Phosphorylation and Dephosphorylation of Purified Phospholamban and Associated Phosphatidylinositides†

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ABSTRACT: Phospholamban, the putative regulator for the calcium pump, was purified to apparent homogeneity and in high yields from canine cardiac sarcoplasmic reticulum membranes. Purified phospholamban migrated with an apparent M_r of 27 000 in alkaline sodium dodecyl sulfate–polyacrylamide gels, and upon boiling in 7.5% sodium dodecyl sulfate, it dissociated into a lower molecular weight component of 5500–6000. Purified phospholamban contained 0.62 ± 0.09 μmol of lipid P_i/mg of protein, and the major phospholipids were phosphatidylserine (34%), phosphatidylcholine (22%), sphingomyelin (17%), phosphatidylinositol (13%), and phosphatidylethanolamine (9%). Phospholamban was phosphorylated by cAMP-dependent protein kinase to a level of 207 nmol of P_i/mg, and this would indicate an incorporation of 1 mol of phosphate/mol of protein, assuming a molecular weight of 5500 for phospholamban. Phosphorylation of phospholamban could be reversed by a "phospholamban phosphatase" isolated from canine cardiac cytosol. Phospholipids associated with the purified phospholamban were also phosphorylated in the presence of the catalytic subunit of cAMP-dependent protein kinase, and the maximal phosphate incorporation was 4 nmol/mg of protein. The main phospholipids phosphorylated were phosphatidylinositol 4-monophosphate and phosphatidylinositol 4,5-bisphosphate. Phosphorylation of phospholipids was inhibited by the heat-stable inhibitor protein of the cAMP-dependent protein kinase, and it could be also reversed by the phospholamban phosphatase. Thus, phosphorylation and dephosphorylation of both protein and phospholipids in phospholamban may be involved in regulation of the calcium pump in cardiac sarcoplasmic reticulum.

Calcium transport by cardiac sarcoplasmic reticulum (SR)[1] appears to be regulated through phosphorylation of phospholamban, a polymeric proteolipid. Phospholamban is phosphorylated by cAMP-dependent protein kinase (EC 2.7.1.37, ATP:protein phosphotransferase), and this results in increased velocity of calcium transport by SR vesicles, which reflects an increased affinity of the transport protein for calcium and an increased turnover of elementary steps in the Ca^{2+}, Mg^{2+}-ATPase (EC 3.6.1.3, ATP phosphohydrolase) reaction sequence (Kranias et al., 1980b; Tada & Katz, 1982; Mandel et al., 1983). Phospholamban is also phosphorylated by an endogenous calcium–calmodulin-dependent protein kinase, and this phosphorylation is associated with an increased rate of calcium transport as well (LePeuch et al., 1979; Kranias et al., 1980a; Kirchberger & Antonetz, 1982; Davis et al., 1983). Recently, a third protein kinase, a calcium–phospholipid-dependent one, isolated from rat brain, was also shown to phosphorylate phospholamban, and it was suggested that this may provide another control mechanism for SR function (Movsesian et al., 1984).

The stimulatory effects of protein kinases on calcium transport can be reversed by a SR-associated protein phosphatase activity, which dephosphorylates both the cAMP-dependent and the calcium–calmodulin-dependent sites on phospholamban (Kranias, 1985). Thus, protein kinases and protein phosphatases may modulate the state of phosphorylation of phospholamban and that would reflect on an overall regulation of calcium transport by cardiac SR.

However, besides protein phosphorylation, phosphorylation of phospholipids has also been implicated in the regulation of membrane function. A special class of phospholipids, the polyphosphoinositides, was shown to activate the Ca^{2+}-ATPase activity in skeletal SR (Varsanyi et al., 1983) in purified reconstituted systems (Carafoli & Zurini, 1982) and in erythrocytes (Redman, 1972). Several reports have indicated that formation of polyphosphoinositides may be stimulated by cAMP-dependent protein kinases in membrane systems such as rabbit cardiac SR (Enyedi et al., 1984), lymphocyte plasma membranes (Sarkadi et al., 1983), and pig granulocyte plasma membranes (Farkas et al., 1984).

In the present study we examined the stimulation of polyphosphoinositide formation in a highly purified phospholamban preparation. Phospholamban was isolated from canine cardiac SR by a reliable procedure, which yielded high quantities of the purified protein and enabled us to carry out studies on phospholipids. Phosphorylation of phospholamban by the catalytic subunit of the cAMP-dependent protein kinase resulted in phosphate incorporation in the protein moiety and increased formation of phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4-monophosphate. Reversal of phosphorylation of both protein and polyphosphoinositides was mediated by a cardiac "phospholamban phosphatase". These findings indicate that phosphorylation and dephosphorylation reactions of both protein and polyphosphoinositides may be

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[1] Abbreviations: SR, sarcoplasmic reticulum vesicles; PLB, phospholamban; cAMP, adenosine 3′,5′-monophosphate; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DOC, sodium deoxycholate; DTT, dithiothreitol; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; P_i, inorganic phosphate; Z, Zwittergent 3-14.

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involved in the regulation of cardiac SR function.

Experimental Procedures

Materials

All biochemical reagents were of "chemical pure grade". Zwittergent 3-14 (*N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate) and acquacide II were purchased from Calbiochem Behring Diagnostics. ATP was purchased from Boehringer Mannheim, and [γ-^{32}P]ATP was purchased from New England Nuclear. Protein molecular weight standards were obtained from Bio-Rad, and phospholipid kits were purchased from Serdary Research Laboratories. High-performance thin-layer silica gel 60 plates were obtained from E. Merck Darmstadt, and silica gel H plates impregnated with 7.5% magnesium acetate were purchased from Analtech. Solvents used for phospholipid analysis and chromatography were HPLC grade.

Methods

Miscellaneous Methods. Cholic acid was recrystallized before being used. Phospholamban phosphatase was isolated from canine cardiac cytosol, and the detailed procedure for its purification will be described elsewhere. Briefly, the phosphatase was purified by sequential chromatography on DEAE-Sephacel, polylysine agarose, heparin agarose, Mono Q HR 10/10, and Superose 6 gel filtration.

