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A Cytosolic Protein Activator of Cardiac Sarcolemmal Phosphoinositide Phospholipase C

Eugene E. Quist, Patricia Powell, Carolyn Quist and Sheila Page

**Department of Pharmacology
Texas College of Osteopathic Medicine
3500 Camp Bowie Blvd.
Fort Worth, Texas 76107**

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Ca^{2+} dependent polyphosphoinositide phospholipase C (PLC) activity in cardiac sarcolemma hydrolyzed both endogenous and exogenous phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) with an associated increase in inositol bisphosphate (IP_2). Dialyzed cytosol and certain fractions of cytosol isolated by anion echange or gel filtration chromatography activated sarcolemmal PLC activity by approx. 100 %. The PLC activator eluted with an apparent molecular weight of 160 Kdal on a Sephacryl 300 column and was destroyed by heat or trypsin treatment. Exogenous ^3H-PIP_2 was not hydrolyzed by cytosolic fractions containing sarcolemmal PLC activator. These studies demonstrate that the polyphosphoinositide PLC in cardiac sarcolemma is regulated by a cytosolic protein.

Activation of polyphosphoinositide phospholipase C by various receptors coupled to G proteins has been postulated to have widespread importance in signal transduction (1,2). The breakdown products of PIP and PIP_2 hydrolysis, diacylglycerol and inositol phosphates, are activators of protein kinase C and Ca^{2+} mobilizers, respectively (1,2). In cardiac tissue, activation of muscarinic cholinergic receptors with carbachol stimulates PLC mediated breakdown of PIP and PIP_2 (3,4). Recent studies by Jones et al. (5) in saponin permealized myocytes demonstrated that PLC is activated by micromolar concentrations of Ca^{2+} and by G protein activators. These observations are consistent with studies in a number of other tissues where PLC has been shown to be coupled to a G_p protein (6-9). The cellular location of receptor regulated PLC may vary in different tissues (6). In cardiac tissue, Low et al. (10) reported the presence of multiple cytosolic forms of a PLC which hydrolyzed phosphatidylinositol (PI). More recently, Schwartz and Halverson (11,12) found that PLC activity which degraded PIP and PIP_2 was primarily located in a crude total membrane preparation from rat ventricles whereas the PI-PLC was completely cytosolic. In the present study, more highly purified cardiac sarcolemma were found to possess a Ca^{2+} stimulated PLC activity which could be stimulated by a cytosolic protein.

Abbreviations used: DTE, dithioerythritol; EGTA, ethylene glycol Bis(β-aminoethyl ether)-N, N'-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; IP, inositol phosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, polyphosphoinositide phospholipase C.

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MATERIALS AND METHODS

Materials. [γ-^{32}P]-ATP (600 Ci/mM) was purchased from ICN, Irvine, CA. Phosphatidyl[2-^{3}H]inositol-4-monophosphate (1.0 Ci/mM) and phosphatidylinositol [2-^{3}H]inositol 4,5-bisphosphate (1.0 Ci/mM) were from Amersham, Arlington Heights, Il. Phosphatidylinositol 4-monophosphate(bovine brain), phosphatidylinositol 4,5-bisphosphate (bovine brain), trypsin, Type XI and soybean trypsin inhibitor were from Sigma Chem. Co. St. Louis, MO.

Preparation of Cardiac Sarcolemma. Sarcolemmal membranes were isolated from canine cardiac ventricles by a procedure recently described in detail by our laboratory (13). Briefly, mongrel dogs were anesthetized with intravenous Surital and the heart was quickly removed and placed in cold isotonic saline and 0.1 mM EGTA. The tissue was minced with scissors, suspended in 10 mM HEPES, pH 7.4, 2.0 mM $MgCl_2$, 0.5 mM dithiothreitol and 0.1 mM EGTA and homogenized with a polytron. The homogenates were filtered through 437 micron nylon mesh and layered on a 8 % and 22.5 % w/v sucrose step gradient. After centrifugation for 60 min at 70,000 x g the top cytoplasmic fraction was collected and dialyzed against 10 mM Tris HCl, pH 7.6, 0.1 mM EGTA and 0.5 mM DTE. The sarcolemmal fraction located at the sucrose interface was collected and washed with the homogenization buffer above and centrifuged for 30 min at 70,000 x g. The sarcolemma were resuspended with homogenization buffer and stored at -80° C.

