Biochimica et Biophysica Acta, 332 (1974) 221–232

BBA 76530

ISOLATION AND CHARACTERIZATION OF SMOOTH MUSCLE CELL MEMBRANES*

MECIA M. OLIVEIRA and SIMONE HOLZHACKER

Department of Biophysics and Physiology, Escola Paulista de Medicina 04023 São Paulo, S.P. (Brazil)

(Received July 3rd, 1973)

(Revised manuscript received September 25th, 1973)

SUMMARY

A method for the isolation of guinea pig ileum smooth muscle cell membranes is described. The plasma membrane fraction possessed a (Na^+, K^+)-ATPase which was inhibited by ouabain. The Mg^{2+}-dependent ATPase of the membrane fraction was stimulated by 1 μM Ca^{2+}. A basal ATPase, not dependent on Mg^{2+}, was directly stimulated by Ca^{2+} in the range of 1 μM to 1 mM.

The isolated membranes contracted in response to the following substances: ATP, angiotensin II and some of its analogs, bradykinin, acetylcholine and histamine. The contractility was inhibited by ouabain and chlorambucil-angiotensin II, but not by cytochalasin B. No contraction was produced by AMP, angiotensin I and adrenaline.

INTRODUCTION

There is great interest in the isolation of smooth muscle cell membranes, not only for studies of interaction with drugs and hormones, but also for experiments leading to the knowledge of the initial events of selective membrane permeability to ions in the phenomenon of muscular contraction.

As an approach to the study of angiotensin interaction with cell structures, we have isolated and studied the properties of the plasma membrane from smooth muscle cells of guinea pig ileum. The method is a modification of published methods for preparations from skeletal muscle [1,2], cardiac muscle [3] and uterine muscle [4].

MATERIALS AND METHODS

Preparation of membranes

Guinea pigs (200–600 g) were killed with a blow to the head, the ileum quickly removed and washed with Tyrode's solution. A washed 10–15-cm segment of the intestine was drawn over a glass rod and the mesentery removed with scissors. A scalpel incision was made through the muscular layer, which receded slightly and

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

* Dedicated to Professor J. R. Valle on his 65th birthday.

was detached gently by blunt dissection [5]. By histological inspection the dissected muscle was free from mucosa and consisted of circular and longitudinal layers. This isolated muscle (20% of the whole ileum) maintained the pharmacological properties of the intact ileum, responded to angiotensin II and was inhibited by its specific antagonist chlorambucil-angiotensin II [6].

The muscle was cut in small pieces and homogenized in a 1:6 (w/v) suspension of cold buffer (0.020 M Tris–maleate, 0.25 M sucrose and 1 mM EDTA), pH 7.4, for 30 s at high speed in a "Virtis" microhomogenizer. All the operations were performed at 5 °C. Phase microscopy was used to evaluate the effectivity of cell disruption as well as all subsequent procedures.

The resultant suspension was filtered through cheesecloth, the residue rehomogenized and the filtrates pooled and centrifuged at $650 \times g$ for 10 min. The pellet was suspended in 3 vol. of the initial buffer, homogenized in a Potter–Elvehjem homogenizer and centrifuged at $164 \times g$ for 5 min. After repeating this step five times, the pellet was suspended in the same volume of buffer and incubated at 37 °C, for 30 min, in an oscillating water bath. The incubation at this temperature was found to be necessary for the complete extraction of cytoplasm inclusions.

The sample was then centrifuged at $164 \times g$ for 5 min and the pellet taken up in 10 vol. of 0.01 mM EDTA solution (free acid, pH 7.4 with Tris). After shaking vigorously and further homogenizing, pellets were obtained by centrifugation at $1000 \times g$ for 5 min. These pellets were washed in the EDTA solution another 6 to 7 times to extract all the actomyosin. In some preparations a further purification was required and 20 ml of the membrane suspension in EDTA were layered over 20 ml of the initial Tris–sucrose buffer and centrifuged at $40 \times g$ for 1 min. The supernatant was rich in emptied cell envelopes. These were collected as a pellet after centrifugation at $4000 \times g$ for 10 min. The membranes thus obtained were kept in EDTA or deionized water and suspended in the appropriate buffer for enzymatic determinations.

The mitochondrial fraction was obtained by centrifuging the first supernatant at $16000 \times g$ for 12 min. After adjusting this supernatant to 0.6 M with KCl it was spun at $48000 \times g$ for 70 min to obtain a microsomal fraction. Both fractions were washed in fresh buffer and centrifuged at the same speed. The subcellular fractions were checked for homogeneity by electron microscopy.

