition the uptake was independent of Na^+/K^+-ATPase (but was dependent on the presence of ATP), and presumably involves a Ca^{2+}-ATPase. The stimulation of Ca^{2+} uptake by cyclic AMP-dependent protein kinase was, however, dependent on the Na^+/K^+-ATPase. This suggests the presence of a Ca^{2+}/Na^+ exchange [4,24] which is responsible for the additional uptake observed on preincubation.

When the vesicles were incubated with [γ-^{32}P]ATP and cyclic AMP-dependent protein kinase, and ^{32}P-incorporation into protein examined by polyacrylamide gel electrophoresis, 4 definite bands of radioactivity associated with the vesicles were observed (fig.3). The major band has M_r 11 000, the others having M_r 21 000, 44 000 and 110 000. There was no phosphorylation of sarcolemmal proteins in the absence of cyclic AMP with or without the addition of cyclic AMP-dependent protein kinase. Phosphorylation of a band of M_r 44 000 in sarcolemmal vesicles from rat aorta, associated with an increased Ca^{2+}

uptake has been reported [13]. However, phosphorylation of the M_r 11 000 protein was not reported. In sarcolemmal vesicles from heart, the major phosphorylated protein has $M_r \sim 11\,000$ [25,26], which is very similar to that seen here.

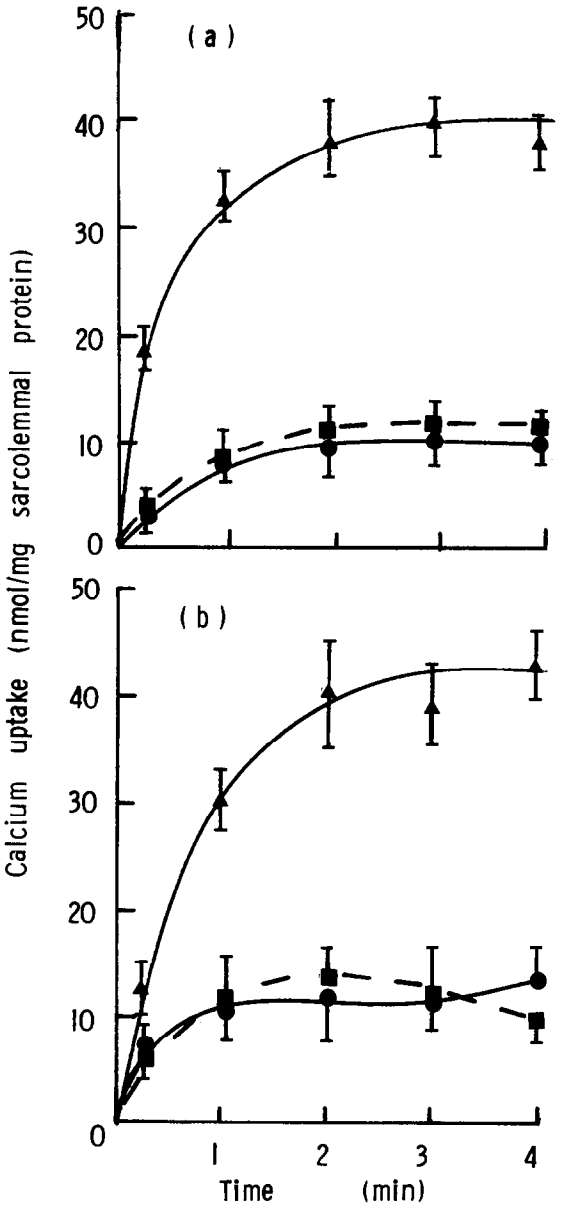

Fig.2. Stimulation of Ca^{2+} uptake into sarcolemmal vesicles by cyclic AMP-dependent protein kinase. Vesicles were preincubated as in section 2 in the presence (▲,●) or absence (■) of cyclic AMP-dependent protein kinase for 10 min. Ca^{2+} uptake was assayed in the presence (a) or absence (b) of oxalate. (●) The effect of adding 10^{-8} M ouabain to the assay. Bars indicate 2 SEM ($n = 5$).

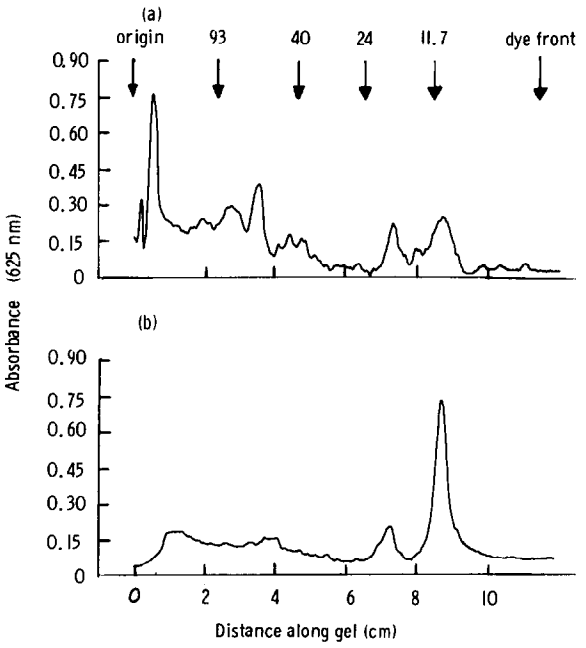

Fig.3. Distribution of protein (a) and ^{32}P (b) in sarcolemmal vesicles following incubation with [γ-^{32}P]ATP and cyclic AMP-dependent protein kinase and electrophoresis on 12.5% polyacrylamide [17]. The positions of proteins of known kM_r values are shown by vertical arrows: 93, phosphorylase; 40, aldolase; 24, chymotrypsinogen; 11.7, cytochrome *c*.