Polyphosphoinositide Labeling. Sarcolemma (250 μg) were incubated at 25° C in (2.5 ml final volume) 10 mM $MgCl_2$, 10 mM HEPES, pH 7.4, 0.1 mM EGTA, 2.5 mM NaN_3, 200 μg alamethacin and 0.1 mM [γ-^{32}P]-ATP (180 μCi). After 20 min., 35 ml of cold 0.1 mm EGTA, 10 mM HEPES, pH 7.4, 0.12 mM KH_2PO_4 and 1.0 mM dithioerythreitol was added and the tube was centrifuged for 20 min at 70,000 x g at 50° C. Membranes were resuspended with 1.7 ml of 10 mM HEPES pH 7.4 and 200 μg of alamethacin with a motor driven Potter-Elvehjem homogenizer.

Phospholipase C Assays. Sarcolemmal membranes (8 μg) prelabeled with ^{32}P were incubated at 25° C in a final volume of 0.25 ml in 10 mM HEPES, pH 7.4, 1.0 mM $MgCl_2$, 0.2 mM NaN_3, 0.1 mM EGTA, 1.0 mM ATP, 25 mM NaCl, 25 mM KCl and varying amounts of $CaCl_2$. Free Ca ion concentration was calculated as previously described (14). In studies using exogenous tritiated PIP_2, sarcolemmal membranes (5 μg) were incubated 20 min at 30° C in a final volume of .05 ml in 10 mM HEPES, pH 7.4, 1.0 mM $MgCl_2$, 1.0 mM ATP, 1.0 mM NaN_3, 0.05 mM ouabain and 100 μM ^{3}H-PIP_2 (0.05 μCi). Reactions were stopped with 1.0 ml of chloroform:methanol:HCl (20:40:1). After 15 min, 0.4 ml of chloroform and 0.15 ml of water were added. Following centrifugation at 1000 x g for 5 min., 0.8 ml of the top phase was neutralized by the addition of 3.0 ml of 38 mM Trizma base. Tubes were centrifuged for 10 min at 1500 x g and 3.3 ml was pipeted onto 0.6 ml anion exchange columns (AG 1-X8,200-400 mesh, formate form, BioRad). Columns were washed twice with 5.0 ml of 0.1 M formic acid and 0.2 M ammonium formate to remove ^{32}Pi. IP_2 and IP_3 were eluted with 5.0 ml each of 0.1 M formic acid/0.4 M ammonium formate and 0.1 M formic acid/0.8 M ammonium formate, respectively (14,15). Fractions were counted in 8.0 ml of Ecolume. In some studies, PIP and PIP_2 in the chlorofrom phase was separated by thin layer chromatography, detected by autoradiography and counted (13).

Miscellaneous Methods. Dialyzed cytosol (2 ml) was added to a 1 ml DEAE Sephacel column. The eluant was collected and the column was further washed with 6 ml of 50 mM NaCl, 20 mM Tris HCl pH 8.0 and 0.01 mM EGTA. The PLC activator was eluted from the column with 4.0 ml of similar solution containing 250 mM NaCl. Protein was determined by the method of Peterson (16). Some samples of dialyzed cytosol or the 250 mM NaCl DEAE Sephacel were heated for 30 min at 65° C or incubated 30 min at 30° C with 25 μg of trypsin. Trypsin was inhibited with 75 μg of soybean trypsin inhibitor after the incubation and controls with buffer, trypsin and trypsin inhibitor were also tested on PLC activity in sarcolemma.

Dialyzed cytosol was concentrated 3 fold by ultafiltration with an Omega membrane (Pharmacia, 10 Kdal cutoff) and 0.75 ml was applied to a 1.5 cm x 50 cm Sephacryl 300 or 200 column equilibrated with 100 mM NaCl, 0.01 mM EGTA, 10 mM Tris HCl, pH 7.6 and 0.2 mM DTE. Fractions were eluted with the same buffer. The

column was calibrated with molecular weight markers; β amylase, 200 Kdal.; aldolase, 154 Kdal.; hexokinase, 96 Kdal.; bovine serum albumin, 68 Kdal.; ovalbumin, 45 Kdal.; chymotrypsinogen 25 Kdal, and cytochrome C, 12.5 Kdal.