Isolation of Sarcoplasmic Reticulum. Cardiac sarcoplasmic reticulum (SR) was prepared as previously described by Harigaya and Schwartz (1968) and Kranias et al. (1982) with the following modifications. The minced cardiac muscle was homogenized for 6 × 25-s intervals in a Sorval Omni mixer at maximal speed (16 000 rpm). The homogenate was subsequently centrifuged at 3000*g* for 10 min, and the supernatant was filtered through four layers of cheesecloth and recentrifuged at 12000–13000*g* for 20 min. The resulting supernatant was again filtered through glass wool and centrifuged at 12000–13000*g* for 20 min. This step was repeated once, and the supernatant was further processed as previously described (Kranias et al., 1982). The final pellet (SR) was resuspended in 0.25 M sucrose, 10 mM Tris-HCl, pH 8, 1 mM DTT, and 1 mM histidine (medium III) at a protein concentration of 10–20 mg/mL. This preparation was then rapidly frozen in liquid nitrogen and stored at −90 °C. The Ca^{2+}-ATPase activity in the SR preparations was assayed by the linked enzyme method (Albers et al., 1968), and it was on the average 95–110 μM P_i (mg of protein)$^{-1}$ h^{-1}. Sarcolemmal and mitochondrial contaminations were less than 5% and 3%, respectively, in these SR preparations.

Phosphorylation Assays. Incorporation of radioactivity from [γ-^{32}P]ATP into phosphoproteins was determined by SDS–polyacrylamide slab gel electrophoresis (Prozio & Pearson, 1977) and consecutive autoradiography. Generally, 30–60 μg of SR membranes, calsequestrin, SR without calsequestrin, SR without extrinsic proteins, 8–10 μg of phospholamban crude fraction, 5–10 μg of phospholamban-rich deoxycholate extract, and 0.5–1.0 μg of column fractions and purified phospholamban fractions were added to buffer containing 50 mM phosphate, pH 7, 10 mM $MgCl_2$, 0.5 mM EGTA, 1 mM DTT, and 15 units of catalytic subunit of cAMP-dependent protein kinase. The samples (40 μL) were preincubated at 30 °C for 7 min, and the phosphorylation reactions were started by addition of 50 μM ATP (final concentration). After incubation for 7 min at 30 °C, the reactions were terminated with 20 μL of SDS stop solution containing 62.5 mM Tris (pH 6.8), 3% SDS, 7% glycerol, 5% β-mercaptoethanol, and a trace of bromophenol blue used as a tracking dye (Borbely et al., 1985). When samples were to be boiled for 5 min at 100 °C, 50 μL of SDS stop solution was added containing 62.5 mM Tris-HCl (pH 6.8), 40 mM dithiothreitol, 15% SDS, 20% glycerol, and a trace of bromophenol blue (Jones et al., 1985). Samples were loaded on SDS–polyacrylamide gels, and after electrophoresis, the radioactive proteins were identified by autoradiography. The high and low molecular weight forms of phospholamban were cut, and the degree of phosphate incorporation was quantitated by liquid scintillation spectrometry.

In dephosphorylation experiments, pure phospholamban (0.5–1 μg) was phosphorylated as described above except that 25 mM histidine buffer (pH 7) was used and the [γ-^{32}P]ATP was 10 μM. Phosphorylation of phospholamban proceeded at 30 °C, and at 7 min phospholamban phosphatase (0.086 μg/assay) was added. The incubation time continued for 30 s to 15 min, and reactions were terminated by SDS stop solution.

Stoichiometric phosphorylation of proteins was quantitated by SDS gel electrophoresis according to the method of Manalan and Jones (1982) and trichloroacetic acid precipitation. Phosphorylation of the samples was carried out as described above except that 35 units of catalytic subunit of cAMP-dependent protein kinase and 370–400 μM [γ-^{32}P]ATP were present in 100 μL of final reaction volume. In some cases 0.42% (w/w) Triton X-100 detergent was used, as indicated in the text. Reactions were terminated and processed as previously described (Kranias et al., 1980) except that bovine serum albumin was used (1 mg/mL) as carrier protein. The purity of radioactive ATP ([γ-^{32}P]ATP) was determined according to the method of Johnson and Walseth (1979), and it was taken into consideration for estimating the degree of phosphate incorporation in proteins.

Phospholipid Analysis. The total lipid extracts were prepared by the following two (a and b) procedures modified from previously described methods [(a) Bligh and Dyer (1959); (b) Jolles et al. (1981)], and the lipid classes were separated by the method of Rouser (Rouser et al., 1969). In the first procedure (a), the sarcoplasmic reticulum (200 μg) or pure phospholamban (100–200 μg) was thoroughly mixed and stirred with 2 mL of methanol for about 5 min. Chloroform (1 mL) was added, and the solution was incubated under shaking at 37 °C for 15 min. The samples were then cooled to room temperature and subsequently mixed with 1 mL of chloroform and 1 mL of distilled water or 1 mL of 0.1 M KCl. At this point, the samples were kept in the refrigerator overnight to obtain phase separation. The organic solvent layer, containing the lipid material, was evaporated under vacuum, and the lipids were redissolved in a small volume of chloroform–methanol (19:1 v/v). In the second procedure (b), the total lipids of sarcoplasmic reticulum (200 μg) or pure phospholamban (100–200 μg) were extracted with chloroform–methanol–13 M HCl (200:100:0.75 v/v) for 15 min at 37 °C. A biphasic system was formed by addition of 0.6 mL of 0.6 M HCl. The samples were kept at 4 °C overnight, mixed for 1 min, and then centrifuged at 2200*g* for 5 min to separate the two phases. The organic phase was dried under a stream of nitrogen, and the lipids were redissolved in a small volume of chloroform–methanol–water (75:25:2 v/v). Aliquots of lipid extracts and phospholipid standards were applied on silica gel 60 or silica gel H plates, and separation occurred by use of the method described by Gentner et al. (1981). In some cases, the phospholipid extracts were also subjected to two-dimensional thin-layer chromatography according to the

method of Rouser et al. (1960).

Phospholipid spots were visualized by exposure to char reagent composed of 50% (w/w) sulfuric acid and heated at 180 °C for 30 min. Identification was obtained by running standards simultaneously. The phospholipid spots were scraped, and the phosphorus group of phospholipids was determined as described by Rouser and Fleischer (1967). Total phosphorus determination was performed by the method of Hess and Derr (1975).

Fatty Acid Determination. The fatty acid composition of the lipids of purified phospholamban was determined as described by Bydlowski et al. (1987). Lipid extracts were saponified, and fatty acids were methylated and quantitated as their methyl esters by gas–liquid chromatography using methyl heptadecanoate as an internal standard.