3.3. *General discussion*

Sarcolemmal vesicles produced by homogenisation of the tissue would be expected to be a mixture having either the extracellular surface or the cytoplasmic surface on the outside of the vesicles [4]. However, only those with the cytoplasmic side outwards would have the ATP-binding sites of transport systems accessible to added ATP. Ca^{2+} uptake into vesicles as measured in this study would therefore be equivalent in the intact tissue to transport of Ca^{2+} out of the cell. The stimulation of Ca^{2+} uptake into sarcolemmal vesicles on incubation with cyclic AMP-dependent protein kinase, would, if occurring in vivo, result in a decrease in [Ca^{2+}] in the cytoplasm and a reduction in tension. This is consistent with the observation that relaxation in vascular smooth muscle is associated with increase in cyclic AMP [5,6].

We have no evidence at the amount as to which of the phosphorylated proteins is responsible for the stimulation of Na^+/K^+-ATPase. When aortic strips are incubated with $^{32}P_i$, several proteins are phosphorylated [27,28]. Work is in progress to determine

whether the proteins phosphorylated in the sarcolemmal vesicles are also phosphorylated in vivo, and how this is affected by hormones.

Acknowledgements

We would like to thank Mr G. J. Belsham and Dr R. M. Denton for help with the development of the Percoll density gradient centrifugation. K. J. B. is in receipt of a Science Research Council CASE Studentship, in co-operation with Beechams Pharmaceuticals Ltd.

References

[1] Godfraind, T. and Kaba, A. (1969) Brit. J. Pharmac. 36, 549–560.
[2] Bohr, D. F. (1973) Circ. Res. 32, 665–672.
[3] Van Breeman, C. (1977) J. Physiol. (Lond) 272, 317–329.
[4] Van Breemen, C., Aaronson, P. and Loutzenhiser, R. (1979) Pharm. Rev. 30, 167–208.
[5] Axelsson, K. L., Wikberg, J. E. S. and Anderson, R. G. G. (1979) Life Sci. 24, 1779–1786.
[6] Nilsson, K. S., Andersson, R. G. G., Borgesson, S., Mackerlova, L., Mohme-Lundholm, E. and Lundholm, L. (1977) in: Excitation–contraction coupling in smooth muscle (Casteels, R. et al. eds) pp. 189–197, Elsevier/North-Holland, Amsterdam, New York.
[7] Krebs, E. G. and Beavo, J. A. (1979) Annu. Rev. Biochem. 48, 923–959.
[8] Illiano, G. and Cuatrecasas, P. (1971) J. Biol. Chem. 246, 2472–2479.
[9] Li, C. C. and Hochstadt, J. (1976) J. Biol. Chem. 251, 1175–1180.
[10] Wei, J.-W., Janis, R. A. and Daniel, E. E. (1976) Circ. Res. 39, 133–140.
[11] Wuytack, F., Landon, E., Fleischer, S. and Herdman, J. G. (1978) Biochim. Biophys. Acta 540, 253–269.
[12] Sands, H., Penberthy, W., Meyer, T. A. and Jorgensen, R. (1976) Biochim. Biophys. Acta 445, 791–801.
[13] Bhalla, R. C., Webb, R. C., Singh, D. and Brock, T. (1978) Am. J. Physiol. 234, M508–M514.
[14] Belsham, G. J., Denton, R. M. and Tanner, M. J. A. (1980) Biochem. J. 192, 457–467.
[15] Harigaya, S. and Schwarz, A. (1969) Circ. Res. 25, 781–794.
[16] Brostom, M. A., Reimann, E. M., Walsh, D. A. and Krebs, E. G. (1970) Adv. Enz. Regul. 8, 191–203.
[17] Laemmli, U. K. (1970) Nature 227, 680–685.
[18] Bers, D. M. (1979) Biochim. Biophys. Acta 555, 131–146.
[19] Edwards, M. J. and Maguire, M. H. (1970) Mol. Pharmacol. 6, 641–648.
[20] Mircheff, A. K. and Wright, E. M. (1976) J. Memb. Biol. 28, 309–333.
[21] Green, D. E., Mii, S. and Kohout, P. M. (1955) J. Biol. Chem. 217, 551–567.
[22] Bergmeyer, H.-U., Bernt, E. and Hess, B. (1965) in: Methods of Enzymatic Analysis (Bergmeyer, H.-U. ed) pp. 736–743, Verlag-Chemie, Weinheim.
[23] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[24] Blaustein, M. P. (1977) Am. J. Physiol. 232, C165–C173.
[25] Will, H., Wollenberger, A., Levchenko, T. S. and Levitsky, D. O. (1979) in: Cyclic nucleotides and protein phosphorylation in cell regulation (Krause, E.-G. et al. eds) pp. 135–146, Pergamon Press, Oxford.
[26] Lamers, J. H. J. and Stinis, J. T. (1980) Biochim. Biophys. Acta in press.
[27] Barron, J. T., Barany, M., Barany, K. and Sorti, R. V. (1980) J. Biol. Chem. 255, 6239–6244.
[28] Murray, K. J. and England, P. J. (1980) Biochem. J. in press.