RESULTS

Studies with ^{32}P Labeled substrates. Preincubation of sarcolemma with ^{32}P ATP resulted in labeling or synthesis of PIP and PIP_2 from endogenous PI by membrane bound kinase activities (13). Alamethacin was included in both the prelabeling and PLC assay medium to increase membrane permeability to small molecules and cytoplasmic proteins (13,17). Incubation of prelabeled and washed sarcolemma resulted in a time dependent increase in labeled IP_2 in the presence of 3.5 μM Ca^{2+} (Fig.1). In the absence of Ca^{2+}, IP_2 levels did not change over this time period. Labeled IP3 levels did not change in the presence or absence of Ca^{2+} because these membranes contain a Mg^{2+} dependent IP_3 phosphatase which rapidly converts IP_3 to IP2. Similar phosphatase activities have previously been reported in erythrocyte (14,15) and synaptosomal membranes (18). Therefore IP_2 accumulation reflects both released IP_2 and IP_3 from PIP and PIP_2. This observation is further substantiated by studies which show that Ca^{2+} stimulates a time dependent decrease in both PIP and PIP_2 in prelabeled cardiac sarcolemma (Fig. 2). It was found that adding aliquots of dialyzed cytosol with the SL increased the rate and extent of IP_2 release (Fig. 1) providing 1.0 mM ATP was included in the medium to inhibit inositol polyphosphate phosphatase activity. In the absence of ATP, the cytosolic phosphatase activity masked Ca^{2+} stimulated IP_2 production by rapidly dephosphorylating IP_2 released from PIP and PIP_2. The effect of cytosol on PIP and PIP_2 breakdown could not be tested directly because heme proteins greatly interfere with the extraction of PIP and PIP_2. This problem was overcome by removing the heme containing proteins and the phosphatase activity from cytosol on a small DEAE Sephacel column (See Methods). It was found that proteins which did not bind to the column at low ionic strength or the fraction eluting with 50 mM NaCl had no effect on cardiac sarcolemmal PLC activity (Table I). However in the absence of ATP, addition of 50 mM NaCl fractions reduced sarcolemmal PLC-IP_2 production indicating the presence of cytosolic phosphatase activity. Addition of desalted aliquots of the 250 mM NaCl column fraction with sarcolemma increased Ca^{2+}-stimu-

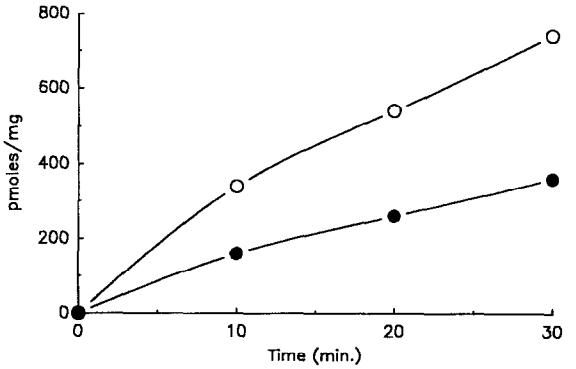

Fig.1. Ca^{2+} stimulated release of ^{32}P-IP_2 in cardiac sarcolemmal membranes in the absence (●) and presence (○) of 0.025 ml of dialyzed cytosol.

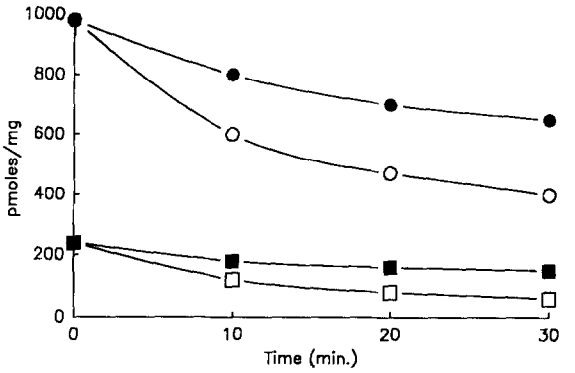

Fig. 2. Ca^{2+} stimulated decreases in sarcolemmal PIP(●) and PIP_2 (■). Ca^{2+} stimulated decreases in sarcolemmal PIP (○) and PIP_2 (□) in the presence of 0.05 ml of the 250 mM NaCl DEAE Sephacel activator fraction (See Methods and Table I).

lated breakdown of PIP and PIP_2 (Fig, 2) without interfering with extraction. This fraction also increased IP_2 production similar to the dialyzed cytosol (Fig.1) in the presence and absence of ATP (Table I).