Phosphorylation of Phospholipids in SR and Pure Phospholamban. Membrane fractions and pure phospholamban were phosphorylated as described above, and the reactions were terminated and processed by two different methods. In the first method, 2 mL of ice-cold chloroform–methanol–13 M HCl (200:100:0.75 v/v) was added to the samples according to the method of Shaikh and Palmer (1977). Extraction of phospholipids was performed as described above under the second procedure (Jolles et al., 1981). After extraction, the samples were dried and redissolved in a small volume of chloroform–methanol–water (75:25:2 v/v). Aliquots of redissolved samples were subjected to high-performance thin-layer plates. Polyinositides were separated with chloroform–methanol–4 N NH_4OH (9:7:2 v/v) and 1 mM EDTA (Gonzalez-Sastre & Folch-Pi, 1968). In the second method, 2 mL of hexane–2-propranol–concentrated HCl (300:200:4 v/v) was added to the samples, and the phospholipids were extracted according to the method of Volpi et al. (1983). Phospholipids were redissolved in a small volume of hexane–2-propanol (3:2 v/v), and aliquots of samples were loaded on silica gel 60 glass plates. Chromatograms were developed, in one dimension, in chloroform–methanol–20% methylamine (60:36:10 v/v) (Bell et al., 1982). The developed plates were dried at room temperature and processed for autoradiography. The identified spots were scraped from the plates and counted in a liquid scintillation spectrometer. The inositol-containing phospholipids were identified by running the standards on the same plate as the sample of extracted lipids.

In dephosphorylation studies, pure phospholamban (4–10 μg) was phosphorylated and dephosphorylated, as described above. The reactions were terminated by chloroform–methanol–13 M HCl, and the samples were treated for phospholipid extraction, as described above.

Inhibition of Phosphate Incorporation by the Heat-Stable Protein Kinase Inhibitor. Purified phospholamban (2 or 6.4 μg) was phosphorylated in the presence of 15 or 35 units of catalytic subunit of cAMP-dependent protein kinase. After 7 min, the process was terminated by addition of 2.5 mL of 10% trichloroacetic acid or 2.0 mL of ice-cold chloroform–methanol–13 M HCl. When the heat-stable protein kinase inhibitor was used, all conditions were similar to those described above except that 73 μg of inhibitor protein was present (1 μg of inhibitor protein can inhibit 0.6 phosphorylating unit of cAMP-dependent protein kinase) (Ashby & Walsh, 1972).

Protein Assay. The protein concentration was measured by the methods of Lowry et al. (1951) and Schaffner and Weissman (1973).

Polyacrylamide Gel Electrophoresis of Proteins. Gel electrophoresis was performed on 10–18% linear SDS–polyacrylamide gradient gels (Laemmli, 1970; Borbely et al., 1985). The following standard proteins were used: phosphorylase *b*, 92 500; bovine serum albumin, 66 000; ovalbumin, 45 000; carbonic anhydrase, 31 000; soybean trypsin inhibitor, 21 500; lysozyme, 14 400; aprotinin, 6 500.

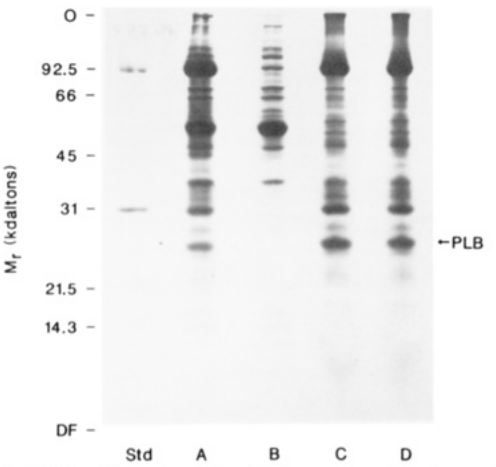

FIGURE 1: SDS–polyacrylamide gel electrophoresis of sarcoplasmic reticulum (A), carbonate-solubilized calsequestrin (B), SR without calsequestrin (C), and SR without other extrinsic proteins (D). Cardiac SR was treated with 100 mM sodium carbonate to solubilize calsequestrin and then with deoxycholate at 0.01 mg/mg of protein to solubilize other extrinsic proteins. Sixty micrograms from each fraction was solubilized by SDS sample buffer containing 3% SDS and subjected to SDS–PAGE (10–18% gradient gels) as described under Methods. Gels were stained with Coomassie Blue. PLB, phospholamban; O, origin; DF, dye front.

Antibody Methods. Antiserum to phospholamban was produced by injecting 150 μg of pure phospholamban intradermally into a rabbit twice, followed by two intramuscular injections of 50 μg of phospholamban each. Phospholamban was emulsified in Freund's complete adjuvant for all of the above injections. Western blots were accomplished by standard techniques (Towbin et al., 1979).

RESULTS

Isolation of Pure Phospholamban from Canine Cardiac Sarcoplasmic Reticulum Vesicles. The aim of this procedure was to extract the different proteins (e.g., calsequestrin and extrinsic glycoproteins) except phospholamban from cardiac sarcoplasmic reticulum vesicles (SR) before solubilization with detergent. Purification of phospholamban involved four steps, as described below.

First Step: Extraction of Calsequestrin from Cardiac Sarcoplasmic Reticulum (SR). Freshly thawed SR vesicles were first pelleted at 100000*g* for 40 min. The pellets were resuspended in cold 100 mM Na_2CO_3 (pH 11.4) at a protein concentration of 3–5 mg/mL, incubated on ice for 30 min, and then centrifuged at 4 °C for 40–60 min at 165000*g*. The resulting supernatant was highly enriched in calsequestrin (Figure 1B) and did not contain any detectable levels of phospholamban (Figure 2). The membrane pellet was gently homogenized and washed (165000*g*, 40 min) 3 times with ice-cold 0.25 M sucrose, 10 mM Tris-HCl (pH 8), 1 mM DTT, and 1 mM histidine (medium III) to remove the remaining calsequestrin. The washed pellet (SR without calsequestrin) was rehomogenized in medium III, and it appeared to be enriched (about 2.5-fold in the absence of Triton X-100 and 3–4-fold in the presence of Triton X-100) in phospholamban (Table I and Figures 1C and 2B).

Second Step: Extraction of Other Extrinsic Proteins from Sarcoplasmic Reticulum. The extrinsic glycoproteins were solubilized with low concentrations of sodium deoxycholate (0.01 mg/mg of protein) in the presence of 1 M potassium

Table I: Purification and Recovery of Phospholamban from Canine Cardiac Sarcoplasmic Reticulum[a]

prepn	fraction	total protein (mg)	$^{32}P_i$ incorpn (nmol of P_i/mg) ± Triton X-100		$^{32}P_i$ incorpn (total nmol of P_i) ± Triton X-100		recovery (%) ± Triton X-100		purifcn (x-fold) ± Triton X-100	
			−	+	−	+	−	+	−	+
1	sarcoplasmic reticulum (SR)	71	3.52	4.39	250	312				
2		93	4.18	5.09	389	474	100	100	1	1
3		63	4.03	4.87	254	306				
1	SR without calsequestrin	42	9.64	16.40	405	689	162	221	2.7	3.7
2		57	9.55	17.46	544	995	140	210	2.3	3.4
3		40	10.16	15.24	406	610	160	199	2.5	3.1
1	SR without extrinsic proteins	40	13.35	22.11	534	884	213	284	3.8	5.0
2		55	12.71	21.59	699	1188	179	250	3.0	4.0
3		38	13.71	18.98	521	722	205	235	3.4	3.9
1	phospholamban-rich DOC extract	9.25	35.25	41.28	314	382	126	123	10.0	9.0
2		11.15	35.51	47.58	396	530	102	112	8.5	9.0
3		8.00	38.22	49.11	306	404	120	128	9.5	10.0
1	pure phospholamban	1.50	170	203	255	303	100	97	48	46
2		2.11	164	212	347	447	89	94	39	42
3		1.41	180	206	253	291	99	95	45	42

[a] Stoichiometric phosphorylation of protein was measured by the trichloroacetic acid precipitation method. The samples were phosphorylated with [γ-^{32}P]ATP in the presence of the catalytic subunit of cAMP-dependent protein kinase ± Triton X-100 as described under Methods. Protein concentration was determined by the method of Schaffner and Weissman (1973).

