Phosphorylation of phosphatidylinositol by transverse tubule vesicles and its possible role in excitation-contraction coupling

Cecilia Hidalgo*+°, M. Angélica Carrasco*, Karin Magendzo* and Enrique Jaimovich*+

*Departamento de Fisiologia y Biofisica, Facultad de Medicina, Universidad de Chile and +Centro de Estudios Científicos de Santiago, Casilla 16443, Santiago 9, Chile

Received 31 March 1986; revised version received 28 April 1986

Phosphorylation of phosphatidylinositol to phosphatidylinositol 4-monophosphate and to phosphatidylinositol 4,5-bisphosphate was demonstrated in transverse-tubule membranes isolated from frog skeletal muscle using [γ-^{32}P]ATP as substrate. At millimolar concentrations of Mg^{2+} both phosphorylation reactions were completed within 15 s at 25°C. Isolated sarcoplasmic reticulum vesicles phosphorylated phosphatidylinositol to phosphatidylinositol 4-phosphate with a lower specific activity than the transverse tubules, and lacked the ability to produce phosphatidylinositol 4,5-bisphosphate. These findings show, for the first time, that isolated transverse-tubule membranes carry out one of the steps required to sustain a role for inositol trisphosphate as the physiological messenger in excitation-contraction coupling in skeletal muscle. The finding that 0.5 mM tetracaine apparently inhibits the phosphorylation of phosphatidylinositol 4-phosphate to phosphatidylinositol 4,5-bisphosphate also supports a role for these intermediates in excitation-contraction coupling.

(Frog skeletal muscle) *Transverse tubule* *Lipid phosphorylation* *Phosphatidylinositol* *Excitation-contraction coupling*

1. INTRODUCTION

The early events in the process of E-C coupling take place at the level of the T-tubule membrane. The depolarization of the T-tubule triggers the cascade of reactions that causes calcium release from the SR and the ensuing muscle contraction.

Vergara et al. [1] have recently proposed that $InsP_3$ is the chemical messenger responsible for E-C coupling in skeletal muscle (for opposing views, see [2,3]). According to Vergara et al. [1] electrical stimulation of the muscle fiber produces a transient depolarization of the T-tubule membrane which stimulates the hydrolysis of PtdIns 4,5-P_2 by a phosphodiesterase to form diacylglycerol and $InsP_3$; the latter by binding to a specific receptor in the SR membrane would cause calcium release. Central to this proposed mechanism of E-C coupling is the presence in the T-tubule membrane of PtdIns and the enzyme systems responsible for the conversion of PtdIns to PtdIns 4,5-P_2, the membrane-bound precursor of the soluble $InsP_3$.

Our approach to study E-C coupling has been to isolate and characterize the membranes that participate in this process. Thus, we have isolated

° To whom correspondence should be addressed at CECS, Casilla 16443, Santiago 9, Chile; on leave from the Department of Muscle Research, Boston Biomedical Research Institute and the Department of Neurology, Harvard Medical School, Boston, MA, USA

Abbreviations: E-C coupling, excitation-contraction coupling; $InsP_3$, inositol 1,4,5-trisphosphate; PtdIns, phosphatidylinositol; PtdIns 4-P, phosphatidylinositol 4-phosphate; PtdIns 4,5-P_2, phosphatidylinositol 4,5-bisphosphate; T-tubule, transverse tubule; SR, sarcoplasmic reticulum

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

highly purified T-tubule and SR membranes from frog skeletal muscle [4,5] using several different criteria to study the origin, purity and sidedness of the vesicular preparations [4–6]. Frog skeletal muscle was chosen as a source of membranes since most physiological studies have been carried out in amphibian muscle.

In this work we show that T-tubule vesicles, which are mostly sealed and with the cytoplasmic side out [4], have the enzyme systems that phosphorylate endogenous PtdIns to PtdIns 4-P and to PtdIns 4,5-P_2. In addition, our results suggest that tetracaine inhibits the phosphorylation of PtdIns 4-P to PtdIns 4,5-P_2. These findings are consistent with the model of E-C coupling proposed by Vergara et al. [1], although by themselves they do not constitute enough evidence to prove this model.

