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PHOSPHORYLATION OF A PANCREATIC ZYMOGEN GRANULE MEMBRANE PROTEIN BY ENDOGENOUS CALCIUM/PHOSPHOLIPID-DEPENDENT PROTEIN KINASE

ROBERT W. WRENN

Department of Anotomy, School of Medicine, Medical College of Georgia, Augusta, GA 30912 (U.S.A.)

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The occurrence of phospholipid-sensitive calcium-dependent protein kinase (referred to as C kinase) and its endogenous substrate proteins was examined in a membrane preparation from rat pancreatic zymogen granules. Using exogenous histone H_1 as substrate, C kinase activity was found in the membrane fraction. The kinase was solubilized from membranes using Triton X-100 and partially purified using DEAE-cellulose chromatography. An endogenous membrane protein ($M_r \approx 18000$) was found to be specifically phosphorylated in the combined presence of Ca^{2+} and phosphatidylserine. Added diacylglycerol was effective in stimulating phosphorylation of exogenous histone by the partially purified C kinase, but had no effect upon phosphorylation of the endogenous 18 kDa protein by the membrane-associated C kinase. Phosphorylation of the 18 kDa protein was rapid (detectable within 30 s following exposure to Ca^{2+} and phosphatidylserine), and highly sensitive to Ca^{2+} ($K_a = 4\ \mu M$ in the presence of phosphatidylserine). These findings suggest a role for this Ca^{2+}-dependent protein phosphorylation system in the regulation of pancreatic exocrine function.

Introduction

The importance of Ca^{2+} as a regulator of exocrine pancreatic secretion is well established [1,2]. Although the precise biochemical pathways by which Ca^{2+} exerts its effects are unclear, one possible mechanism is through the phosphorylation of endogenous pancreatic proteins by Ca^{2+}-activated protein kinases, with subsequent alterations in protein functions and, ultimately, cellular activity such as secretion. The presence of a Ca^{2+}-dependent protein kinase in brain utilizing phospholipid (preferentially phosphatidylserine) rather than calmodulin as a cofactor was first reported by Takai et al. [3]. We subsequently showed this phospholipid-sensitive Ca^{2+}-dependent protein kinase and its endogenous substrate proteins to be distributed in several tissues, including whole pancreas [4–6]. More recent studies have located C kinase and its substrate proteins to the pancreatic acinar cell population [7]. We report here the presence of C kinase, and a specific endogenous substrate protein for this kinase, in a membrane preparation from rat pancreatic zymogen granules. Since it has been suggested that activation of C kinase is associated with increases in membrane phospholipid turnover and intracellular diacylglycerol levels [8], both of which are intermediate steps in the introduction of pancreatic exocrine secretion [9], the presence of this protein phosphorylation system in the zymogen granule membrane suggests a possible pathway for the regulatory effects of Ca^{2+} on acinar cell function.

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*',-tetraacetic acid; SDS, sodium dodecyl sulfate.

Materials

Phosphatidylserine (bovine brain), lysine-rich histone (type III-S, corresponding to histone H_1), 1,2-diacylglycerol (diolein), aprotinin, and marker proteins used for M_r determination of endogenous substrate proteins in SDS-polyacrylamide gel electrophoresis (phosphorylase *b*, 94000; bovine serum albumin, 68 000; ovalbumin, 43 000; α-chymotrypsinogen, 25 000; soybean trypsin inhibitor, 21 000; and cytochrome *c*, 13 000) were from Sigma.

Methods

Male Wistar rats (150–200 g) were killed by decapitation. Pancreatic zymogen granules were isolated as described by Meldolesi et al. [10], with the proteinase inhibitor aprotinin (100 μg/ml) present throughout the preparation procedure. The purity of the preparation (less than 2% contamination by mitochondria) was verified by electron microscopy. Granules were sonicated in 0.25 M sucrose (pH 6.2) containing aprotinin (100 μg/ml) and membranes collected by centrifugation at $100000 \times g$ for 60 min. Membranes were then resuspended in ice-cold 20 mM Tris-HCl (pH 7.5), containing 2 mM EDTA, 50 mM 2-mercaptoethanol and 100 μg/ml aprotinin (solution A).