Table II: Effect of Sodium Deoxycholate on Extraction of Phospholamban[a]

fraction	total protein	$^{32}P_i$ incorpn (mol of P_i/mg)	$^{32}P_i$ incorpn (total nmol of P_i)	recovery (%)	purification (x-fold)
SR membrane vesicles (SR)	70	3.05	214	100	1
	85	3.24	276	100	1
SR without calsequestrin	43	9.01	388	181	3
	62	8.06	500	182	2.5
SR without extrinsic proteins	42	12.13	510	238	4.0
	61	10.88	663	241	3.4
	Extracts Obtained from Deoxycholate-Treated Samples				
0.05 mg of DOC/mg of protein	0.91	5.83	5.3	2.5	2.0
	1.06	5.01	5.3	1.9	1.5
0.1 mg of DOC/mg of protein	1.22	9.11	11	5.2	3.0
	1.77	8.81	15.6	5.6	2.7
0.3 mg of DOC/mg of protein	4.31	23.09	100	47	7.6
	6.25	21.49	134	49	6.6
0.5 mg of DOC/mg of protein	6.70	28.17	188	88	9.2
	9.71	25.00	243	88	7.7
0.7 mg of DOC/mg of protein	8.62	28.60	246	115	9.0
	12.50	27.06	388	122	8.0
1.0 mg of DOC/mg of protein	9.40	25.02	235	109	8.0
	13.62	22.47	306	111	7.0

[a] Stoichiometric phosphorylation of protein was measured by the trichloroacetic acid precipitation method. The samples were phosphorylated with [γ-^{32}P]ATP in the presence of the catalytic subunit of cAMP-dependent protein kinase. Protein concentration was determined by the Lowry method (Lowry et al., 1951). Results are presented for two different preparations.

chloride (pH 8) (MacLennan, 1970). The suspension was centrifuged, and the obtained pellet (SR without extrinsic proteins) was enriched 3.5–5-fold in phospholamban (Table I and Figures 1D and 2C).

Third Step: Extraction of a Phospholamban-Rich Fraction. The pellets of SR without extrinsic proteins were rehomogenized in medium III (6–10 mg/mL) and then dialyzed against 0.25 M sucrose, 10 mM Tris-HCl (pH 8), and 1 mM histidine (buffer A). The dialysates were made 1 M in KCl and 1 mM in dithiothreitol and incubated at room temperature for 10–15 min under stirring. Various concentrations of sodium deoxycholate (pH 8; 10% w/v) were then added to determine conditions under which phospholamban could be selectively extracted. The suspensions were further incubated for 20 min at 4 °C and centrifuged (165000*g*; 90–120 min), and the opalescent supernatants were dialyzed against buffer A. Results indicated that phospholamban was not selectively extracted at low concentrations (0.05–0.2 mg/mg of protein) of sodium deoxycholate. Optimum extraction of phospholamban occurred with 0.5–0.7 mg of sodium deoxycholate/mg of protein (Table II). The specific activity of extracted phospholamban was maximal at 0.7 mg of sodium deoxycholate/mg of protein, and this activity decreased when the detergent concentration was increased to 1.0 mg/mg of protein (Table II). The Ca^{2+}-ATPase did not appear to be solubilized in appreciable amounts at sodium deoxycholate concentrations up to 0.7 mg/mg of protein (data not shown). Thus, on the basis of these findings, we chose 0.7 mg of deoxycholate/mg of protein for selective solubilization of phospholamban, and the fraction obtained is referred to as the phospholamban-rich DOC extract.

Fourth Step: Further Purification of Phospholamban by Sulfhydryl Group Affinity Chromatography. The phospholamban-rich DOC extract was dialyzed against 300 volumes of buffer A, changed 3 times, and subsequently dialyzed against 200 volumes of 0.25 M sucrose, 30 mM histidine (pH 7), and 66.6 mM $CaCl_2$ overnight at 4 °C with one change. After dialysis, phospholamban ghosts were solubilized by

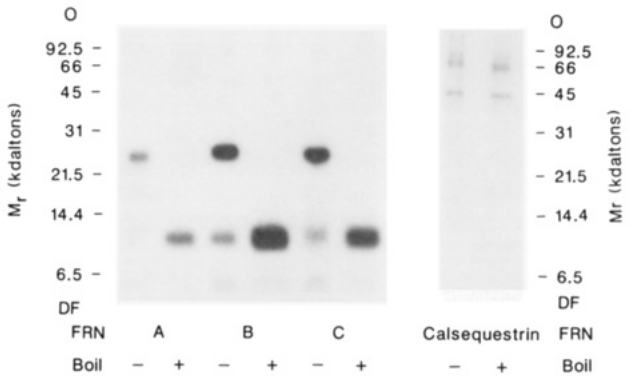

FIGURE 2: Effect of removal of different membrane proteins from cardiac sarcoplasmic reticulum on enrichment of phospholamban. SR was treated with sodium carbonate (SR without calsequestrin) and then with deoxycholate (SR without extrinsic proteins) as described in Figure 1. Each fraction (60 μg) was phosphorylated by the catalytic subunit of cAMP-dependent protein kinase in the presence of [γ-^{32}P]ATP as described under Methods. Half of the samples were placed in a boiling water bath for 5 min just prior to electrophoresis [boil (+)]. The samples were then subjected to SDS–PAGE. Phosphorylated proteins were determined by autoradiography using Kodak X-Omat RP X-ray film (24-h exposure time). (A) Original SR; (B) SR without calsequestrin; (C) SR without extrinsic proteins; (calsequestrin) sodium carbonate extracted supernatant fraction.