2. MATERIALS AND METHODS

T-tubule and SR membranes were isolated from frog skeletal muscle as described in [4]. Phosphorylation of the isolated membranes was carried out under the following conditions: 0.1 mg protein were incubated in a final volume of 0.1 ml, at 25°C, with a solution containing 0.1 M KCl, 20 mM Tris/maleate, pH 7.0, and variable concentrations of ATP and Mg^{2+}, as specified in the table and figure legends. High specific activity [γ-^{32}P]ATP was diluted with unlabeled ATP to a final specific activity of 0.5 Ci/mmol. The reaction was initiated by addition of [γ-^{32}P]ATP, after previous incubation of the membranes for 5 min in the reaction solution, and stopped by addition of 1.0 ml of 1 N HCl, followed by addition of 2 ml chloroform:methanol (1:1, v/v) as in [7]. After vortex-mixing for 30 s, the organic phase was collected and evaporated under a stream of nitrogen. The extraction procedure was repeated once more by adding to the evaporated sample 0.5 ml of 1 N HCl and 1.0 ml chloroform:methanol (1:1). The organic phase was collected, evaporated to a volume of 50 μl, and the component phospholipids resolved by one-dimensional thin-layer chromatography using silica gel G plates (Merck). The composition of the solvent system used was chloroform:methanol:4 N ammonia (9:7:2). Phospholipids were visualized by staining with iodine vapor; radioactive incorporation of ^{32}P into lipids was visualized by autoradiography. The amount of phosphorus present in each iodine-stained spot was determined as described [8], after scraping the spots from the plates. The amount of ^{32}P incorporated into phospholipids was determined by liquid scintillation counting of material scraped from the plates. Protein was determined by the procedure of Hartree [9] using bovine serum albumin as standard. The phosphorylated derivatives of PtdIns (PtdIns 4-P and PtdIns 4,5-P_2) were obtained from Sigma. [γ-^{32}P]ATP was prepared as in [10] by phosphorylating ADP with ^{32}P obtained from the Comisión Chilena de Energía Nuclear.

3. RESULTS

It has been previously shown that T-tubule membranes isolated from either rabbit [11,12] or chicken [13] skeletal muscle contain 5–6% PtdIns

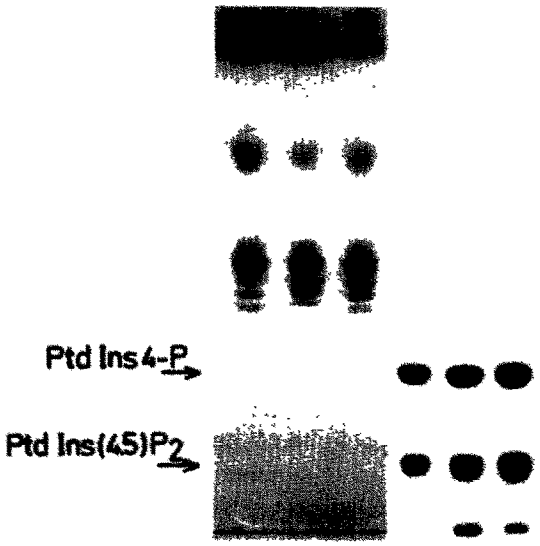

Fig.1. Phosphorylation of T-tubule membrane lipids by [γ-^{32}P]ATP. (Left) Major phospholipid components of the T-tubule membrane after extraction and thin-layer chromatography as indicated in the text. The phospholipids were visualized with iodine vapor. Arrows indicate the positions of the standards of PtdIns 4-P and PtdIns 4,5-P_2. (Right) Autoradiogram of the same plate. The phosphorylation conditions for the three samples shown were (from left to right) 0.5, 1.0 and 2.0 mM ATP, respectively. The concentration of Mg^{2+} used was 5 mM in all cases. The reaction was stopped after 1 min.

relative to the total phospholipids. The T-tubules isolated from frog muscle also contain 4% PtdIns (P. Donoso, personal communication). Thus we investigated whether the T-tubules isolated from frog muscle contain the kinases that phosphorylate PtdIns to PtdIns 4-P and PtdIns 4,5-P_2.