Actomyosin from intestinal muscle was prepared according to Mallin [7]. Protein was determined by the method of Lowry et al. [8], using bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis

Gels of 5% acrylamide + 0.1% bisacrylamide were polymerized with ammonium persulfate and *N,N,N′,N′*-tetramethyl ethylenediamine as catalyst in a 0.05 M barbital buffer, pH 8.6, with sodium dodecylsulfate 0.1%. Solubilized membranes (1% sodium dodecyl sulfate plus 1 mM β mercaptoethanol) and actomyosin, both samples with 150 μg of protein, were electrophoresed at a current of 3 mA/tube for 4 h. After electrophoresis the gels were stained with 0.1% Coomassie blue in 12% trichloroacetic acid, 3.6% sulfosalicylic acid and 40% methanol, at 70 °C for 3 h. Destaining was obtained with several changes of 5% acetic acid in 40% methanol.

Enzymatic assays

ATPase (ATP phosphohydrolase, EC 3.6.1.3) was assayed as follows. For Mg^{2+}-dependent ATPase activity an aliquot of each particulate fraction (650–150 μg protein) was incubated for 5 min at 37 °C with 1 mM ATP, 3 mM $MgCl_2$ and 10 mM Tris–maleate buffer (pH 7.4), in a 0.5-ml volume. At the end of the incubation the reaction was stopped with 10% trichloroacetic acid and the P_i measured according to the procedure of Fiske and SubbaRow [9]. For (Na^+, K^+)-ATPase activity 120 mM NaCl and 12 mM KCl were added to the incubation mixture. The increment in activity was an index of Na^+–K^+ activation. For Ca^{2+}-activated ATPase a different set of experiments was done and the conditions are listed under Results.

Controls with and without cell fractions, and with and without ATP were performed. All experiments were done in duplicate. The hydrolysis of ATP was linear within 5 min of reaction time and was a function of cell fraction protein in the range of 50–150 μg.

Glucose-6-phosphatase (D-glucose 6-phosphate phosphohydrolase, EC 3.1.3.9) was determined by the method of Hubscher and West [10].

Succinate-cytochrome *c* reductase (E.C. 1.3.9.1) was assayed as described previously [11]. The amount of cytochrome *c* reduced during the reaction was calculated by applying the extinction coefficient of $2.10 \cdot 10^{-4}$ cm^2/mmole to the observed increase in absorbance at 550 nm.

Contractility tests

The method used was essentially the same described by Carvalho et al. [12]. The sarcolemma suspension was kept at room temperature, in either deionized water, 0.01 mM EDTA or 1 mM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA). Drops of this suspension were placed under a cover slip on a glass slide. The test solutions were added in the periphery of the cover slip so that they would diffuse under it and mix with the sarcolemmal suspension. These preparations were viewed and photographed with a Zeiss photomicroscope, using Kodak high contrast copy film. Tests were performed at least in five different samples of the same preparation and in three different preparations.

Materials

Cytochrome *c* (grade III), ATP, EGTA, ouabain and glucose 6-phosphate were obtained from Sigma; Tris and EDTA were purchased from Merck Darmstadt. Acrylamide, bis-(*N*,*N'*-methylene-bis-acrylamide), Coomassie brilliant blue and sodium dodecylsulfate were obtained from BIO-RAD Laboratories and 2-mercaptoethanol from Eastman Kodak.

The peptides were synthesized by the method of solid phase as described by Stewart and Young [13]. The synthesis of chlorambucil-angiotensin II and of the other angiotensin analogs are described elsewhere [14].

Cytochalasin B was gift from Professor G. C. Mueller, University of Wisconsin.

RESULTS

Isolation of plasma membrane

The methods developed by McCollester [1] and Peter [2] for the isolation of plasma membrane from skeletal muscle were adapted for intestinal smooth muscle.

The procedure essentially consisted of homogenization of the isolated muscle, differential centrifugation, incubation at 37 °C (designed to disrupt internal structure) and extraction of the cellular components and actomyosin by diluted EDTA solution. EDTA (not EGTA) in homogenizing and washing solutions was important for the preservation of (Na^+,K^+)-ATPase of the membranes.

The sarcolemmal fraction consisted in large part of ghosts, smaller and more elongated than those of skeletal muscle sarcolemma, and membrane fragments.

