Kidney International, Vol. 49 (1996), pp. 251–254

Elevation of cytosolic calcium of rat cardiac myocytes in phosphate depletion

YI-BIN ZHANG, MIROSLAW SMOGORZEWSKI, ZHENMIN NI, HA-YOUNG OH, HUNG-HSIANG LIOU, and SHAUL G. MASSRY

Division of Nephrology, University of Southern California, Los Angeles, California, USA

Elevation of cytosolic calcium of rat cardiac myocytes in phosphate depletion. Phosphate depletion is associated with a rise in cytosolic calcium ($[Ca^{2+}]i$) of cells and such a derangement is responsible in major part for organ dysfunction in phosphate depletion (PD). Cardiac function is impaired in PD, and it is possible that PD is also associated with rise in $[Ca^{2+}]i$ of cardiac myocytes. The present study examined the effect of PD on $[Ca^{2+}]i$ of cardiac myocytes and explored the mechanisms that may lead to the rise in their $[Ca^{2+}]i$. The $[Ca^{2+}]i$ of cardiac myocytes began to rise and ATP content began to fall at the third week of PD. After six weeks of PD, the values of $[Ca^{2+}]i$ were significantly higher ($P < 0.01$) and those of ATP content were significantly lower ($P < 0.01$) than in control (PW) rats. The V_{max} of Ca^{2+}-ATPase and Na^+,K^+-ATPase as well as the Na^+-Ca^{2+} exchange were significantly lower ($P < 0.01$) in PD than in PW animals. The data of the present study are consistent with the notion that the rise in $[Ca^{2+}]i$ of cardiac myocytes of PD rats is due to a decrease in calcium efflux out of them.

Experimental and clinical data have demonstrated that phosphate depletion (PD) is associated with depressed myocardial performance in rats [1] and dogs [2], and with impaired myocardial function manifested by elevated end diastolic pressure and reduced stroke volume in humans [3]. The mechanisms underlying these abnormalities in myocardial function in PD are not fully understood.

PD is associated with a reduction in ATP content of almost every organ studied including red blood cells [4], polymorphonuclear leukocytes [5, 6], kidney [7], pancreatic islets [8] and brain synaptosomes [9]. Brautbar et al [10] reported that the ATP content of the heart is also reduced in PD. The reduction in ATP content in these various organs has been considered a major cause underlying the derangement in organ dysfunction in PD animals and humans [11].

Other studies have shown that PD is also associated with an elevation in the basal levels of cytosolic calcium ($[Ca^{2+}]i$) of many cells including polymorphonuclear leukocytes [6], pancreatic islets [8] and brain synaptosomes [9]. It is of interest that the prevention of the rise in the basal levels of $[Ca^{2+}]i$ of these cells resulted in normalization of their functional derangements and significant improvement or normalization of their ATP content [6, 12–14], despite the same degree and duration of PD. These observations assigned a primary role for the rise in the basal levels of $[Ca^{2+}]i$ in the genesis of cell dysfunction in PD.

It is possible that PD also causes a rise in $[Ca^{2+}]i$ of the cardiac myocytes and such an abnormality would contribute to the genesis of myocardial dysfunction in PD. The present study examined the effect of PD on $[Ca^{2+}]i$ of cardiac myocytes and explored the pathways through which PD may raise the basal levels of $[Ca^{2+}]i$ of these cells.

Methods

A total of 127 male Sprague-Dawley rats weighing 145 to 175 (168 ± 1.6) g were used. The animals were housed in individual cages and were allowed free access to deionized water at all times. The rats were divided into two groups. The first group was fed a low phosphorus diet (phosphorus content 0.03%, ICN., Chemicals, Cleveland, OH, USA) and was designated phosphate-depleted (PD) rats. The second group received control diet (phosphorus content, 0.99%, Wayne Research Animals, Bartonville, IL, USA) in a quantity adjusted to maintain their body wt equal to that of the PD animals; these rats were designated pair-weighed (PW). The other component of the two diets were comparable (protein 20 vs. 24% and fat 4.5 vs. 4.5%). Calcium content was lower in the low phosphorus diet (0.06%) than in the control diet (1.48%); this is necessary, otherwise marked hypercalcemia may develop and endanger the life of the PD animals. Blood samples were withdrawn from the tail vein under light ether anesthesia before and weekly for six weeks after the feeding of low phosphate diet (PD rats), and before and at six weeks after feeding with regular diet (PW rats). These blood samples were used for the measurements of plasma calcium, phosphorus and creatinine.