The sarcolemmal PLC activator appears to be present in relatively high concentrations in cytosol since only small aliquots (diluted 5 fold during homogenization) produced a maximal activation of sarcolemmal PLC (Fig. 3). Pretreatment of dialyzed

TABLE I

DEAE Sephacel Chromatography of Cardiac Cytosol: Separation of the PLC Activator and Inositol Polyphosphate Phosphatase[a]

Fraction	[Protein] (μg/ml)	^{32}P-IP_2 (cpm)	[ATP] (mM)
0 NaCl	215	300	0
0 NaCl	215	320	1
50 mM NaCl	1500	0	0
50 mM NaCl	1500	280	1
250 mM NaCl	280	560	0
250 mM NaCl	280	620	1
Dialyzed Cytosol	3150	0	0
Dialyzed Cytosol	3150	592	1
Control	0	295	0
Control	0	315	1

[a]Sarcolemma, pre-labeled with ^{32}P, was incubated 20 min at 25° C with 0.025ml/0.25 ml of the fractions indicated or without any additions (ie. Control). Other conditions: 1.0 mM $MgCl_2$, 10 mM HEPES, pH 7.4, 25 mM NaCl, 25 mM KCl, 0.2mM NaN_3, 8 μg sarcolemma, 6 μg alamethacin, 0.1 mM EGTA and 0.1 mM $CaCl_2$, $[Ca^{2+}]$ = 3.5 μM.

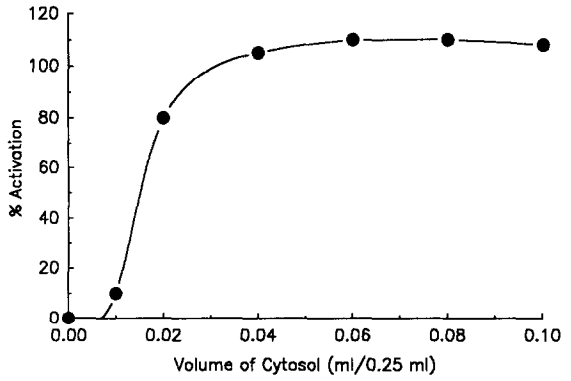

Fig. 3. Activation of ^{32}P-IP_2 release from PIP and PIP_2 by increasing concentrations of dialyzed cytosol.

cytosol or the 250 mM NaCl DEAE Sephacel fraction for 30 min at 65° C or with trypsin at 30° C abolished the ability of these fractions to activate sarcolemmal PLC, providing evidence that the cytosolic activator is probably a protein (not shown).

Studies were also done to determine if the cytosolic activator protein is a PLC activity which could utilize sarcolemmal PIP and PIP_2. Sarcolemma prelabeled with ^{32}P were preincubated for 30 min at 65° C to inactivate PLC activity. In these membranes, Ca^{2+} in the presence or absence of dialyzed cytosol did not activate IP_2 release (not shown) suggesting that the activator fraction is not a PLC activity. However, this study did not rule out the possibility that heat denaturation obstructed access of the membrane substrates to a cytosolic PLC. This possibility was examined below using exogenous substrate.

The molecular weight of the activator protein was estimated by chromatography of dialyzed cytosol on Sephacryl 200 (not shown) and 300 columns (Fig. 4). Fractions eluting with a Ve/Vo of 1.25 corresponding to an apparent molecular weight of approximately 160 Kdal activated sarcolemmal PLC activity. Fractions eluting at molecular

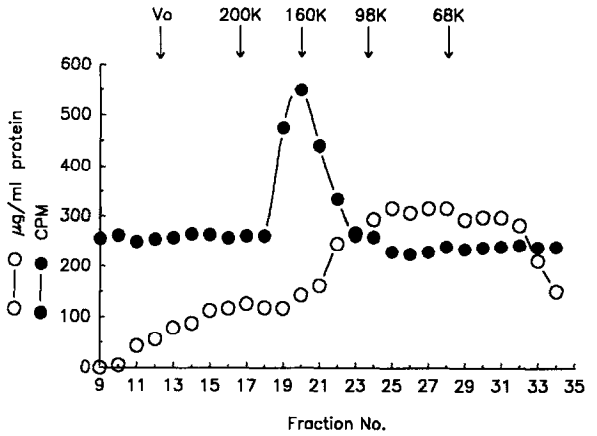

Fig. 4. Molecular weight estimation of the sarcolemmal PLC cytosolic activator on a Sephacryl 300 column. Effects of fractions on the release of IP_2(●) from sarcolemmal ^{32}P labeled PIP and PIP_2.