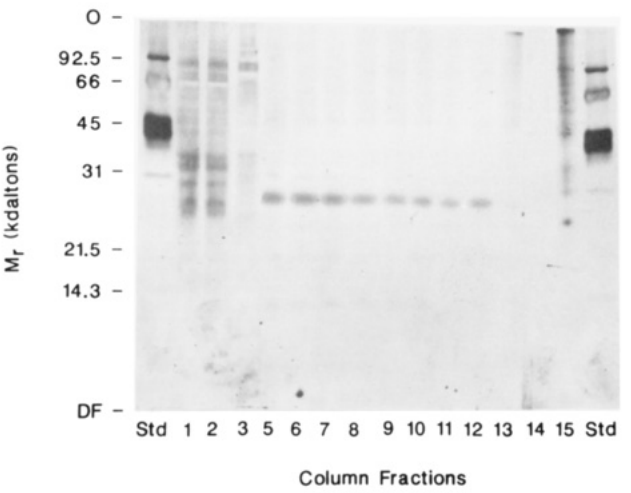

FIGURE 3: SDS–polyacrylamide gel electrophoresis of *p*-(hydroxymercuri)benzoate agarose column fractions. Phospholamban (8 mL) in 1% Zwittergent 3-14 (v/v) was applied to a *p*-(hydroxymercuri)benzoate agarose column (5-mL bed vol; 0.782 cm^2) preequilibrated in 10 mM MOPS (pH 7.0)–0.1% Zwittergent 3-14 (Z)–0.5 M NaCl. The column was washed with 20 mL of the above buffer, and 10-mL fractions were collected (fractions 1, 2). The column was then washed with 20 mL of the same buffer but in the absence of NaCl, and 10-mL fractions were collected (fractions 3, 4). Phospholamban was eluted with 25 mL of 10 mM MOPS (pH 7.0)–0.1% Z–20 mM DTT (fractions 5–9) followed by 15 mL of 10 mM MOPS (pH 7.0)–0.25% Z–20 mM DTT (fractions 10–12), and 5-mL fractions were collected. Remaining proteins were eluted with 20 mL of 10 mM MOPS (pH 7.0)–0.25% Z–20 mM DTT–0.5 M NaCl, and 5-mL fractions were collected (fractions 13–17). Protein in each column fraction was determined by the method of Schaffner and Weissman (1973). Aliquots (0.5 μg) of the column fractions were solubilized by SDS sample buffer and subjected to SDS gel electrophoresis and silver staining as described under Methods. Std, molecular weight standards.

addition of Zwittergent 3-14 (10% w/v), under continuous stirring, to give a final concentration of 1% Zwittergent 3-14 (v/v). The clear sample was then applied to a column of *p*-(hydroxymercuri)benzoate agarose, which was preequilibrated with 0.1% Zwittergent 3-14 and 10 mM MOPS (pH 7). The volume of the sample to the bed volume of the column was 1.5–2.0. Pure phospholamban was eluted by a stepwise approach using selective elution methods in combination with sulfhydryl group specific adsorbent, as previously described (Jones et al., 1985). On the basis of silver staining (Figure 3) and autoradiography, following SDS gel electrophoresis of column fractions, the fractions (5–12) enriched in pure phospholamban were collected and concentrated. Both the high and low M_r forms of phospholamban were detected by SDS gel electrophoresis (data not shown).

Phosphate incorporation in the various fractions, obtained during purification of phospholamban, was determined by three different procedures: trichloroacetic acid precipitation of samples phosphorylated in the absence or in the presence of Triton X-100 or by counting the phosphorylated bands after they were cut from SDS gels. Pure phospholamban appeared to incorporate 191 nmol of P_i/mg of protein, estimated by the gel electrophoresis method. Similar incorporation was observed when phosphorylation was estimated by the trichloroacetic acid method (Table I). The average of phospholamban phosphorylation in three different preparations was 171 ± 5 nmol P_i/mg of protein, and inclusion of Triton X-100 increased the phosphorylation level to 207 ± 3 nmol of P_i/mg. Assuming a molecular weight of 27 000 for phospholamban, the stoichiometry of phosphate incorporation is calculated to be 5.5 mol of P_i/mol of protein in the presence of Triton X-100. The purification of phospholamban appeared to be about 44-fold (Table I), but this is an underestimation since the sarcoplasmic reticulum preparation, used for isolation of phospholamban, was phosphorylated to a high level (3.9 ± 0.2 nmol of P_i/mg of SR in the absence of Triton X-100). An average of 2.2 ± 0.6% of original SR protein was recovered as pure phospholamban by this procedure, and the yield was about 96% with respect to original SR (Table I).

Immunolocalization of Phospholamban in Cardiac SR. Purified phospholamban was highly antigenic in rabbits. The resulting antiserum reacted specifically with the high and low molecular weight forms of phospholamban, as determined by the western blotting method (data not shown). Immunohistochemical studies revealed that phospholamban was mainly localized in the SR and nuclear membranes in the canine cardiac muscle (Young et al., 1987).

Dephosphorylation of Purified Phospholamban by Phospholamban Phosphatase. Pure phospholamban was phosphorylated by [γ-^{32}P]ATP and the catalytic subunit of cAMP-dependent protein kinase. The ATP concentration used in these studies was low (10 μM) because ATP is an inhibitor of the phospholamban phosphatase. ^{32}P incorporation into phospholamban increased up to 5 min at 30 °C, and it subsequently remained constant (data not shown). At 7 min, phospholamban phosphatase was added, and this resulted in dephosphorylation of both the high and low molecular weight forms of phospholamban (Figure 4).

Phospholipid and Fatty Acid Composition of Purified Phospholamban. Pure phospholamban contained 0.62 ± 0.09 μmol of lipid P_i/mg (n = 4), while canine cardiac SR contained 2.64 ± 0.10 μmol of lipid P_i/mg (n = 5), when the protein concentration was determined by the Amido Black method (Schaffner & Weissman, 1973). When the protein concentration of SR was estimated by the Lowry method (Lowry et al., 1951), it was found that the lipid phosphorus was 1.45 ± 0.03 μmol/mg (n = 5), in agreement with previous observations (Gross, 1985). The distribution of the individual phospholipids was markedly different in pure phospholamban compared to that in SR membranes. Phosphatidylserine comprised 34% of the total phospholipid in purified phospholamban, followed by phosphatidylcholine (22%), sphingomyelin (17%), phosphatidylinositol (13%), and phosphati-

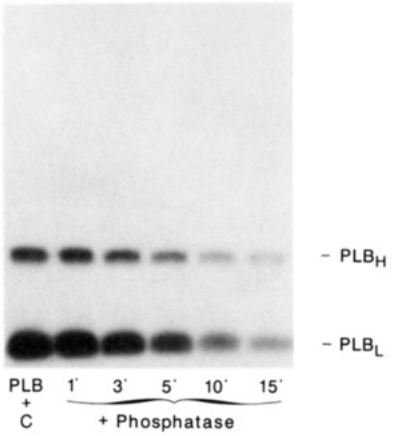

FIGURE 4: Autoradiography of pure phospholamban dephosphorylated by a phospholamban phosphatase. Phospholamban (1 μg) was incubated with 10 μM [γ-^{32}P]ATP in the presence of 15 units of the catalytic subunit of cAMP-dependent protein kinase (PLB + C) as described under Methods. At 7 min, phospholamban phosphatase (0.08 μg) was added. The incubation time was continued for 1, 3, 5, 10, and 15 min, and the samples were then subjected to SDS–PAGE followed by autoradiography as described under Methods. PLB$_H$, high M_r form of phospholamban; PLB$_L$, low M_r form of phospholamban.