Groups of PD rats were sacrificed weekly for six weeks for the measurement of cytosolic calcium $[Ca^{2+}]i$ and ATP content of the cardiac myocytes. These parameters were evaluated only after six weeks in PW rats. The V_{max} and Km of Ca^{2+}-ATPase, V_{max} of Na^+,K^+-ATPase and the Na^+-dependent Ca^{2+} exchange were measured after six weeks in both the PD and PW rats.

Cardiac myocytes were isolated from ventricles using a modification of the methods of De Young et al [15] and Haworth et al [16]. The details of the isolation technique used in our laboratory have been reported [17]. $[Ca^{2+}]i$ in cardiac myocytes was measured by Fura 2 AM using the Perkin-Elmer fluorometer model LS 5B (Perkin-Elmer, Norwalk, CT, USA). The details of this methodology have also been reported from our laboratory [17].

Received for publication June 12, 1995
and in revised form August 9, 1995
Accepted for publication August 10, 1995

The dissociation constant for Ca^{2+}-Fura 2 was assumed to be 225 nM and calculation of $[Ca^{2+}]i$ was made using the Grynkiewicz equation [18].

ATP content of cardiac myocytes was estimated with an high-performance liquid chromatography (Shimadzu Scientific Instrument, Japan) system using 15-C Axiom C18 column (5-μm particle size; Cole Scientific, Calabasas, CA, USA).

Measurement of Na^+,K^+-ATPase and Ca^{2+}-ATPase activities were made using sarcolemmal vesicles of ventricles prepared according to the method described by Pitts [19]. The details of these measurements were reported previously from our laboratory [20].

The Na^+-Ca^+ exchange was measured in sarcolemmal vesicles as Na^+-dependent Ca^{2+} uptake by a modification of the method described by Bersohn et al [21]. An aliquot of 5 μl of sarcolemmal vesicles which were suspended in NaCl-MOPS solution prior to freezing were added to 245 μl of uptake media containing 160 mM KCl, ^{45}Ca 0.3 μCI (specific activity 10 to 40 mCi/mg calcium; Amershan, Arlington Heights, IL, USA), 10 μM $CaCl_2$, 20 mM MOPS (pH 7.4). After different times of 5,10,15,30,60 and 90 seconds, 30 μl of 10 mM EGTA (pH 7.4) were added and followed by 1 ml of ice-cold 1 mM EGTA-KCl-MOPS to stop the reaction. An aliquot of 1 ml was then taken from the mixture and passed through millipore membrane filter (0.45 μm, Millipore Corp., Bedford, MA, USA). The membranes were subsequently washed twice with 2 ml of ice cold 1 mM EGTA-KCl-MOPS. The membranes were then placed in scintillation vials containing 10 ml of scintillation fluid (Ecolite, ICN, Irvine, CA, USA) and counted with Beckman LS 7000 scintillation counter. K^+-loaded vesicles were treated in a similar manner. The Na^+-dependent Ca^{2+} uptake activity was calculated as the difference between the uptake by Na^+-loaded vesicles and K^+-loaded vesicles.

The measurement of calcium in the plasma was made by Perkin-Elmer atomic absorption spectrophotometer, model 503, and those of plasma phosphorus and plasma creatinine by an autoanalyzer (Technicon Instrument Corp., Tarrytown, NY, USA). Protein content of the sarcolemmal vesicles was measured by the method of Bradford [22]. Statistical analysis was done with paired and unpaired Student's *t*-test. Data are presented as mean ± 1 SE.