Incubation of T-tubule membranes with [γ-^{32}P]ATP results in incorporation of ^{32}P into only two phospholipid components, which have the same R_f values as PtdIns 4-P and PtdIns 4,5-P_2 (fig.1). In contrast, incubation of SR with [γ-^{32}P]ATP results in incorporation of ^{32}P into only one phospholipid component with the same R_f as PtdIns 4-P (table 1). Thus, the T-tubule membranes but not the SR membranes have the ability to phosphorylate PtdIns 4-P to PtdIns 4,5-P_2, the membrane-bound precursor of $InsP_3$.

The time course of incorporation of ^{32}P into PtdIns 4-P and PtdIns 4,5-P_2 shows rapid labeling of both phospholipids in the T-tubule membrane (fig.2). Both reactions are complete within 1 min, although labeling at 20 mM Mg^{2+} was faster and higher than at 0.5 mM Mg^{2+} (fig.2).

Table 1

Phosphorylation of phosphatidylinositol by transverse-tubule and sarcoplasmic reticulum membranes

	pmol per μmol lipid P_i	
	PtdIns 4-P	PtdIns 4,5-P_2
Sarcoplasmic reticulum	12.1	<1
Transverse tubules	17.4	15.5

Incorporation of ^{32}P into PdtIns 4-P and PtdIns 4,5-P_2 in membranes was measured after 1 min incubation with 0.5 mM Mg^{2+} and 0.5 mM [γ-^{32}P]ATP

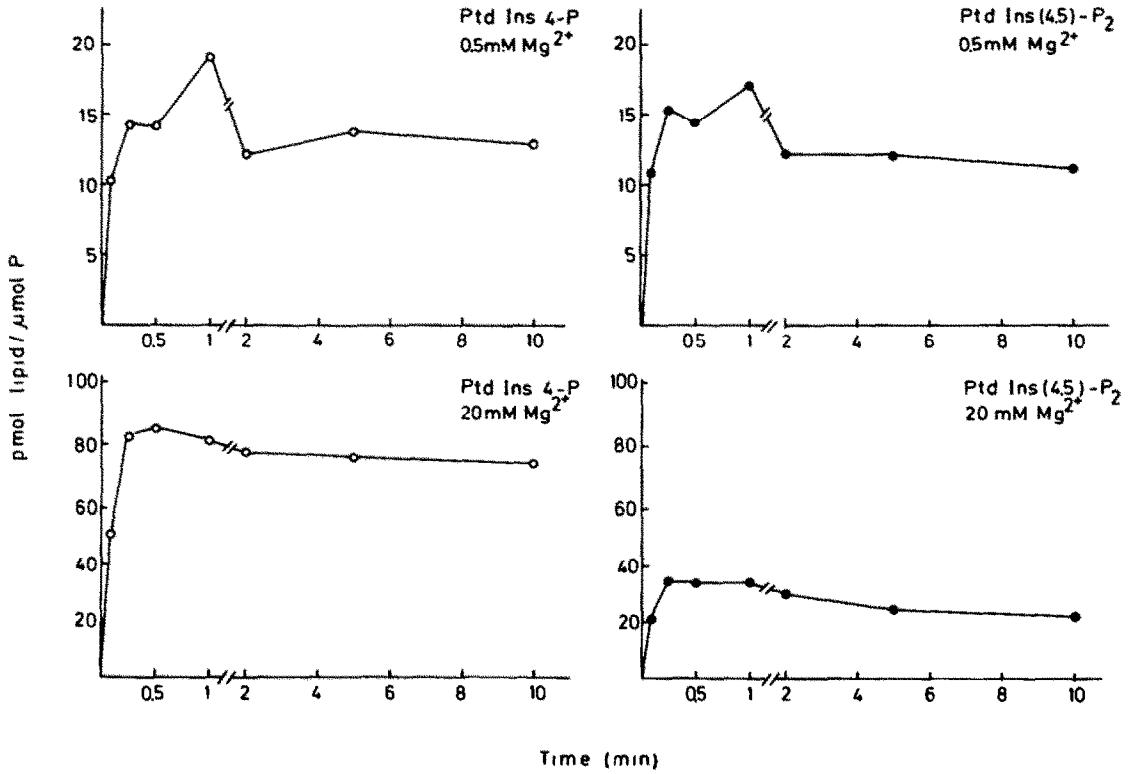

Fig.2. Time course of ^{32}P incorporation into phospholipids. (Left) Incorporation of ^{32}P into PtdIns 4-P at 0.5 mM (top) and 20 mM (bottom) Mg^{2+}. (Right) Incorporation of ^{32}P into PtdIns 4,5-P_2 at 0.5 mM (top) and 20 mM Mg^{2+} (bottom). The reaction was carried out with 0.5 mM [γ-^{32}P]ATP. The results are expressed in nmol lipid phosphorus per μmol membrane lipid phosphorus. (○) PtdIns 4-P, (●) PtdIns 4,5-P_2.