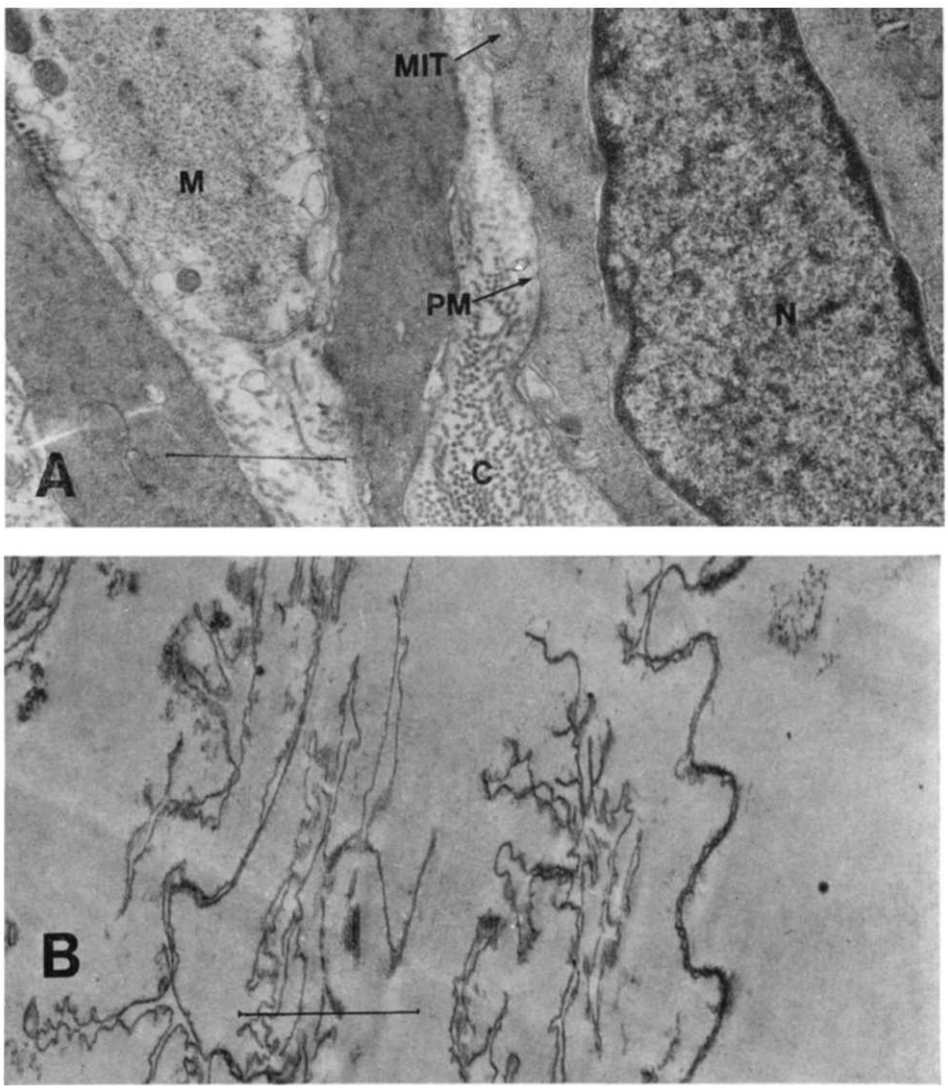

Fig. 1. Electron micrograph of guinea pig intestinal smooth muscle and isolated sarcolemma. The specimens were fixed in 2% glutaraldehyde, post fixed in 1% OsO_4, with 0.5% uranyl acetate and embedded in araldite. The ultrathin sections were stained with uranyl acetate and lead acetate. (A) Intact muscle showing: N, nucleus; Mit, mitochondria; M, myofilaments; C, collagen; PM, plasma membrane. (B) Plasma membrane fraction. Bars represent 1 μm.

Our preparations were not visibly contamined with subcellular components when examined by phase contrast optics and electron microscopy (Fig. 1). In acrylamide gel electrophoresis the sodium dodecyl sulfate-solubilized membranes showed different bands from that of actomyosin extracted from the same muscle (Fig. 2). This indicates that a reasonable purification of the smooth muscle cell ghosts was attained.

In 30 preparations using the method described here, the average protein yield of the membrane fraction was 1.0% (S.E. = 0.12) of the initial homogenate.