Table III: Distribution of Individual Fatty Acids in Purified Phospholamban[a]

	distribution (%)
myristic acid (14:0)	2.3
palmitic acid (16:0)	20.0
palmitoleic acid (16:1)	0.8
stearic acid (18:0)	16.2
oleic acid (18:1)	21.5
linoleic acid (18:2)	8.7
linolenic acid (18:3)	25.2
arachidic acid (*n*-eicosanoic acid) (20:0)	1.2
eicosatrienoate (20:3)	2.8
arachidonic acid (eicosatetraenoic acid) (20:4)	1.3
unsaturated/saturated fatty acids	1.51

[a] Fatty acids were analyzed as described under Methods. The results are expressed in percent of total fatty acid (two different preparations).

dylethanolamine (9%). In SR membranes, the major phospholipid constituents were phosphatidylcholine (61%), phosphatidylethanolamine (23%), phosphatidylinositol (9%), phosphatidylserine (3%), and sphingomyelin (2%), in agreement with previous observations (Chamberlain et al., 1983).

The individual fatty acids of the phospholamban phospholipids were identified and quantitated as their methyl esters by gas–liquid chromatography. The composition of fatty acids is shown in Table III.

Phosphorylation of Phospholipids in Pure Phospholamban. Phosphate incorporation into phospholipids occurred upon incubation of purified phospholamban with the catalytic subunit of cAMP-dependent protein kinase in the presence of [γ-^{32}P]ATP. Phosphorylation of phospholipids increased with time up to 5 min at 30 °C (Figure 5), and it subsequently remained constant up to 10 min. When higher concentrations of ATP were used (up to 400 μM), there was an increase in the rate and the level of lipid phosphorylation (data not shown). The maximal phosphate incorporation into phospholipids of the purified phospholamban was 4 nmol/mg, and this represents about 2% of the total phosphate incorporated in pure phospholamban (Table I). Phosphorylation of phospholipids was inhibited up to 85% by the heat-stable inhibitor protein of the cAMP-dependent protein kinase, while phosphorylation of phospholamban was inhibited up to 90% under identical conditions.

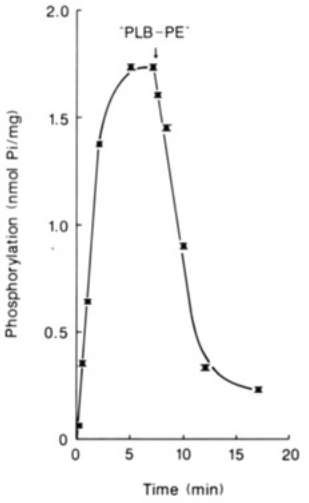

FIGURE 5: Phosphorylation and dephoshorylation of phospholipids in pure phospholamban. Pure phospholamban (8 μg) was incubated with 10 μM [γ-^{32}P]ATP in the presence of 15 units of the catalytic subunit of cAMP-dependent protein kinase. At 7 min, phospholamban phosphatase (PLB-PE) (0.08 μg) was added, and the incubation time was continued up to 17 min. The lipid phosphorylation reaction was terminated by addition of acidified chloroform–methanol, and ^{32}P incorporation into lipids was determined as described under Methods. Results represent the mean ± SE for three determinations.

Table IV: Phosphorylation of Polyphosphoinositides during Purification of Phospholamban[a]

	phosphate incorpn (pmol of P$_i$/mg of protein)			
	PIP		PIP$_2$	
fraction	A	B	A	B
SR	49	39	63	35
SR without calsequestrin	61	76	341	405
phospholamban-rich DOC extract	3645	2581	ND[b]	ND[b]
phospholamban	2206	2173	2152	1481

[a] Acidic chloroform–methanol extracts containing the ^{32}P-labeled lipids were obtained and analyzed by thin-layer chromatography, and phosphate incorporation was determined as described under Methods. Results are expressed as picomoles of phosphate incorporated into PIP (phosphatidylinositol 4-monophosphate) and PIP$_2$ (phosphatidylinositol 4,5-bisphosphate). (A, B) Two different preparations. [b] ND, not detected.

It has been previously reported that an endogenous phosphatase can cause rapid dephosphorylation of both protein and polyphosphoinositides in rat brain (Jolles et al., 1981). In the present study, a phospholamban phosphatase was used to catalyze dephosphorylation of the purified phospholamban (Figure 4). To determine whether dephosphorylation of phospholipids could also occur under identical conditions, the purified phospholamban was phosphorylated by the catalytic subunit of the cAMP-dependent protein kinase, in the presence of low [γ-^{32}P]ATP concentration (10 μM; see above), and at 7 min, an exogenous phospholamban phosphatase was added to the system. Addition of the phosphatase was associated with dephosphorylation of the phospholipids (Figure 5).

The major phospholamban phospholipids phosphorylated in the presence of the cAMP-dependent protein kinase in pure phospholamban were identified as phosphatidylinositol 4-monophosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP$_2$). Equal amounts of phosphate appeared to be incorporated into both PIP (450 pmol of P$_i$/min) and PIP$_2$ (350 pmol of P$_i$/min), shown in Table IV, and addition of the phospholamban phosphatase was associated with dephosphorylation of both PIP and PIP$_2$. When phospholipid phosphorylation in the various fractions obtained during isolation

of phospholamban was determined, it was observed that phosphorylation of both PIP and PIP_2 occurred in the SR membranes, and extraction of calsequestrin was associated with increased phosphorylation of both PIP and PIP_2 but specifically PIP_2 (Table IV). Phosphorylation of the deoxycholate-extracted phospholamban-rich sample occurred on PIP, and there was no detectable phosphate incorporation into PIP_2 (Table IV), indicating that subsequent phosphorylation of PIP was inhibited at this step. In some cases, there was a small amount of radiolabeled material that did not migrate from the origin in the thin-layer chromatography system. This was scraped from the silica gel, and it was eluted with chloroform–methanol–concentrated HCl (250:500:3 v/v) by using the Brautigan method (Brautigan et al., 1985). The samples appeared free of protein as indicated by the lack of a peak around 262–282 nm. However, the ultraviolet spectrum of the eluant had an absorbance maximum around 254–265 nm, marking the presence of nonincorporated nucleotides at the origin.