C kinase was assayed as described recently [4,5,7] in a final volume of 0.2 ml, containing Tris-HCl (pH 7.5), 25 mM; lysine-rich histone, 40 μg; $MgCl_2$, 10 mM; phosphatidylserine, 5 μg; EGTA, 25 μM; with or without $CaCl_2$, 0.5 mM. The reaction was initiated by addition of [γ-^{32}P]ATP, 1 nmol, containing $0.5–1.0 \cdot 10^6$ cpm, and was carried out for 5 min at 30°C.

Phosphorylation of endogenous substrate proteins was carried out in a reaction mixture (0.2 ml) containing 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 25 μM EGTA, sample proteins (10–15 μg), 20 μM [γ-^{32}P]ATP, containing about $4 \cdot 10^7$ cpm; in the presence or absence of Ca^{2+}, phospholipid or calmodulin as indicated. Incubations were carried out at 30°C for 3 min. The phosphorylation reaction was terminated by addition of 0.1 ml stop solution (30 mM Tris-HCl (pH 7.8)/9% SDS/15% glycerol/0.05% Bromphenol blue) followed by placing the tubes in boiling water for 3 min. Mercaptoethanol (20 μl) was added to each tube and samples were kept overnight at 4°C. SDS-polyacrylamide gel electrophoresis and autoradiography of the phosphorylated proteins was carried out as described earlier [5,6] using 12% acrylamide separating gels and 5% stacking gels, both containing 0.1% SDS. Amounts of ^{32}P incorporated into substrate proteins were quantified using a scanning densitometer (E-C Apparatus Co., Model 910) interfaced to a microcomputer (Apple II plus, Adalab interface, Interactive Microware, State College, PA, U.S.A.) for peak integration.

Calmodulin was prepared by the fluphenazine affinity method [11]. [γ-^{32}P]ATP was prepared by the method of Post and Sen [12] or Walseth and Johnson [13]. Protein was determined by the method of Bradford [14]. Free Ca^{2+} concentrations were determined using Ca^{2+}-EGTA association constants of Solaro and Shiner [15].

Results

Soluble and membrane fractions from isolated zymogen granules were assayed for Ca^{2+}-activated protein kinase activity in the absence and presence of phosphatidylserine, using histone H_1 as substrate (Table I, A). In the combined presence of Ca^{2+} and phosphatidylserine, a significant increase in protein kinase activity was noted in the granule membrane fraction, but not in the soluble fraction. No Ca^{2+}-dependent stimulation of protein kinase activity was seen in the absence of phospholipid in these fractions. Elimination of histone from the assay reaction mixture prevented the detection of the membrane-associated kinase activity (data not shown). Neither cyclic-AMP-dependent nor cyclic-GMP-dependent protein kinase activity was observed in either fraction (data not shown).

C kinase activity was recovered from detergent-solubilized membranes (Table I, B) and partially purified using DEAE-cellulose (Fig. 1). For phosphorylation of histone H_1, this preparation had an absolute requirement for added phosphatidylserine (Fig. 2), with half-maximal activation noted at a Ca^{2+} concentration of approx. 200 μM (Fig. 2). Diacylglycerol submaximally activated the kinase,

TABLE I

EFFECTS OF Ca^{2+} AND PHOSPHATIDYLSERINE ON PROTEIN KINASE ACTIVITY (A) IN RAT ZYMOGEN GRANULE SOLUBLE AND MEMBRANE FRACTIONS AND (B) RECOVERED FROM DETERGENT-SOLUBILIZED ZYMOGEN GRANULE MEMBRANES

(A) Soluble and membrane fractions (4–7 μg protein/sample) were prepared as described from isolated zymogen granules. (B) Membranes were solubilized in solution A (Methods) containing 0.3% Triton X-100 and 2.5 mM EGTA for 1 h on ice. Protein thus solubilized from the membrane was recovered by centrifugation (105 000 × g, 1 h) and used as the enzyme source. Final concentration of Triton X-100 in the assay reaction mixture was 0.0075%. Protein kinase activity was measured in the absence and presence of phosphatidylserine (PS; 5 μg) and Ca^{2+} (0.5 mM). All groups were assayed in triplicate. Results given represent data from two or three experiments (mean ± S.E.).