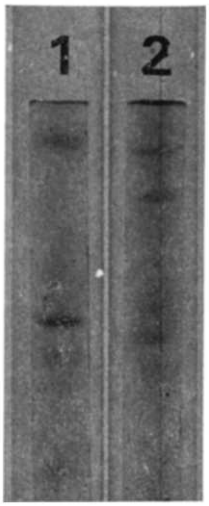

Fig. 2. Polyacrylamide gel electrophoresis. (1) Actomyosin extracted from intestinal muscle. (2) Sodium dodecylsulfate-solubilized intestinal muscle plasma membranes (details in text).

Enzymatic characterization

The enzymatic analysis of the different subcellular fractions of the intestinal smooth muscle are shown in Table I. The (Na^+, K^+)-ATPase was found only in

TABLE I

DISTRIBUTION OF ENZYME ACTIVITIES IN SMOOTH MUSCLE SUBCELLULAR FRACTIONS

The enzymatic activities are expressed in the following units: ATPase, μmole P_i per min per mg protein; Succinate cytochrome *c* reductase, nmole cytochrome *c* reduced per min per mg protein; Glucose-6-phosphatase, μmoles P_i per 15 min per mg protein. The values for the sarcolemma fraction represent the mean of 14 preparations ± S.E., and the ones for the other fractions the mean of 5 preparations ± S.E.

	ATPase			Succinate cytochrome *c* reductase	Glucose-6-phosphatase
	Mg^{2+}	Mg^{2+} (Na^+,K^+)	$\frac{Mg^{2+}\ (Na^+,K^+)}{Mg^{2+}}$		
Homogenate	0.231 ± 0.040	0.219 ± 0.040	0.9	1.5 ± 0.97	0.056 ± 0.027
Sarcolemma	0.077 ± 0.017	0.154 ± 0.029	2.0	0	0
Mitochondria	0.754 ± 0.212	0.705 ± 0.150	0.9	11.97 ± 2.57	0.041 ± 0.028
Microsomes	0.544 ± 0.094	0.526 ± 0.069	0.9	6.3 ± 0.75	0.470 ± 0.106

the sarcolemmal fraction, with an average stimulation of 2-fold in the presence of 120 mM Na^+ and 12 mM K^+. Both cations were necessary for this effect. The Na^+ plus K^+ stimulation was suppressed by 0.5 mM ouabain.

These results suggest that our sarcolemma fraction is composed of plasma membranes, since the presence of $(Na^+, K)^+$-ATPase in the surface membrane of cells, as well as the role of this enzyme in the selective transport of cations, is well established [15,16].

Variation of ATP concentration from 0.125 to 1 mM, in the presence of 3 mM $MgCl_2$, followed Michaelis–Menten kinetics with an apparent K_m of 1.24 mM in the absence, and of 0.87 mM in the presence of 120 mM Na^+ and 12 mM K^+. The enzymatic reactions were done within 4 h of the membrane purification. The (Na^+,K^+)-ATPase activity was lost after 24 h at either 4 °C or −20 °C. The Mg^{2+}-dependent ATPase kept its activity for at least 5 days at 4 °C.

The membrane fraction did not have either succinate cytochrome *c* reductase or glucose-6-phosphatase activities, thus suggesting no significant contamination by mitochondria or microsomes, as also demonstrated by electron microscopy.

Ca^{2+}-activated ATPase

An ATPase activated by Ca^{2+} (1 mM) in the absence of Mg^{2+} has been observed in plasma membranes of skeletal [2] and cardiac [3, 17] muscles. We have investigated the same activity in plasma membranes of smooth muscle cell.