Results

Both PD and PW rats grew at the same rate and the body wt of these animals after six weeks of the study was not different (267 ± 4.2 vs. 274 ± 4.8 g). There was slight but significant ($P < 0.01$) decrease in the plasma concentration of phosphorus [from 7.8 ± 0.35 to 6.9 ± 0.30 mg/dl ($P < 0.01$) after six weeks] in the PW rats, but the plasma level of calcium in these animals did not show a significant change. In the PD rats, there was a significant ($P < 0.01$) decrement in the plasma levels of phosphorus (from 8.0 ± 0.18 to 4.1 ± 0.11 mg/dl) and significant ($P < 0.01$) increment in the plasma levels of calcium (from 9.9 ± 0.20 to 11.5 ± 0.10) after six weeks of PD. Plasma concentrations of creatinine did not change.

Figure 1 depicts the changes in $[Ca^{2+}]i$ and ATP content of the cardiac myocytes during the six weeks of phosphate depletion. The values of $[Ca^{2+}]i$ during the first and second weeks of PD were not different from those observed PW rats or from those noted in cardiac myocytes from normal rats previously reported from our laboratory [17]. However, beginning with week three of PD, the basal levels of $[Ca^{2+}]i$ began to rise progressively,

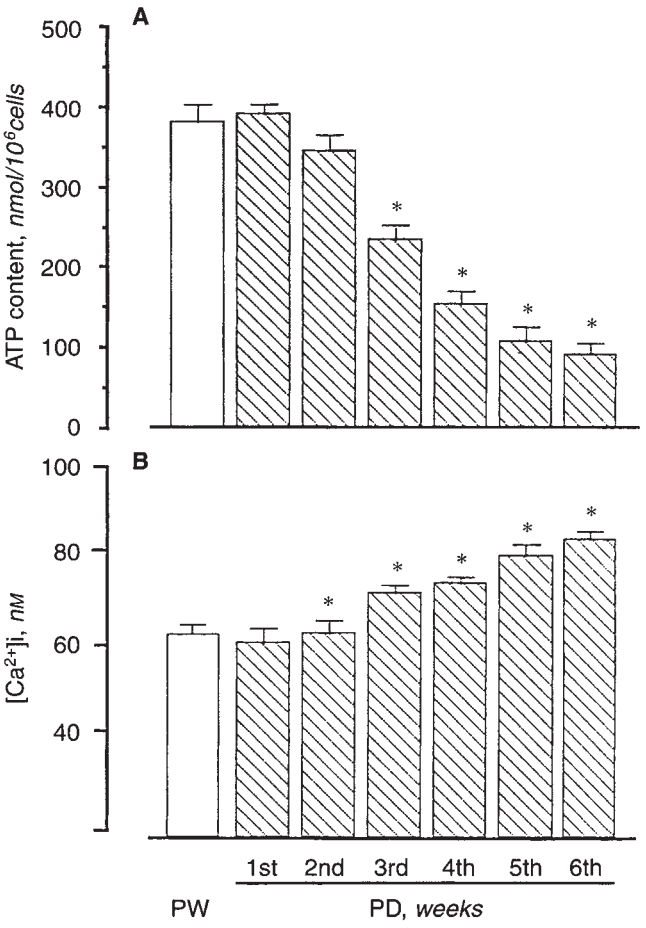

Fig. 1. *Basal levels of $[Ca^{2+}]i$ of cardiac myocytes during the evolution of PD over a period of six weeks* (**A**). The open column provides the data in PW rats and the shaded columns in PD animals. Each column represents the mean value of 7 to 9 rats and the brackets denote 1 SE. *$P < 0.01$ versus first and second weeks of PD or PW. The ATP content of cardiac myocytes during the evolution of PD over a period of six weeks (**B**). The open column provides the data in PW rats and the shaded columns in PD animals. Each column represents the mean value of 6 to 9 rats and the brackets denote 1 SE. *$P < 0.01$ versus first and second weeks of PD or PW.

reaching a value of 83 ± 7.4 nM at week 6 of PD. The values of $[Ca^{2+}]i$ at weeks 3, 4, 5, and 6 of PD were significantly higher than those at weeks 1 and 2 of PD. At week 6 of PD the basal levels of $[Ca^{2+}]i$ were significantly ($P < 0.01$) higher than those in cardiac myocytes of PW rats (63 ± 1.8 nM).