Fraction	Protein kinase activity (pmol ^{32}P/min)			
	−PS		+PS	
	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$
(A)				
Soluble	2.24 ± 0.21	4.19 ± 0.38	3.97 ± 0.23	3.87 ± 0.44
Membrane	2.08 ± 0.35	2.06 ± 0.35	3.32 ± 0.23	5.16 ± 0.28 [a]
(B)				
Membrane (solubilized)	3.88 ± 0.28	3.76 ± 0.18	3.94 ± 0.40	6.23 ± 0.45 [a]

[a] Significantly different ($P < 0.01$, two-tailed Student's t-test) from the corresponding group in the absence of Ca^{2+}.

both in the presence of Ca^{2+} (0.5 mM) and in its absence (Fig. 2).

The possible existence of endogenous substrate protein(s) for this Ca^{2+}/phospholipid-dependent kinase was subsequently examined in the zymogen granule membrane (Fig. 2). One granule membrane protein (18 kDa protein; $M_r = 18\,300 \pm 900$, based on eight determinations) was specifically phosphorylated in the combined presence of Ca^{2+} and phosphatidylserine (Fig. 3, lane 4). Silver

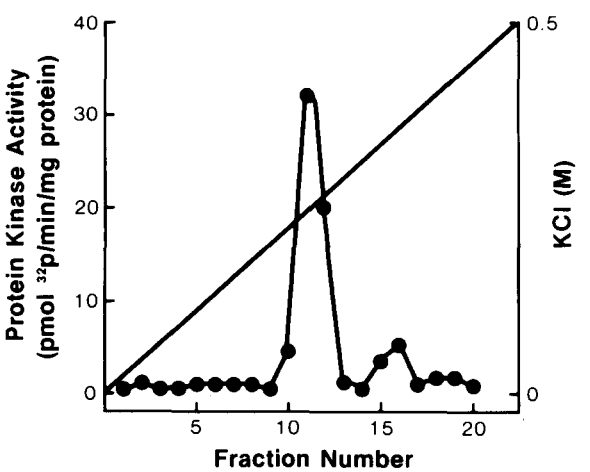

Fig. 1. Ion-exchange chromatography of Ca^{2+}/phospholipid-dependent protein kinase recovered from solubilized zymogen granule membranes. Solubilized membranes (80–90 μg protein) were prepared as described in Table I and charged to a small column of DEAE-cellulose (0.5 cm × 1.5 cm). The column was washed with solution A (Methods) and protein kinase activity eluted using a linear gradient (0–0.5 M) of KCl in solution A. Fraction size was 0.5 ml.

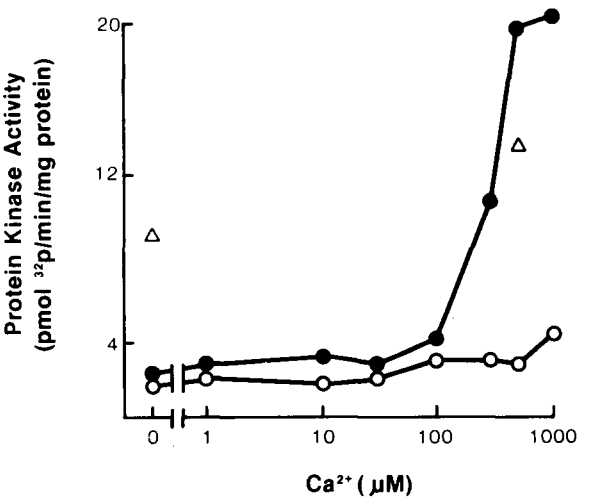

Fig. 2. Effect of varying Ca^{2+} concentration on protein kinase activity partially purified from pancreatic zymogen granule membranes. C kinase prepared by DEAE-cellulose chromatography (Fig. 1) was assayed in the presence (●) or absence (○) of phosphatidylserine (PS; 5 μg) or in the presence of 1,2-diolein (△; 0.5 μg). Lysine-rich histone was used as substrate.