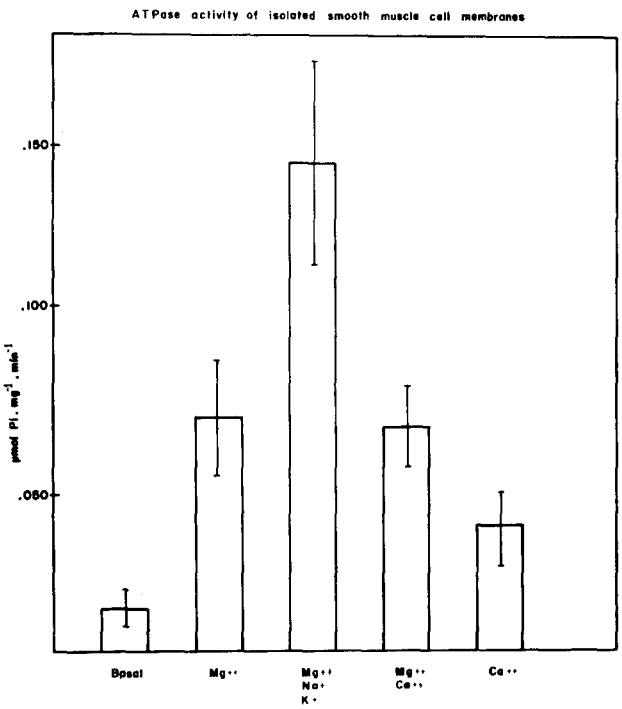

Fig. 3. Cation requirements for ATPase activity of isolated smooth muscle sarcolemma. The values represent the mean of 7 preparations ±S.E. Conditions of reaction described under Methods. Calcium was added as 1 mM $CaCl_2$.

Within the concentration range of 0.01 to 1 mM Ca^{2+} in the reaction mixture, we observed a stimulation of the basal ATPase activity, but when Mg^{2+} was present in the medium this stimulation did not occur, and some inhibition was detected Fig. 3 shows a summary of the action of different cations on the ATPase activity of the smooth muscle sarcolemma.

Another set of experiments was done, in which the concentration of Ca^{2+} was lowered by the use of EGTA in the mixture. The concentration of Ca^{2+} was 7.11 μM, as calculated from the value of $3.95 \cdot 10^{-6}$ M for the dissociation constant [18]. This value for Ca^{2+} in the mixture is known to activate Mg^{2+}-ATPase and the "calcium pump" in skeletal muscle microsomes [19]. In Table II are listed the experiments with different fractions of smooth muscle cells. In those experimental conditions an activation of the Mg^{2+}-dependent ATPase by Ca^{2+} was observed only in the microsomal and mitochondrial fraction.

TABLE II

Ca^{2+}-ACTIVATED ATPase IN SUBCELLULAR FRACTIONS OF SMOOTH MUSCLE CELLS

The incubation medium consisted of 10 mM Tris-maleate buffer (pH 7.1), 4 mM $MgCl_2$, 4 mM potassium oxalate, 0.2 mM $CaCl_2$, 0.3 mM EGTA and 1 mM ATP. For determination of Mg^{2+}-dependent ATPase $CaCl_2$ and potassium oxalate were omitted and the concentration of EGTA was 0.5 mM. The reaction, in a volume of 1 ml, was started with the particulate fraction (100 μg of protein), incubated for 5 min at 37 °C and stopped by millipore filtration. The release of P_i was determined as described in Methods.

Experiment No.	Fraction	ATPase activity (μmole $P_i \cdot mg^{-1} \cdot min^{-1}$)		
		Mg^{2+}-dependent	Total (Mg^{2+} plus Ca^{2+})	Ca^{2+}-activated
1	Sarcolemma	0.120	0.120	0
	Mitochondria	1.230	1.200	0
	Microsomes	0.870	1.200	0.430
2	Sarcolemma	0.040	0.040	0
	Mitochondria	0.742	0.765	0.023
	Microsomes	0.920	1.040	0.120
3	Sarcolemma	0.065	0.065	0
	Mitochondria	0.412	0.489	0.066
	Microsomes	0.489	0.594	0.105
4	Sarcolemma	0.120	0.120	0
	Mitochondria	0.930	1.000	0.070
	Microsomes	0.770	1.200	0.430

In the sarcolemmal fraction, a stimulation of Mg^{2+}-dependent ATPase was observed only when Ca^{2+} was lowerd to 1 μM (Fig. 4). Higher Ca^{2+} concentrations failed to produce that effect. However, the basal ATPase activity was stimulated by low (Fig. 4) and high Ca^{2+} concentrations (Fig. 3).