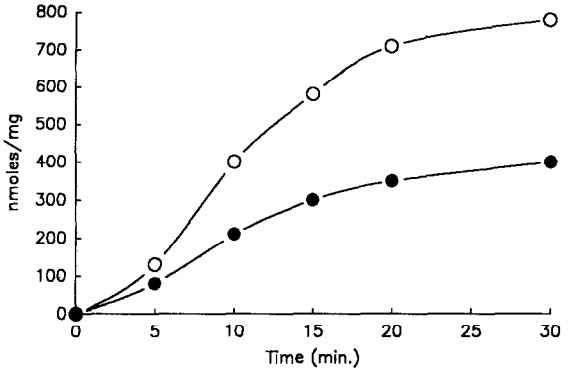

Fig. 5. Time course of IP_2/IP_3 release from ^3H-PIP_2 in the absence (●) and presence (O) of the 250 mM NaCl DEAE Sephacel activator fraction (See Methods and Table I).

weights as low as 10 Kdal had no effect on sarcolemmal PLC activity. The molecular weight estimation of the activator from the Sephacryl 200 and 300 column were almost identical but the Sephacryl 300 column was used in later studies because of the proximity of the PLC activator to the Vo (Ve/Vo =1.17) on a Sephacryl 200 column.

Studies with Exogenous Substrates. To further investigate the nature of the cytosolic activator, the ability of sarcolemma, cytosolic fractions, or both to hydrolyze exogenous tritiated PIP or PIP_2 was examined. Addition of 15 μM PIP_2 with sarcolemma resulted in a time dependent increase in IP_2/IP_3 for up to 20 min at 25° C in the presence of Ca^{2+} (Fig. 5). The rate of hydrolysis was approximately 1000 fold greater with the exogenous substrate possibly because of greater amounts and easier accessibility of exogenous substrate to the sarcolemmal PLC. Under the same ionic conditions, dialyzed cytosol or the 250 mM NaCl DEAE Sephacel fraction did not hydrolyze tritiated PIP_2 in the absence of sarcolemma. Therefore the cytosolic fractions from cardiac tissue contain negligible amounts of polyphosphoinositide PLC activity in agreement with the study of Shwertz and Halverson (11). Addition of cytosolic fraction with the SL increased IP_2/IP_3 release by approximately 125 % (Fig. 5). Therefore the cytosolic activator protein can increase the ability of sarcolemmal PLC to utilize exogenous as well as endogenous PIP_2. Essentially identical results were obtained using PIP as a substrate (not shown) although the specific activity of PLC activity was approximately 50 % less than with PIP_2. Collectively, these studies suggest that the cytosolic protein which activates sarcolemmal PLC activity is a co-factor of membrane bound PLC rather than a separate PLC activity.

DISCUSSION

The results of this investigation demonstrate that purified cardiac sarcolemmal membranes possess a Ca^{2+} activated PLC activity which hydrolyzes both PIP and PIP_2. Of greater significance is the observation that cardiac cytosol contains a protein which activates sarcolemmal PLC activity. The high molecular weight and the sensitivity of this activator to trypsin digestion and heat treatment identify the PLC activator as a protein. How this activator protein increases sarcolemmal PLC activity or its physiological relevance is currently unknown. It does not regulate PLC analogous to a G protein for in a preliminary report we found that the activator increased the Vmax of PLC activity without affecting the affinity of PLC for Ca^{2+} (19). In contrast the G protein activator NaF increases both the Vmax and the Ca^{2+} affinity of cardiac sarcolemmal PLC

(19). This observation is consistent with studies in other tissue membranes where G protein activators generally increase the Ca^{2+} affinity of PLC (6-10). The cytosol or cytosolic activator fractions did not hydrolyze PIP_2 and therefore do not contain PLC activities which would act additively with sarcolemmal PLC. The cytosolic protein activator is most likely a co-factor protein for membrane bound PLC activity. The activator protein appears to be present in excessive amounts in cytosol suggesting that sarcolemmal PLC would be maximally activated providing sufficient Ca^{2+} were present. This does not rule out the possibility that other regulatory pathways are present in heart cells which could influence the levels or efficacy of the PLC activator. To our knowledge a similar protein activator of PLC has not been reported in the cytosol of heart or in other cell types. Studies will be initiated to examine if cardiac cytosol from other species or tissues possess a similar activator. Fukui et al. (20) reported the existence of amine activator in the cytosol of liver which in contrast to the protein activator reported here, did not bind to anion exchange columns. Because the cytosolic protein PLC activator could have important physiological significance in regulating phosphoinositide turnover in cardiac and possibly other tissues, studies are in progress to purify and characterize this protein.

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