Effects of pertussis toxin on α_1-agonist-mediated phosphatidylinositide turnover and myocardial cell hypertrophy in neonatal rat ventricular myocytes

J. S. Karliner, T. Kagiya and P. C. Simpson

Cardiology Section (III C), Veterans Administration Medical Center, 4150 Clement Street, San Francisco (California 94121, USA), and Cardiovascular Research Institute and the Department of Medicine, University of California, San Francisco (California, USA)

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Summary. In neonatal rat ventricular myocytes pretreatment with pertussis toxin did not affect 1 μM (−)-norepinephrine stimulation of inositol phosphates or myocardial cell hypertrophy as measured either by protein radiolabelling or by myocardial cell protein content. Thus guanine nucleotide protein(s) ADP-ribosylated by pertussis toxin do not play a role in two α_1-adrenoceptor-mediated processes, phosphatidylinositide turnover and induction of myocardial cell hypertrophy.

Key words. α_1-Adrenoceptor; pertussis toxin; inositol phosphates; myocardial hypertrophy.

Myocardial cell growth without proliferation occurs in the postnatal and adult heart. There has been much recent interest in stimulants of this process of hypertrophy. Among the substances that have been studied are a variety of growth factors, hormones and neurotransmitters. The naturally occurring neurotransmitter (−)-norepinephrine stimulates myocardial cell growth in neonatal rat ventricular myocytes through its action on the α_1-adrenoceptor[1,2]. There is considerable evidence that α_1-adrenoceptor signal transduction is initiated by the binding of (−)-norepinephrine to cell surface receptors resulting in membrane phosphatidylinositide hydrolysis via activation of a specific phospholipase C[3–7]. This process results in a rapid increase in cytosolic free calcium and an increase of other products of phosphoinositide hydrolysis, particularly inositol trisphosphate, which acts to mobilize intracellular calcium, presumably from sarcoplasmic reticulum. In many systems, activation of phospholipase C also leads to formation of diacylglycerol, which in turn activates a calcium-phospholipid-dependent protein kinase, protein kinase C[8].

A family of guanine nucleotide binding proteins, the G proteins, transduce extracellular signals that are detected by adrenergic receptors on the cell surface into cellular responses[9]. These G proteins contain specific sites for ADP-ribosylation by bacterial toxins, such as pertussis toxin and cholera toxin. Pertussis toxin covalently modifies the α_i-subunit of the guanine nucleotide inhibitory protein G_i which results in its inactivation[10]. Both pertussis toxin sensitive and pertussis toxin insensitive guanine nucleotide regulatory proteins have been described[11–14]. Since α_1-adrenergic agonism results in both myocardial hypertrophy and in phosphatidylinositol turnover, we asked whether pertussis toxin influences (−)-norepinephrine-induced myocardial cell growth and inositol phosphate production in neonatal ventricular myocytes.

Materials and methods

Cell culture. Cultures were composed of single, isolated cells prepared from hearts of one-day-old rats as described previously[1,2]. On culture day 1, after overnight attachment, those cultures designated to receive (−)-norepinephrine as a growth stimulant were placed in 8 ml of a serum-free medium containing 10 μg/ml insulin and 10 μg/ml transferrin. Medium was routinely changed on day 4. At the time of this medium change, cultures treated with (−)-norepinephrine received 2 μM of this agent in serum-free medium for 36–72 h. Other cultures designated to be used for phosphatidylinositide measurements were maintained in 5% calf serum throughout the entire time of culture. After a medium change on day 4, measurements of inositol phosphates after stimulation with (−)-norepinephrine were carried out as described below in medium containing 5% calf serum.

Cell yield was 3–5 million per heart, of which over 90% were viable. All cultures were kept at 37 °C in humidified air with sufficient CO_2 (about 1%) to maintain pH 7.3. The cultures contained > 90% myocardial cells and cell numbers were constant over time and in response to (−)-norepinephrine or pertussis toxin as determined by counting cells in the dishes. Average cell density was 200/mm^2.

Phosphatidylinositide hydrolysis. Phosphatidylinositide hydrolysis was monitored by measuring inositol phosphate accumulation in the presence of LiCl, an inhibitor of the enzyme that converts inositol-1-phosphate (IP_1) to inositol. A minor modification of the procedure of Masters et al.[15] was used. Phosphatidylinositides were radiolabelled by incubating myocardial cells with myo-[2-^{3}H] inositol, 5–10 μCi/ml, 16,5 Ci/mmol, for 18–20 h. Prior to initiating the reaction, medium was removed by aspiration and cells were washed 3 times with 1 ml of Hanks balanced salt solution. Reactions were initiated by addition of (−)-norepinephrine and 10 mM LiCl. Incubation

with 1 μM (−)-norepinephrine was for 5 or 10 min at 37 °C. When antagonists were used, they were added just prior to addition of (−)-norepinephrine. To terminate the incubation, the drug solution was rapidly removed by aspiration, and 1 ml of 10% trichloroacetic acid (TCA) at 4 °C was added. The contents were removed from the dishes using a rubber spatula and centrifuged at 350 × g for 10 min, and the supernatant stored at − 20 °C until assay several days later. No differences were found when fresh and frozen preparations were compared.