Microscopic studies on the contractility of the isolated sarcolemma

The ghosts observed in our sarcolemmal fraction contracted radially upon addition of ATP, in a manner similar to that described for skeletal muscle

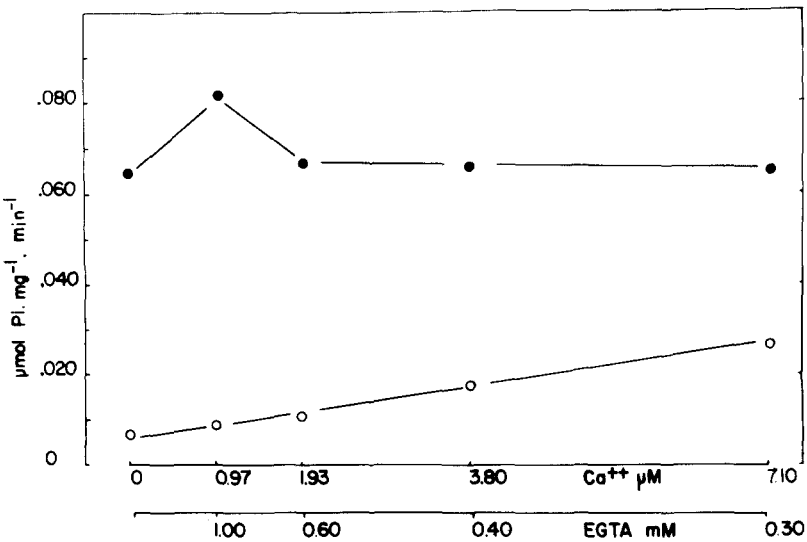

Fig. 4. Effect of low Ca^{2+} concentration on the ATPase of smooth muscle sarcolemma. Conditions of reaction are the same described in Table II. EGTA in the medium as marked in the graph. The Ca^{2+} concentration was calculated as described [18]. ○—○, without Mg^{2+}; ●—●, in the presence of Mg^{2+}.

sarcolemma [12]. The first sign of contractile response under microscopic observation was a change in refringence of the membrane, appearing as a thickening of the wall. This phenomenon was temperature-dependent, no contractile response being observed at 5 °C.

Our membrane preparation was essentially free of contaminating actomyosin as demonstrated by electron microscopy and acrylamide gel electrophoresis. Nevertheless, to minimize the risk of a possible interference by actomyosin, all the contraction tests were done in a Ca^{2+}-free medium, such as deionized water, 0.01 mM EDTA or 1 mM EGTA.

Other substances were found to induce contractile response of the membranes. These contractions were morphologically indistinguishable from those produced by ATP. Table III lists the results observed with several substances. Angiotensin I was without effect, while angiotensin II elicited a strong contraction within the concentration range of 1 μM to 1 mM (Fig. 5). The heptapeptide des[Asp^1]angiotensin II was still effective, but the hexapeptide des[Asp^1, Arg^2]angiotensin II was not.

Acetylcholine and histamine, which have a contractile effect upon isolated guinea pig ileum, were both efficient in contracting the membranes. Adrenaline had no visible effect.

As an attempt to understand the contractile phenomenon we have used three different types of inhibitors. The first one was cytochalasin B, a fungal metabolite which is known to inhibit the function of contractile microfilament systems of cells [20]. Therefore, if an eventual contamination by filaments of actomyosin in the membrane were responsible for the contractile process, this would be inhibited by cytochalasin B.

Cytochalasin B, dissolved in 1% dimethylsulfoxide, was used in the concen-

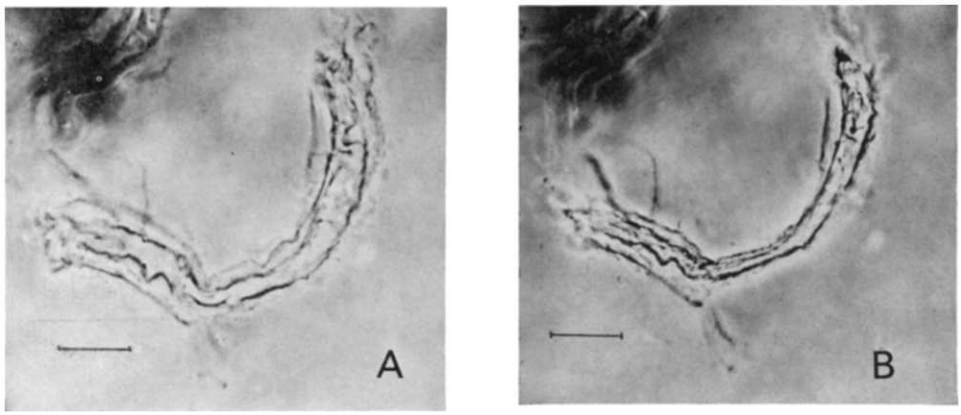

Fig. 5. Phase contrast micrograph of sarcolemma isolated from guinea pig ileum smooth muscle. (A) Normal appearance of sarcolemma. (B) Same specimen contracted by 1 mM angiotensin II. Bars represent 20 μm.