Discussion

In this study we report the formation of polyphosphoinositides upon phosphorylation of purified phospholamban by the catalytic subunit of cAMP-dependent protein kinase. Phospholamban was isolated by a procedure that resulted in high yields of the protein in a highly phosphorylatable state. The unique aspect of this isolation procedure was that various proteins, such as calsequestrin and other extrinsic proteins, were initially extracted from SR prior to solubilization of phospholamban, and the isolation procedure was relatively simple and fast compared to previous methods (Inui et al., 1985; Jones et al., 1985). The purified phospholamban could be phosphorylated to a level of 205 nmol of P_i/mg of protein, which would indicate a stoichiometric incorporation of about 5 mol of P_i/mol of protein, assuming a molecular weight of 27 000 for phospholamban. Analysis of the phospholipid content in phospholamban revealed that there were 0.62 ± 0.09 μmol of lipid P_i/mg of phospholamban, while the cardiac sarcoplasmic reticulum membranes contained about 4-fold higher levels of lipid P_i/mg of protein. Furthermore, the phospholipids associated with phospholamban were mainly acidic phospholipids such as phosphatidylserine and phosphatidylinositol. These acidic phospholipids and specifically phosphatidylinositol may play a role in the regulation of phospholamban phosphorylation. It has recently been shown that phosphorylation of phospholamban may be stimulated by either an endogenous factor, which is extracted by organic solvents, or by phosphatidylinositol (Suzuki & Wang, 1987). The maximal phosphate incorporation in phospholamban, in the presence of the extracted factor or exogenous phosphatidylinositol, was 5 mol of P_i/mol of protein (Suzuki & Wang, 1987). This level of incorporation is similar to the level obtained in the present study, suggesting that the phospholamban preparation isolated by our method probably contains saturating amounts of phosphatidylinositol. However, it is not clear whether phosphatidylinositol itself or polyphosphoinositides, formed during phosphorylation reactions, may control the degree of phospholamban phosphorylation. In the present study, it was observed that phosphorylation of phospholamban by the catalytic subunit of cAMP-dependent protein kinase was associated with increased formation of polyphosphoinositides (PIP and PIP_2). Previously, formation of polyphosphoinositides by the cAMP-dependent protein kinase was reported to occur in a sarcoplasmic reticulum preparation of rabbit heart (Enyedi et al., 1984), in lymphocyte plasma membranes (Sarkadi et al., 1983), and in a plasma membrane preparation of pig granulocytes (Farkas et al., 1984). Phosphorylation of phosphoinositides has been suggested to regulate the permeability of the membrane (Michell et al., 1977; Michell, 1982; Berridge, 1984) or regulate the Ca^{2+}-ATPase activity in skeletal sarcoplasmic reticulum (Varsanyi et al., 1983) and in erythrocyte plasma membranes (Redman, 1972; Buckley & Hawthorne, 1972). This possible regulation of Ca^{2+} pumping through the phosphoinositides has also been implicated in short-term memory (Penniston, 1983).

Phosphorylation of polyphosphoinositides in the purified phospholamban was dependent on the presence of the catalytic subunit of cAMP-dependent protein kinase. Furthermore, phosphorylation of polyphosphoinositides was inhibited in the presence of the heat-stable cAMP-dependent protein kinase inhibitor, and the extent of inhibition was similar for both the protein and the polyphosphoinositide moieties. It is not presently known whether formation of the polyphosphoinositides was mediated directly by the catalytic subunit of cAMP-dependent protein kinase or by endogenous phosphatidylinositide protein kinase(s), which may be regulated by cAMP-dependent phosphorylation. Recently, we have observed that the catalytic subunit of cAMP-dependent protein kinase could also phosphorylate pure phosphatidylinositol, to a low extent (0.12 pmol/min), but only PIP_2 was detected in contrast to the phosphorylation of pure phospholamban, where both PIP and PIP_2 were observed. The reason for this discrepancy is not known, but it may be due to differences in the nature of the substrate present either in liposomes (pure PI) or in the protein–lipid form (pure phospholamban).

Phosphorylation of phospholamban and associated polyphosphoinositides could be reversed by the phospholamban phosphatase isolated from canine cardiac cytosol. Dephosphorylation of the polyphosphoinositides in phospholamban may occur either by the protein phosphatase activity or by a phosphodiesterase activity associated with, or exhibited by, the phosphatase preparation. Previously, an endogenous phosphatase was shown to dephosphorylate both the protein and the phosphatidylinositol monophosphate and bisphosphate in a fraction from rat brain that contained both membranes and soluble enzymes (Jolles et al., 1981).

The polyphosphoinositides associated with pure phospholamban were only detected upon labeling with [^{32}P]phosphate, and it is not known whether phospholamban contains only the radioactive ones or unlabeled polyphosphoinositides as well. Although the polyphosphoinositides formed constitute only minor amounts, compared to phosphatidylinositol (about 5%), it is possible that phosphorylation/dephosphorylation of these phospholipids changes the polarity of phospholamban, and this is associated with alterations in its conformational shape. Such changes in the conformation of phospholamban may play a key role in the phosphorylation of phospholamban by the protein kinases and/or in the interaction of phospholamban with the calcium pump. The manner by which phospholamban regulates the Ca^{2+}-ATPase is not known yet, but it has been suggested that there is a physical interaction of the two proteins, which is associated with inhibition of the Ca^{2+}-ATPase (Inui et al., 1986; Suzuki & Wang, 1986). Phosphorylation of phospholamban by protein kinase(s) relieves this inhibition, resulting in activation of the Ca^{2+} pump. It is possible that both formation of polyphosphoinositides, which are tightly associated with phospholamban, and phosphorylation of the protein moiety are responsible for the observed stimulatory effects on the SR Ca^{2+}-ATPase. Previously, phosphorylation of phospholamban was shown to occur in perfused beating hearts during the peak of the inotropic response to β-adrenergic agonists (Lindemann et al., 1983; Kranias et al., 1985).

Recently, we have observed that β-adrenergic stimulation of perfused beating guinea pig hearts was also associated with increased phosphate incorporation in phosphatidylinositol monophosphate and bisphosphate in SR membranes (Jakab et al., 1988). However, it remains to be seen whether the increased polyphosphoinositide formation is associated with functional alterations in cardiac SR function, which may be involved, at least in part, in mediating the effects of β-adrenergic agents in the mammalian heart.