The ATP content of cardiac myocytes after weeks 1 and 2 of PD were not different from those noted in PW rats or from normal cardiac myocytes previously reported from our laboratory [17]. The ATP content began to display a progressive fall between weeks 3 and 6 of PD, reaching a value of 91 ± 13.4 nmol Pi/10^6 cells by the end of week 6 of PD (Fig. 1). The ATP content of the cardiac myocytes after six weeks of PD was markedly and significantly ($P < 0.01$) lower than that found after six weeks in PW rats.

After six weeks of PD the V_{max} of Ca^{2+}-ATPase of the sarcolemmal vesicles of the cardiac myocytes of (2.8 ± 1.74 μmol Pi/hr/mg protein) was significantly lower ($P < 0.01$) than that in

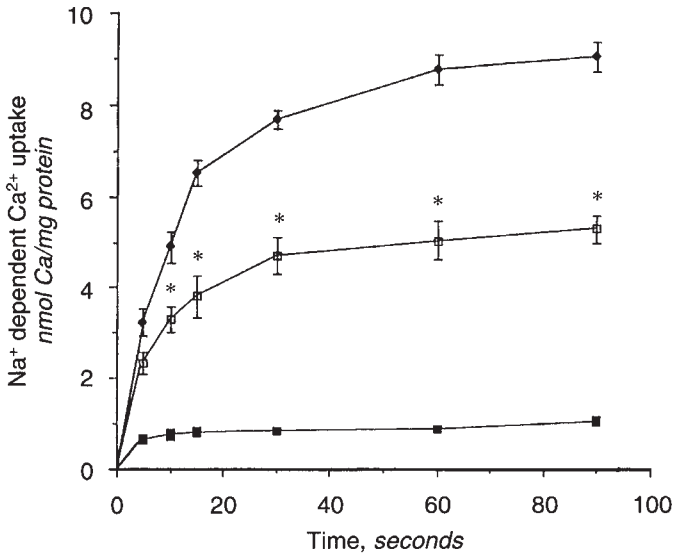

Fig. 2. *Time course of Na^+-dependent ^{45}Ca uptake by sarcolemmal vesicles of cardiac myocytes in PW and PD rats.* Symbols are: (♦) PW; (□) PD; (■) KCl. Each datum point represent the mean of 9 rats and the brackets denote ± 1 SE.

PW rats (6.4 ± 0.82 μmol/Pi/hr/mg protein). In contrast, the Km of this enzyme in these two groups of rats was not different (0.29 ± 0.02 vs. 0.27 ± 0.01 μM). The values of the V_{max} and Km of Ca^{2+} ATPase in the PW rats were not different from those found in normal rats (6.1 ± 0.29 μmol Pi/hr/mg protein and 0.28 ± 0.037 nM, respectively) previously reported from our laboratory [17].

The V_{max} of the Na^+,K^+-ATPase of the sarcolemmal vesicles of the cardiac myocytes after six weeks of PD (7.5 ± 0.62 μmol Pi/hr/mg protein) was significantly lower ($P < 0.01$) than in PW rats (16.7 ± 0.5 μmol/hr/mg protein). This latter value was not different from that found in normal rats (16.9 ± 0.73 μmol/hr/mg protein) previously reported from our laboratory [17].

The Na^+-dependent ^{45}Ca uptakes by cardiac sarcolemmal vesicles in PD and PW rats are shown in Figure 2. PD rats displayed significantly lower ($P < 0.01$) levels of uptake at all times of the uptake study.

Discussion

The results of the present study demonstrated that PD is associated with a significant rise in $[Ca^{2+}]i$ of cardiac myocytes, and this change becomes apparent in week 3 of PD and progressed with the duration of PD. Our data are similar to those found in brain synaptosomes [23], pancreatic islets [24] and polymorphonuclear leukocytes [6]. Taken together, our observations and those previously reported support the notion that an elevation in $[Ca^{2+}]i$ in PD is a generalized phenomenon.