TABLE III

EFFECT OF SUBSTANCES ON THE CONTRACTILITY OF ISOLATED SARCOLEMMA FROM SMOOTH MUSCLE CELL

Substance	Concentration (mM)	Response
Vasoactive peptides:		
Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu (Angiotensin I)	1	no visible effect
Asp–Arg–Val–Tyr–Ile–His–Pro–Phe (Angiotensin II)	1–0.001	strong contraction
Arg–Val–Tyr–Ile–His–Pro–Phe	1	contraction
Val–Tyr–Ile–His–Pro–Phe	1	no visible effect
Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg (Bradykinin)	1–0.01	strong contraction
Smooth muscle-active substances:		
Acetylcholine	1	contraction
Histamine	1	contraction
Adrenaline	1	no contraction
Adenine nucleotides:		
ATP	10	contraction
AMP	10	no visible effect
Ions of biological significance:		
Na^+	100	no visible effect
K^+	10	no visible effect
Zn^{2+}	5	contraction
Mg^{2+}	10	small contraction
Ca^{2+}	10	small contraction

tration of 50–500 μg/ml of membrane suspension (above 100 μg/ml, it behaved as a fine emulsion) and incubated from 20 min to 6 h. In four different preparations, the presence of this compound did not affect the contractile response elicited by angiotensin II, bradykinin or ATP. It is important to note that incubation for 10 min with 10 μg/ml of cytochalasin B inhibited over 50%, and incubation with 37 μg/ml during 1 h completely inhibited the response of the guinea pig ileum to angiotensin II. An inhibitory effect on intestinal peristalsis has been described previously [20].

The second inhibitor tested was chlorambucil-angiotensin II, a specific and irreversible antagonist of the miocontractile activity of angiotensin II in the guinea pig ileum [6]. This inhibitor, used in a final concentration of 0.3 mM, consistently blocked the contraction elicited by angiotensin II. This would indicate that the contraction produced by angiotensin II in our sarcolemma preparations are due to an interaction with the same receptor involved in angiotensin action upon the guinea pig ileum.

The other inhibitor tested was ouabain which had an inhibitory effect on (Na^+,K^+)-ATPase in our preparation and in a variety of other membrane-bound (Na^+,K^+)-ATPases [16]. When incubated with the membrane suspension for 20 min, 0.5 mM ouabain totally inhibited the contraction elicited by angiotensin II. This was consistently observed in four different preparations and in a minimum of five tests with each preparation. These results suggest the participation of (Na^+, K^+)-ATPase in the sarcolemma contractile activity.

DISCUSSION

We have obtained a plasma membrane preparation, isolated from the guinea pig ileum, which appears to keep a similar structure to that found physiologically, a desirable requisite for further studies with regard to the problem of drug–receptor interaction.

Our sarcolemmal preparations contained a (Na^+,K^+)-ATPase, an enzyme "marker" for plasma membranes, which is regarded as having a function in the selective transport of cations [16]. The values found in our preparations for the activity of that enzyme were similar to those described for sarcolemma preparations from other muscles [2,3,17].

The Mg^{2+}-dependent ATPase of the membrane fraction was stimulated, to about 20% by 1 μM Ca^{2+}. At greater Ca^{2+} concentrations, the stimulation would cease and a slight inhibition would take place. Mg^{2+}-dependent ATPase from microsomes and mitochondria was stimulated by Ca^{2+} in the concentration range of 1–7 μM. The membrane fraction showed another ATPase, not dependent on Mg^{2+}, that was directly stimulated by calcium in the range of 1 μM to 1 mM. With these findings we are not yet in the position of knowing which of these Ca^{2+}-stimulated ATPases are physiologically linked with calcium transport in intestinal smooth muscle. Stam et al. [20] described in heart muscle sarcolemma a Mg^{2+}-dependent ATPase stimulated by low concentrations of Ca^{2+}, which they believe to participate in the control of calcium within the myocardium.

The radial contractions induced in the isolated smooth muscle sarcolemma by several substances appeared to result from a direct effect upon the membrane,

as the preparations seemed free of actomyosin by electron microscopy and acrylamide gel electrophoresis. Furthermore, the contractility tests were done in a medium free of Ca^{2+}, which is necessary for activating the actomyosin system [22,23]. This was indirectly confirmed by the lack of inhibition by cytochalasin B, a substance known to inhibit microfilaments in contractile process in a variety of cells [20].