A study of both phosphorylation reactions as a function of Mn^{2+} concentration yielded apparent K_m values of about 10 mM for PtdIns 4-P and about 5 mM for PtdIns 4,5-P_2. However, it must be considered that while the first reaction has probably a non-limiting concentration of PtdIns as substrate, the second phosphorylation reaction might be restricted by the low concentrations of PtdIns 4-P available.

A similar study as a function of ATP concentration, at a constant Mg^{2+} concentration of 5 mM, showed that the formation of both PtdIns 4-P and PtdIns 4,5-P_2 increased with increasing ATP concentration up to 2 mM, without an apparent saturation (fig.3). Thus, it was not possible to determine an apparent K_m value for ATP under the experimental conditions used. We have previously shown that the T-tubule membranes isolated from frog muscle display an Mg^{2+}-ATPase activity [4] that will hydrolyze ATP during the course of the phosphorylation reaction. Hence, it will be difficult to determine K_m values for ATP unless a constant ATP concentration is maintained during the assay.

We determined the effect of tetracaine, a known inhibitor of E-C coupling [14] on the phosphorylation reactions. We found that addition of increasing amounts of tetracaine produced a significant increase in the amount of PtdIns 4-P formed, without affecting the levels of PtdIns 4,5-P_2. Since

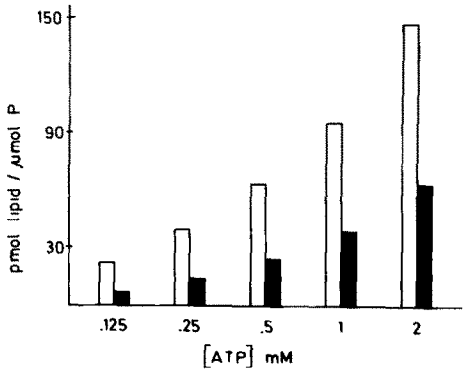

Fig.3. Incorporation of ^{32}P into PtdIns 4-P (empty bars) and PtdIns 4,5-P_2 (solid bars) at different concentrations of [γ-^{32}P]ATP. The reaction was carried out at 5 mM Mg^{2+} and was stopped after 1 min incubation. The concentrations of [γ-^{32}P]ATP used are indicated below the bars.

Table 2

Effect of tetracaine on phosphorylation of phosphatidylinositol by transverse-tubule membranes

	pmol per μmol lipid P_i	
	PtdIns 4-P	PtdIns 4,5-P_2
Control	47.5	14.2
+ 0.1 mM tetracaine	39.3	10.8
+ 0.5 mM tetracaine	74.6	12.8
+ 2.0 mM tetracaine	159.6	13.6

Incorporation of ^{32}P into both phospholipids was measured after 1 min incubation with 5 mM Mg^{2+} and 0.5 mM [γ-^{32}P]ATP

it is likely that the levels of both polyphosphoinositides measured represent steady-state values of intermediates in the cycle of PtdIns, these results suggest that the accumulation of PtdIns 4-P induced by tetracaine reflects inhibition of the kinase that phosphorylates PtdIns 4-P to PtdIns 4,5-P_2 (table 2).

4. DISCUSSION

Our results demonstrate that the T-tubule membranes isolated from frog skeletal muscle phosphorylate PtdIns to PtdIns 4-P, and the latter to PtdIns 4,5-P_2. The amounts of both phosphorylated phospholipids obtained with 5 mM Mg^{2+} and at high ATP concentration (fig.3) are in the range of 150 pmol per μmol lipid phosphorus. This value represents only 0.01% of the total phospholipid content of T-tubules, and about 0.2% of the total PtdIns content. However, since unknown factors might be missing from the isolated membranes, it is conceivable that the T-tubules in the intact cell have the ability to produce higher amounts of both phosphorylated derivatives.