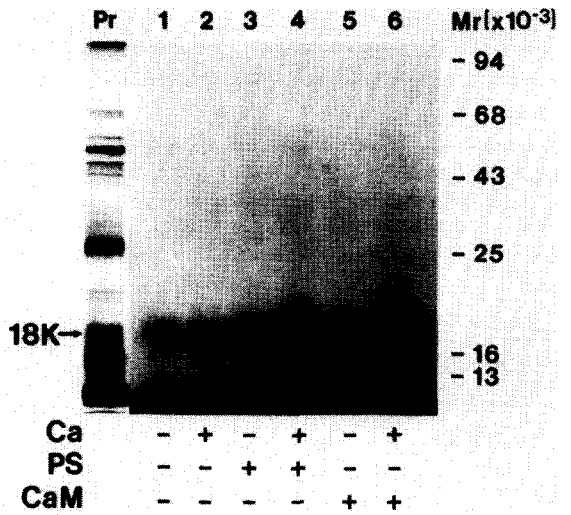

Fig. 3. Autoradiograph showing effects of Ca^{2+} (25 μM), phosphatidylserine (PS; 5 μg) and calmodulin (CaM; 2 μg) on endogenous protein phosphorylation in the zymogen granule membrane. Preparation of the granule fraction, incubation conditions and subsequent SDS-polyacrylamide gel electrophoresis and autoradiography of samples were as described in Methods. The figure presented is representative of three separate experiments. Pr, Silver protein stain.

staining revealed two closely associated proteins corresponding to the 18 kDa substrate (Fig. 3). Whether only one or both proteins in the doublet are phosphorylated by C kinase has not yet been determined. Calmodulin was not effective as a cofactor in supporting this Ca^{2+}-dependent phosphorylation (Fig. 3, lane 6). Neither 8-bromo-cyclic AMP nor 8-bromo-cyclic GMP (1 μM) stimulated phosphorylation of this protein (data not shown).

The effect of various Ca^{2+} concentrations on phosphorylation of the 18 kDa protein was examined in the presence and absence of phosphatidylserine (Fig. 4). In the absence of added phospholipid, a submaximal increase in ^{32}P incorporation into the 18 kDa protein was noted at higher Ca^{2+} concentrations (Fig. 4, lanes 4–5). The presence of exogenous phosphatidylserine resulted in a marked increase in the Ca^{2+} sensitivity of the phosphorylation reaction with a detectable increase in ^{32}P incorporation into the 18 kDa protein at Ca^{2+} concentrations as low as 1 μM (Fig. 4, lane 9). Densitometric analysis (Fig. 5) demonstrated that half-maximal stimulation of ^{32}P

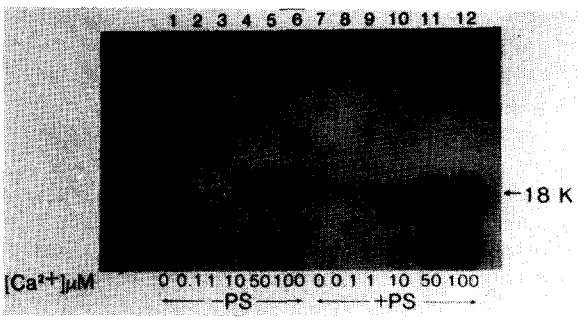

Fig. 4. Effect of various Ca^{2+} concentrations on phosphorylation of endogenous zymogen granule membrane proteins. Membrane preparations were incubated as described in the presence or absence of phosphatidylserine (PS; 5 μg) with the Ca^{2+} concentration indicated. Incubation time was 5 min. Results presented are representative of three experiments.

incorporation into the 18 kDa substrate was noted at approx. 4 μM Ca^{2+} in the presence of phosphatidylserine, while in its absence, approx. 40 μM Ca^{2+} was necessary. The maximum attainable Ca^{2+}-dependent phosphorylation of this protein was at least 2-fold greater in the presence of phosphatidylserine than in its absence (Fig. 5). Exogenous diacylglycerol had no detectable effect

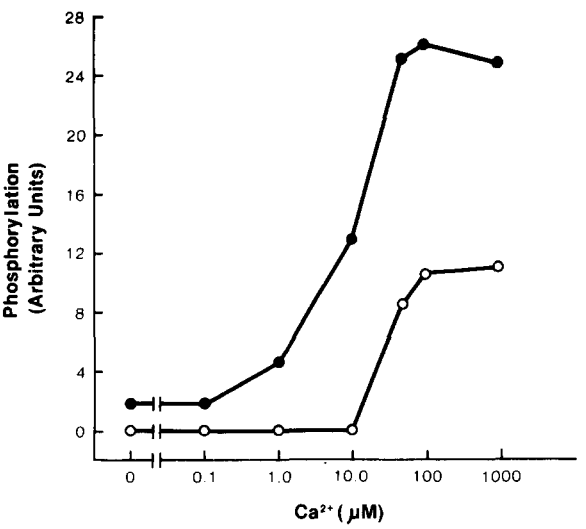

Fig. 5. Effect of various Ca^{2+} concentrations on phosphorylation of the 18 kDa zymogen granule membrane protein in the absence (○, $K_a = 40$ μM) and presence (●, $K_a = 4$ μM) of exogenous phosphatidylserine (PS; 5 μg). Incubation time was 5 min. Incorporation of ^{32}P was quantified by densitometric analysis of the autoradiograph as described in Methods. Results presented are typical of three experiments.