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REFERENCES

Albers, P. N., Wood, G. J., & Siegel, G. J. (1968) *Mol. Pharmacol. 4*, 324–336.
Ashby, C. D., & Walsh, D. A. (1972) *J. Biol. Chem. 247*, 6637–6642.
Bell, M. E., Peterson, R. G., & Eichberg, J. (1982) *J. Neurochem. 39*, 192–198.
Berridge, J. M. (1984) *Biochem. J. 220*, 345–360.
Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–916.
Borbely, G., Suranyi, G., Korcz, A., & Palfi, Z. (1985) *J. Bacteriol. 161*, 1125–1130.
Brautigan, D. L., Randazzo, P., Shriner, C., & Fain, J. N. (1985) *Lipids 20*, 492–495.
Buckley, J. T., & Hawthorne, N. Y. (1972) *J. Biol. Chem. 247*, 7218–7223.
Bydlowski, S. P., Yunker, R. L., & Subbiah, M. T. R. (1987) *Am. J. Physiol. 252*, H14–H21.
Carafoli, E., & Zurini, M. (1982) *Biochim. Biophys. Acta 683*, 279–301.
Chamberlain, B. K., Levitsky, D. O., & Fleischer, S. (1983) *J. Biol. Chem. 258*, 6602–6609.
Davis, B. A., Schwartz, A., Samaha, F. J., & Kranias, E. G. (1983) *J. Biol. Chem. 258*, 13587–13591.
Enyedi, A., Farago, A., Sarkadi, B., & Gardos, G. (1984) *FEBS Lett. 176*, 235–238.
Farkas, G., Enyedi, A., Sarkadi, B., Gardos, G., Nagy, Z., & Farago, A. (1984) *Biochem. Biophys. Res. Commun. 124*, 871–876.
Gentner, P., Bauer, M., & Dieterich, J. (1981) *J. Chromatogr. 206*, 200–204.
Gonzalez-Sastre, F., & Folch-Pi, J. (1968) *J. Lipid Res. 9*, 532–533.
Gross, R. W. (1985) *Biochemistry 24*, 1662–1668.
Harigaya, S., & Schwartz, A. (1968) *Circ. Res. 25*, 781–794.
Hess, H. H., & Derr, J. E. (1975) *Anal. Biochem. 63*, 607–613.
Inui, M., Kadoma, M., & Tada, M. (1985) *J. Biol. Chem. 260*, 3708–3715.
Inui, M., Chamberlain, B. K., Saito, A., & Fleischer, S. (1986) *J. Biol. Chem. 261*, 1794–1800.
Jakab, G., Rapundalo, S. T., Solaro, R. J., & Kranias, E. G. (1988) *Biochem. J. 251*, 189–194.
Johnson, R. A., & Walseth, T. F. (1979) *Adv. Cyclic Nucleotide Res. 10*, 135–167.
Jolles, J., Zwiers, H., Dekker, A., & Wirtz, K. W. A. (1981) *Biochem. J. 194*, 283–291.
Jones, L. R., Simmerman, H. K. B., Wilson, W. W., Guard, F. R. N., & Wegener, A. D. (1985) *J. Biol. Chem. 260*, 7721–7730.
Kirchberger, M. A., & Antonetz, T. (1982) *J. Biol. Chem. 257*, 5685–5691.
Kranias, E. G. (1985) *J. Biol. Chem. 260*, 11006–11010.
Kranias, E. G., Bilezikjian, L. M., Potter, J. D., Piascik, M. T., & Schwartz, A. (1980a) *Ann. N.Y. Acad. Sci. 356*, 279–291.
Kranias, E. G., Mandel, F., Wang, T., & Schwartz, A. (1980b) *Biochemistry 19*, 5434–5439.
Kranias, E. G., Schwartz, A., & Jungmann, R. A. (1982) *Biochim. Biophys. Acta 709*, 28–37.
Kranias, E. G., Garvey, J. L., Srivastava, R. D., & Solaro, R. J. (1985) *Biochem. J. 226*, 113–121.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
LePeuch, C. L., Haiech, J., & DeMaille, J. G. (1979) *Biochemistry 18*, 5150–5157.
Lindemann, J. P., Jones, L. R., Hathaway, D. R., Henry, B. G., & Watanebe, A. M. (1983) *J. Biol. Chem. 258*, 464–471.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
MacLennan, D. H. (1970) *J. Biol. Chem. 245*, 4508–4528.
Manalan, A. S., & Jones, L. R. (1982) *J. Biol. Chem. 257*, 10052–10062.
Mandel, F., Kranias, E. G., & Schwartz, A. (1983) *J. Bioenerg. Biomembr. 15*, 179–194.
Michell, R. H. (1982) *Neurosci. Res. Program Bull. 20*, 338–350.
Michell, R. H., Jafferji, S. S., & Jones, L. M. (1977) *Adv. Exp. Med. 83*, 447–465.
Movsesian, M. A., Nishikawa, M., & Adelstein, R. S. (1984) *J. Biol. Chem. 259*, 8029–8032.
Penniston, J. T. (1983) *Ann. N.Y. Acad. Sci. 402*, 296–303.
Porzio, M. A., & Pearson, A. M. (1977) *Biochim. Biophys. Acta 490*, 27–34.
Redman, C. M. (1972) *Biochim. Biophys. Acta 282*, 123–134.
Rouser, G., & Fleischer, S. (1967) *Methods Enzymol. 10*, 385–406.
Rouser, G., Siakotos, A. N., & Fleischer, S. (1960) *Lipids 1*, 85–86.
Rouser, G., Fleischer, S., & Yamamoto, A. (1969) *Lipids 5*, 494–496.
Sarkadi, B., Enyedi, A., Farago, A., Meszaros, G., Kremmer, T., & Gardos, G., (1983) *FEBS Lett. 152*, 195–198.
Schaffner, W., & Weissman, C. (1973) *Anal. Biochem. 56*, 502–514.
Shaikh, N. A., & Palmer, F. B. St. C. (1977) *J. Neurochem. 28*, 395–402.
Suzuki, T., & Wang, J. H. (1986) *J. Biol. Chem. 261*, 7018–7023.
Suzuki, T., & Wang, J. H. (1987) *J. Biol. Chem. 262*, 3880–3885.
Tada, M., & Katz, A. (1982) *Annu. Rev. Physiol. 44*, 401–423.
Towbin, H., Staehelin, T., & Dordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Varsanyi, M., Tolle, H. G., Heilmeyer, L. M. G., Dawson, R. M. C., & Irvine, F. R. (1983) *EMBO J. 2*, 1543–1548.
Volpi, M., Yassin, R., Naccache, P. H., & Sha'afi, R. I. (1983) *Biochem. Biophys. Res. Commun. 112*, 957–964.
Young, E. F., Ferguson, D. G., & Kranias, E. G. (1987) *Biophys. J. 51*, 350a.