From several experimental observations, Vergara et al. [1] proposed a model whereby $InsP_3$ would be the chemical messenger in E-C coupling. Among these are the findings that $InsP_3$ injected into a skeletal muscle fiber produces contraction, and that increased $InsP_3$ production takes place after tetanic stimulation of the muscle fibers.

The sequence of events in the model proposed by Vergara et al. [1] can be divided into 5 steps. Step

1: the depolarization of the T-tubule membrane stimulates the hydrolysis of PtdIns 4,5-P_2 by a phosphodiesterase to release diacylglycerol and $InsP_3$. Step 2: $InsP_3$ reaches the SR membrane and causes calcium release. Step 3: $InsP_3$ is hydrolyzed to inositol as the end product. Step 4: inositol is incorporated into the T-tubule membrane lipids as PtdIns. Step 5: by successive phosphorylation reactions PtdIns is converted into PtdIns 4,5-P_2, the membrane-bound precursor of $InsP_3$. Of the 5 proposed steps, there is contradictory evidence regarding step 2, since there is no general agreement that $InsP_3$ causes calcium release from SR vesicles [15,16], and there is no direct experimental evidence supporting steps 1, 3 or 4.

Our results demonstrate that step 5 takes place in isolated T-tubule vesicles. Furthermore, the effect of tetracaine on the phosphorylation reactions suggests that this drug, at a concentration as low as 0.5 mM, inhibits the kinase that phosphorylates PtdIns 4-P to PtdIns 4,5-P_2. Thus, the inhibition of E-C coupling produced by tetracaine could be ascribed to the inhibition of the formation of PtdIns 4,5-P_2, which would result in decreased levels of $InsP_3$ after activation of the phosphodiesterase.

In conclusion, while our findings do not by themselves prove that $InsP_3$ is the chemical messenger in E-C coupling in skeletal muscle, they are certainly compatible with the model proposed by Vergara et al. [1].

ACKNOWLEDGEMENTS

This research was supported by National Institutes of Health grant HL23007, by a grant from the Tinker Foundation, Inc. to the Centro de Estudios Científicos de Santiago, and by Universidad de Chile grants DIB-2149 and DIB-2123. We thank Dr Catherine Allende for her kind gift of [γ-^{32}P]ATP.

REFERENCES

[1] Vergara, J., Tsien, R.Y. and Delay, M. (1985) Proc. Natl. Acad. Sci. USA 82, 6352–6356.
[2] Ashley, C.C., Griffiths, P.J., Lea, T.J. and Tregear, R.T. (1985) J. Physiol. 369, 185P.
[3] Somlyo, A.P. (1985) Nature 316, 298–299.
[4] Hidalgo, C., Parra, C., Riquelme, G. and Jaimovich, E. (1986) Biochim. Biophys. Acta 855, 79–88.
[5] Jaimovich, E., Donoso, P., Liberona, J.L. and Hidalgo, C. (1986) Biochim. Biophys. Acta 855, 89–98.
[6] Hidalgo, C. (1986) in: Ionic Channels in Cells and Model Systems (Latorre, R. ed.) Plenum, New York, in press.
[7] Knowles, A.F. and Lawrence, C.M. (1985) Biochem. Biophys. Res. Commun. 129, 220–225.
[8] Rouser, G., Fleischer, S. and Yamamoto, A. (1970) Lipids 5, 494–496.
[9] Hartree, E.F. (1972) Anal. Biochem. 48, 422–427.
[10] Walsitt, T.F. and Johnson, R.A. (1979) Biochim. Biophys. Acta 526, 11–13.
[11] Rosemblatt, M., Hidalgo, C., Vergara, C. and Ikemoto, N. (1981) J. Biol. Chem. 256, 8140–8148.
[12] Lau, Y.L., Caswell, A.H., Brunschwig, J.-P., Baerwald, R.J. and Garcia, M. (1979) J. Biol. Chem. 254, 540–546.
[13] Sumnicht, G.E. and Sabbadini, R.A. (1982) Arch. Biochem. Biophys. 215, 628–637.
[14] Almers, W. and Best, P.M. (1976) J. Physiol. 262, 583–611.
[15] Scherer, N.M. and Ferguson, J.E. (1985) Biochem. Biophys. Res. Commun. 128, 1064–1070.
[16] Volpe, P., Salviati, G., Di Virgilio, F. and Pozzan, T. (1985) Nature 316, 347–349.