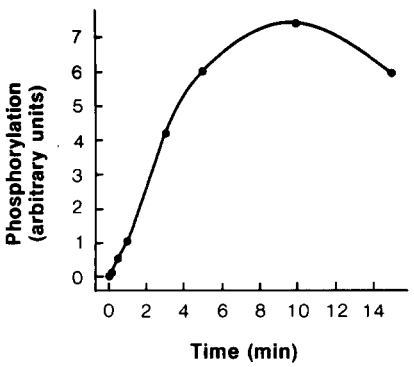

Fig. 6. Ca^{2+}/Phospholipid-dependent phosphorylation of the 18 kDa zymogen granule membrane protein as a function of time. Incubation conditions and subsequent SDS-polyacrylamide gel electrophoresis and autoradiography were as described in Methods. Granule membranes (10–15 μg) were incubated in the combined presence of Ca^{2+} (25 μM) and phosphatidylserine (5 μg) for various times as indicated. Samples were preincubated for 30 s at 30°C prior to addition of [γ-^{32}P]ATP. ^{32}P incorporation was determined by densitometric analysis of the autoradiograph. Results shown are typical of three experiments.

on the Ca^{2+} sensitivity of phosphorylation of this 18 kDa zymogen membrane protein, in the presence or absence of added phospholipid (data not shown).

In time-course studies, measurable Ca^{2+}/phospholipid-dependent phosphate incorporation into the 18 kDa protein was noted within 30 s (Fig. 6). Phosphorylation appeared linear to 5 min and was maximal at 10 min, followed by a gradual decline, possibly due to dephosphorylation by endogenous phosphoprotein phosphatase.

Discussion

The present studies have demonstrated the presence of C kinase and a specific endogenous substrate protein for this kinase in membranes of rat pancreatic zymogen granules. This substrate protein is of similar molecular weight to one of the pancreatic substrates for C kinase we recently reported [6,7] in acinar cell extracts prepared under conditions which would be expected to result in dissolution of the zymogen granule and liberation of the membrane-bound kinase and its substrate(s) into the soluble extract. Lack of phosphorylation in the granule membrane of several higher molecular weight proteins, such as noted in total extracts of acinar cells [6,7], suggests a marked differential distribution of substrates for C kinase within subcellular compartments and may reflect the relative purity of the zymogen granule preparation. The combined presence of Ca^{2+} and phosphatidylserine also resulted in increased ^{32}P incorporation into one or more small proteins or peptides which ran at the dye front on SDS-polyacrylamide gel electrophoresis (Fig. 3, lane 4), as has been noted in other tissues [5–7]. Radiophosphate incorporation noted is evidently indeed into protein and not phospholipid, since phospholipid would be expected to run ahead of the dye front on SDS-polyacrylamide gel electrophoresis [16].

Of additional interest is the differential sensitivity to diacylglycerol of Ca^{2+}/phospholipid-dependent histone phosphorylation and endogenous protein phosphorylation. Added diacylglycerol has been shown to decrease the K_a for Ca^{2+} of C kinase for phosphorylation of histone H_1 [4,17]. In the present studies, C kinase solubilized from granule membranes and partially purified was activated by added diacylglycerol (Fig. 2) while phosphorylation of the 19 kDa protein by membrane-associated kinase was completely insensitive to added diacylglycerol. The latter observation may reflect the presence of endogenous diacylglycerol readily available to the kinase within the hydrophobic region of the granule membrane. This may also be indicated by the increased Ca^{2+} sensitivity of endogenous protein phosphorylation in the intact membrane compared with that of histone phosphorylation by the partially purified enzyme.