An increase in basal levels of $[Ca^{2+}]i$ in cells could be due to increased calcium influx, decrease calcium efflux or both. Previous studies in brain synaptosomes [9, 23] and pancreatic islets [8, 24, 25] of PD rats have shown that both increased influx and decreased efflux of calcium contributed to the elevation of the basal levels of $[Ca^{2+}]i$ in these cells.

In the present study, we have not evaluated calcium influx in cardiac myocytes. However, the results demonstrated significant reduction in calcium efflux from these cells in PD. Calcium exit from cells is mediated directly by Ca^{2+}-ATPase, and Na^+-Ca^{2+} exchanger and indirectly by Na^+,K^+-ATPase [26]. The results of our study showed that the activity of all these calcium pumps are impaired in PD. Thus it appears that in PD, the rise in the basal levels of $[Ca^{2+}]i$ of cardiac myocytes is due to decreased efflux of this ion from these cells.

ATP is required for the normal function of Ca^{2+}-ATPase and Na^+,K^+-ATPase, and the decrease in the ATP content of the cardiac myocytes in PD could be responsible for the reduction in the activity of these pumps and hence to the decrease in calcium exit out of these cells. Indeed, as ATP began to fall during the third week of PD, $[Ca^{2+}]i$ began to rise. This finding suggests that the activity of Ca^{2+}-ATPase and Na^+,K^+-ATPase was already reduced by the third week of PD. Although we did not measure the activity of these enzyme after three weeks of PD, Rios et al [23] found that the V_{max} of Ca^{2+}-ATPase and of Na^+,K^+-ATPase of brain synaptosomes of PD rats were reduced by the third week of PD.

It has been reported that phospholipid contents of cell membrane modulate the activities of Ca^{2+}-ATPase and Na^+,K^+-ATPase [27, 28], and Missiaen et al [29] found, in *in vitro* studies, that the V_{max} of Ca^{2+}-ATPase is directly related to the concentration of phosphatidylinositol and phosphatidylserine. Thus, a reduction of phospholipids of cardiac myocytes in PD may also contribute to the reduced V_{max} of Ca^{2+}-ATPase and Na^+,K^+-ATPase. Indeed, Brautbar et al reported that PD caused a decrease in the content of total phospholipid, phosphatidylcholine and phosphatidylserine of the myocardium [30].

PD *per se* is associated with a fall in ATP content in many cells [31]. However, the rise in the basal levels of $[Ca^{2+}]i$ may also contribute to the magnitude of the decrement of ATP in PD. Previous studies showed that an increment in calcium burden of cells is associated with inhibition of mitochondrial oxidation and ATP production [32–34] and hence with lower ATP content [32–34]. Indeed, the prevention of the rise in basal levels of $[Ca^{2+}]i$ in brain synaptosomes [9] or in polymorphonuclear leukocytes [6] of PD rats was associated either with the normalization or improvement in the ATP contents of these cells, respectively.

The mechanisms responsible for the inhibition of the activity of the Na^+-Ca^{2+} exchanger are not elucidated by the present study. The control of the cardiac Na^+-Ca^{2+} exchanger is complex and not well understood [35]. Deitmer and Ellis [36] reported that the inhibition of Na^+,K^+-ATPase may produce acidosis in cardiac muscle, and a decrease in intracellular pH inhibits the Na-dependent Ca^{2+} efflux [37]. It is possible that the inhibition of Na^+,K^+-ATPase in PD contributes to the inhibition of Na^+-Ca^{2+} exchanger.

It has been previously demonstrated that the elevation in the basal levels of $[Ca^{2+}]i$ in PD plays an important role in organ dysfunction associated with PD [27]. Indeed, the prevention of the elevation of $[Ca^{2+}]i$ by the treatment of PD rats with verapamil prevented the impaired phagocytosis of polymorphonuclear leukocytes [6], the reduction in insulin secretion by pancreatic islets [14], and the impairment in norepinephrine metabolism of brain synaptosomes [13] as well as the decrease in their phospholipid content [12]. It is tempting to propose that the elevation in the basal levels of $[Ca^{2+}]i$ of cardiac myocytes in PD also contribute to the cardiac dysfunction associated with PD.