The contractile phenomenon of smooth muscle sarcolemma was inhibited by ouabain, in the same concentration that was inhibitory for the (Na^+,K^+)-ATPase. Since this enzyme is thought to participate in the selective transport of cations in cell membranes [16] this process might be connected to the contractile activity of the sarcolemma.

The isolation of a "contractile protein" from non-muscular cell membranes such as red blood cells [24] and liver cells [25], has been described. These proteins are involved in membrane movement and permeability, and are related to (Na^+,K^+)-ATPase [26]. With our present findings, we believe that a similar protein might be involved in the contractile process of the smooth muscle cell membranes. Then, the observed sarcolemmal contraction induced by certain substances could be explained as a magnification of the primary event in muscle contraction, namely the membrane conformational change after a chemical stimulus.

The membrane preparation described here may be a useful tool in the study of the initial events of muscular contraction as well as in the study of drug–receptor interaction.

ACKNOWLEDGMENTS

We are grateful to professor A. C. M. Paiva for his interest and helpful discussions during the course of this work. We thank Professor W. Sasso, Mr O. A. Mora and Miss M. M. Vitt, from the Department of Morphology, for the assistance with the electron microscopy.

This investigation was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo (Projeto Bioq/FAPESP) and Conselho Nacional de Pesquisas (CNPq).

REFERENCES

1 McCollester, D. L. (1962) Biochim. Biophys. Acta 57, 427–437
2 Peter, J. B. (1970) Biochem. Biophys. Res. Commun. 40, 1632–1637
3 Stam Jr, A. C., Shelburne, J. W., Feldeman, D. and Sonnenblick, E. H. (1969) Biochim. Biophys. Acta 189, 304–307
4 Caroll, P. M. and Sereda, D. D. (1968) Nature 217, 666–667
5 Ambache, N. (1954) J. Physiol. 125, 53P–55P
6 Paiva, T. B., Paiva, A. C. M., Freer, R. J. and Stewart, J. M. (1972) J. Med. Chem. 15, 6–8
7 Mallin, M. L. (1965) J. Cell. Comp. Physiol. 65, 355–360
8 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
9 Fiske, C. H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–400
10 Hubscher, G. and West, G. R. (1965) Nature 205, 799–800
11 Oliveira, M. M., Weglicki, W. B., Nason, A. and Nair, P. P. (1969) Biochim. Biophys. Acta 90, 146–158
12 Carvalho, A. P., Madeira, V. M. C. and Antunes-Madeira, M. C. (1971) Biochim. Biophys. Acta 234, 210–221

13 Stewart, J. M. and Young, J. D. (1969) Solid Phase Peptide Synthesis, W. H. Freeman and Co., San Francisco
14 Paiva, T. B., Goissis, G., Juliano, L., Miyamoto, M. E. and Paiva, A. C. M. (1973) J. Med. Chem., in the press
15 Kamat, V. B. and Wallach, D. F. (1965) Science 148, 1343–1345
16 Skou, J. C. (1965) Physiol. Rev. 45, 596–617
17 Dietze, G. and Hepp, K. D. (1971) Biochem. Biophys. Res. Commun. 44, 1041–1049
18 De Meis, L. and Hasselbach, W. (1971) J. Biol. Chem. 246, 4759–4763
19 De Meis, L. (1971) J. Biol. Chem. 246, 4764–4773
20 Wessels, N. K., Spooner, B. S., Ash, J. F., Bradley, M. O., Luduena, M. A., Taylor, E. L., Wrenn, J. T. and Yamada, K. M. (1971) Science 171, 135–143
21 Stam, Jr, A. C., Weglicki, W. B., Gertz, E. W. and Sonnenblick, E. H. (1973) Biochim. Biophys. Acta 298, 927–931
22 Ebashi, S. (1972) Nature 240, 217–218
23 Weber, A. and Herz, R. (1963) J. Biol. Chem. 238, 599–605
24 Ohnishi, T. J. (1962) J. Biochem. Tokyo 52, 307–308
25 Neifakh, S. A. and Vasilets, I. M. (1964) Fed. Proc. 24, T561–T562
26 Benedetti, E. L. and Emmelot, P. (1968) in Ultra structure in Biological Systems (Dalton, A. J. and Haguenau, F., eds), Vol. 4, pp. 33–120, Academic Press, New York and London