Although direct evidence relating to stimulus-secretion coupling cannot be obtained in a cell-free system, the present studies provide an intriguing complement to recent reports suggesting protein phosphorylation as a mechanism of action in pancreas of certain secretagogues. Burnham and Williams [18] have reported increased phosphorylation of specific soluble and particulate proteins from carbachol- and cholecystokinin-treated pancreatic acini prelabelled with $^{32}P_i$, and Freedman and Jamieson noted increased phosphorylation of a 29 kDa ribosomal protein in secretagogue-treated pancreatic lobules from rats [19,20]. They also reported secretagogue-enhanced phosphorylation

of acinar cell proteins of $M_r < 20000$, but stated that these effects were not consistently observed. In the current study, we found that the presence of aprotinin, a broad-spectrum protease inhibitor, was necessary throughout zymogen granule membrane preparation for reproducible detection of C kinase activity and phosphorylation of the 18 kDa protein. Other protease inhibitors (such as leupeptin, antipain or soybean trypsin inhibitor) were not effective. Maximal inhibition of native proteinases thus seems vital in studies dealing with endogenous protein phosphorylation in pancreas, and particularly in a zymogen granule fraction, since proteolysis could rapidly alter the primary as well as higher order structure of proteins, rendering them artifactually unsuitable as substrates for protein kinase-catalyzed phosphorylation [21]. Our previous studies [6,7], recently confirmed by Burnham and Williams [22], have suggested the Ca^{2+} / phospholipid-dependent protein phosphorylation system as a potential pathway for the regulatory actions of Ca^{2+} upon pancreatic exocrine function. The localization of this kinase and its substrate protein to the secretory granule membrane would seem to strengthen this contention. Purification and further study of the 18 kDa substrate protein should extend our understanding of Ca^{2+} as a modulator of acinar cell function.

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References

1 Gardner, J.D. (1979) Annu. Rev. Physiol. 41, 55–66
2 Case, R.M. and Clausen, T. (1973) J. Physiol. (Lond.) 235, 75–102
3 Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. and Nishizuka, Y. (1979) J. Biol. Chem. 254, 3692–3695
4 Kuo, J.F., Andersson, R.G.G., Wise, B.C., Mackerlova, L., Salomonsson, I., Brackett, N.L., Katoh, N., Shoji, M. and Wrenn, R.W. (1980) Proc. Natl. Acad. Sci. USA 77, 7039–7043
5 Wrenn, R.W., Katoh, N., Wise, B.C. and Kuo, J.F. (1980) J. Biol. Chem. 255, 12042–12046
6 Wrenn, R.W., Katoh, N. and Kuo, J.F. (1981) Biochim. Biophys. Acta 676, 266–269
7 Wrenn, R.W. (1983) Life Sci. 32, 2385–2392
8 Takai, Y., Kishimoto, A., Kawahara, Y., Minakuchi, R., Sano, K., Kikkawa, U., Mori, T., Yu, B., Kaibuchi, K. and Nishizuka, Y. (1981) Adv. Cyclic Nucleotide Res. 14, 301–313
9 Jones, L.M. and Michell, R.H. (1975) Biochem. J. 148, 479–485
10 Meldolesi, J., Jamieson, J.D. and Palade, G.E. (1971) J. Cell Biol. 49, 109–129
11 Charbonneau, H. and Cormier, M.J. (1979) Biochem. Biophys. Res. Commun. 90, 1039–1047
12 Post, R.L. and Sen, A.K. (1967) Methods Enzymol. 10, 773–775
13 Walseth, T.F. and Johnson, R.A. (1979) Biochim. Biophys. Acta 562, 11–31
14 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254
15 Solaro, R.J. and Shiner, J.S. (1976) Circ. Res. 39, 8–14
16 Campbell, K.P., MacLennan, D.H. and Jorgensen, A.O. (1983) J. Biol. Chem. 258
17 Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. and Nishizuka, Y. (1980) J. Biol. Chem. 255, 2273–2276
18 Burnham, D.B. and Williams, J.A. (1982) J. Biol. Chem. 257, 10523–10528
19 Freedman, S.D. and Jamieson, J.D. (1982) J. Cell Biol. 95, 903–908
20 Freedman, S.D. and Jamieson, J.D. (1982) J. Cell Biol. 95, 909–917
21 Glass, D.B. and Krebs, E.G. (1979) J. Biol. Chem. 254, 9728–9738
22 Burnham, D.B. and Williams, J.A. (1983) Fed. Proc. 42, 456 (abstr.).