Acknowledgments

This work was supported by Grant DK 29955 from the National Institute of Diabetes and Digestive and Kidney Diseases. Dr. Zhang was fellow of the National Kidney Foundation of Southern California and Dr. Ni was a fellow of the National Kidney Foundation, Inc.

Reprint requests to Shaul G. Massry, M.D., Division of Nephrology, University of Southern California, 2025 Zonal Avenue, Los Angeles, California 90033, USA.

References

1. Capasso JM, Aronson RS, Strobeck JE, Sonnenblick EH: Effects of experimental phosphate deficiency on action potential characteristics and contractile performance of rat myocardium. *Cardiovasc Res* 16:71–79, 1982
2. Fuller TJ, Nichols WW, Brenner BJ, Peterson JC: Reversible depression in myocardial performance in dogs with experimental phosphorus deficiency. *J Clin Invest* 62:1194–1200, 1978
3. O'Connor LR, Wheeler WS, Bethune JE: Effect of hypophosphatemia on myocardial performance in men. *N Engl J Med* 294:901–904, 1977
4. Lichtman MA, Miller DR, Cohen J, Waterhause C: Reduced red cell glycolysis 2,3-diphosphoglycerate and adenosine triphosphate concentration, and increased hemoglobin oxygen affinity caused by hypophosphatemia. *Ann Intern Med* 74:562–568, 1971
5. Craddock PR, Yawata Y, Vansanten L, Gilbertstadt S, Jacob H: Acquired phagocyte dysfunction: A complication of hypophosphatemia of parenteral hyperalimentation. *N Engl J Med* 290:1403–1407, 1984
6. Kiersztejn M, Chervu I, Smogorzewski M, Fadda GZ, Alexiewicz J, Massry SG: On the mechanisms of impaired phagocytosis in phosphate depletion. *J Am Soc Nephrol* 2:1484–1489, 1992
7. Kreusser JW, Kurokawa K, Aznar E, Massry SG: Phosphate depletion, effect of renal inorganic phosphorus and adenine nucleotides, urinary phosphate and calcium balance. *Miner Electrol Metab* 5:30–42, 1978
8. Zhou X-J, Fadda GZ, Perna AF, Massry SG: Phosphate depletion impairs insulin secretion by pancreatic islets. *Kidney Int* 39:120–128, 1991
9. Massry SG, Hajjar SM, Koureta P, Fadda GZ, Smogorzewski M: Phosphate depletion increases cytosolic calcium of brain synaptosomes. *Am J Physiol* 260 (Renal Fluid Electrol Physiol 29):F12–F18, 1991
10. Brautbar N, Baczynski R, Carpenter CH, Moser S, Geiger P, Finander P, Massry SG: Impaired energy metabolism in rat myocardium during phosphate depletion. *Am J Physiol* 242 (Renal Fluid Electrol Physiol 11):F699–F704, 1982
11. Massry, SG: The clinical syndrome of phosphate depletion. *Adv Exp Med Biol* 103:301–312, 1978
12. Smogorzewski M, Islam A, Koureta P, Fadda GZ, Massry SG: Reduced phospholipid content of brain synaptosomes in phosphate depletion. *Am J Physiol* 261:742–747, 1991
13. Smogorzewski M, Islam A, Koureta P, Massry SG: Abnormal norepinephrine metabolism in rat brain synaptosomes in phosphate depletion. *Am J Nephrol* 13:43–52, 1993
14. Fadda GZ, Hajjar SM, Zhou X-J, Massry SG: Verapamil corrects abnormal metabolism of pancreatic islets and insulin secretion in phosphate in phosphate depletion. *Endocrinology* 130:193–202, 1992
15. De Young MB, Giannattasio B, Scarpa A: Isolation of calcium-tolerant atrial and ventricular myocytes from adult rat heart. *Meth Enzymol* 173:662–676, 1989
16. Haworth RA, Goknur AB, Warner TF, Berkoff HA: Some determinants of quality and yield in the isolation of adult heart cells from rat. *Cell Calcium* 10:57–62, 1989
17. Smogorzewski M, Zayed M, Zhang Y-B, Roe J, Massry SG: Parathyroid hormone increases cytosolic calcium concentration in adult rat cardiac myocytes. *Am J Physiol (Heart Circ Physiol 33)* 264:H1998–H2006, 1993
18. Grynkiewicz G, Poenie M, Tsien RY: A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 260:3440–3450, 1984
19. Pitts BJ: Steichiometry of sodium-calcium exchange in cardiac sarcolemmal vesicles. *J Biol Chem* 254:6232–6235, 1979
20. Zhang Y-B, Smogorzewski M, Ni Z, Massry SG: Altered cytosolic calcium homeostasis in rats cardiac myocytes in CRF. *Kidney Int* 45:1113–1119, 1994
21. Bersohn MM, Vemuri R, Schuil DW, Weiss RS, Philipson KD: Effect of temperature on sodium-calcium exchange in sarcolemma from mammalian and amphibian hearts. *Biochim Biophys Acta* 1062: 19–23, 1991
22. Bradford M: A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–259, 1976
23. Rios T, Smogorzewski M, Ni Z, Levi E, Massry SG: Sequence of appearances of the metabolic derangements in rat brain synaptosomes during phosphate depletion. *Nephron* 67:54–58, 1994
24. Levi E, Fadda GZ, Ozbasali C, Massry SG: Evolution of metabolic and functional derangements of pancreatic islets in phosphate depletion. *Endocrinology* 131:2182–2188, 1992
25. Fadda GZ, Hajjar SM, Zhou X-J, Massry SG: Verapamil corrects abnormal metabolism of pancreatic islets and insulin secretion in phosphate depletion. *Endocrinology* 130:193–202, 1992
26. Carafoli E: Calcium pumps of the plasma membrane. *Physiol Rev* 71:129–153, 1991
27. Sandermann M Jr: Regulation of membrane enzymes by lipids. *Biochem Biophys Acta* 515:209–237, 1978
28. Yeagle PL: Lipid regulation of cell membrane structure and function. *FASEB J* 3:1833–1842, 1989
29. Missiaen L, Raeymadieos L, Vujtack F, Vrolix M, Desmedt H, Costells R: Phospholipid protein interactions of the plasma-membrane Ca^{2+}-transporting ATPase. *Biochem J* 263:687–694, 1989
30. Brautbar N, Tabernero-Romo J, Coats J, Massry SG: Impaired myocardial lipid metabolism in phosphate depletion. *Kidney Int* 26:18–23, 1984
31. Massry SG, Fadda GZ, Perna AF, Kiersztejn M, Smogorzewski M: Mechanism of organ dysfunction of phosphate depletion: A critical role for a rise in cytosolic calcium. *Miner Electrol Metab* 18:133–140, 1992
32. Baczynski R, Massry SG, Kohan R, Magott M, Saglikes Y, Brautbar N: Effect of parathyroid hormone on myocardial energy metabolism in the rat. *Kidney Int* 27:718–725, 1985
33. Baczynski R, Massry SG, Magott M, El-Belbessi S, Kohan R, Brautbar N: Effect of parathyroid hormone on energy metabolism of skeletal muscle. *Kidney Int* 28:722–727, 1985
34. Trump BE, Berzesky IK: The role of ion deregulation in toxic cell injury. *Adv Mod Environ Toxicol* 14:27–50, 1987
35. Philipson KD: The cardiac Na^+-Ca^{2+} exchanger, in *Calcium and the Heart*, edited by Langer GA, New York, Raven Press Ltd, pp 85–108, 1990
36. Deitmer JW, Ellis DA: Interactions between the regulation of the intracellular pH and sodium activity of sheep cardiac Purkinje fibers. *J Physiol (London)* 307:471–488, 1980
37. Philipson KD, Bersohn MM, Nishomoto AY: Effects of pH on Na^+-Ca^{2+} exchanger in canine sarcolemmal vesicles. *Circ Res* 50:287–293, 1982