The TCA was removed by 5 washes with 4 volumes of diethyl ether. The samples were then applied to 5 × 25 mm columns containing approximately 550 mg of anion exchange resin (Bio-Rad AF 1-x 8, 100–200 mesh, formate form). Columns were washed sequentially with 5 ml of distilled water to remove free ^{3}H-inositol and then with 5 ml of 60 mM Na formate in 5 mM Na tetraborate to remove glycerolphosphoinositol; and then with 8 ml of 200 mM ammonium formate in 100 mM formic acid to collect IP_1.

In separate experiments, IP_1, inositol bisphosphate (IP_2), and inositol trisphosphate (IP_3) were measured in the same sample. Cells were labelled with 5 μCi/ml of myo-[2-^{3}H] inositol; at the time of removal from the dishes the cells were sonicated and TCA was added. After free ^{3}H-inositol, glycerolphosphoinositol and IP_1 were eluted as described above, further elutions were carried out with 400 mM and 1 M ammonium formate respectively, in 100 mM formic acid, to recover IP_2 and IP_3. For each data point the results from 2 dishes were averaged.

Cell protein content. Protein was measured either by asymptotic labelling with radioactive amino acids or by a spectrophotometric assay, as previously described [2]. For the former, culture medium was brought to 0.1 μCi/ml (^{14}C) or 0.5 μCi/ml (^{3}H) with (U^{14}C) phenylalanine, (ring-^{3}H) phenylalanine or (U^{14}C) tyrosine, amino acids that are not metabolized by myocardial tissue. Increasing phenylalanine with constant specific activity had no effect on the pattern of incorporation [2]. Therefore medium 199 which contains 303 mM phenylalanine and 255 mM tyrosine was used without modification. At intervals after the addition of isotopes and (−)-norepinephrine, cell protein, defined as material that was TCA-insoluble and sodium dodecyl sulfate (SDS)-soluble, was taken for liquid scintillation counting. Labelling medium was removed, the attached cells were quickly rinsed with phosphate-buffered saline, treated with 10% TCA at 0 °C for at least 1 h, rinsed 3 times with TCA, and dissolved in 1 ml of 1% SDS at 37 °C. The entire volume of SDS was quantitatively removed into glass vials and counted as a gel in a mixture of 8 ml of water and 10 ml of scintillant at an efficiency of 50–70% for ^{14}C and 20–30% for ^{3}H. Counting error was always less than 2% and usually less than 1%. Cell protein content was also determined by the method of Bradford [16]. Cells were rinsed with saline and dissolved in 0.1% SDS. Duplicate aliquots were taken for assay, using bovine serum albumin as standard. Since cell numbers were the same in cell dishes and the fraction of nonmuscle cells was < 10%, protein (mg) per dish reflects protein content per cell [2].

Pertussis toxin catalyzed ADP-ribosylation. ADP-ribosylation was performed by a modification of the method described by Morris and Bilezikian [17]. Dithiothreitol-activated pertussis toxin (2.5 μg) was incubated with membrane protein (75–100 μg/sample) for 2 h at 37 °C in a 100 μl reaction mixture containing $MgCl_2$, 5 mM; thymidine 20 mM; ATP 0.4 mM; phosphocreatine 10 mM; creatine phosphokinase 10 U/ml; K_3PO_4 250 mM, pH 7.4; ^{32}P-NAD 10 μCi and NAD 10 μM. The reaction was stopped by the addition of 10 mM NAD in 50 mM Tris, 4 μM EDTA, pH 7.4 and centrifuged at 7000 × g for 5 min, washed in this buffer and recentrifuged. SDS-polyacrylamide gel electrophoresis was carried out using the method of Laemmli [18].

Results and discussion

In initial experiments we used IP_1 as a marker for phosphatidylinositide turnover. (−)-Propranolol 1 μM had no effect on 1 μM (−)-norepinephrine stimulation of IP_1. By contrast 1 μM terazosin, a selective α_1-adrenoceptor antagonist, inhibited this response by 95% (n = 3), while 0.1 μM terazosin inhibited the response by 85% (n = 2). Yohimbine, a selective α_2-adrenoceptor an-

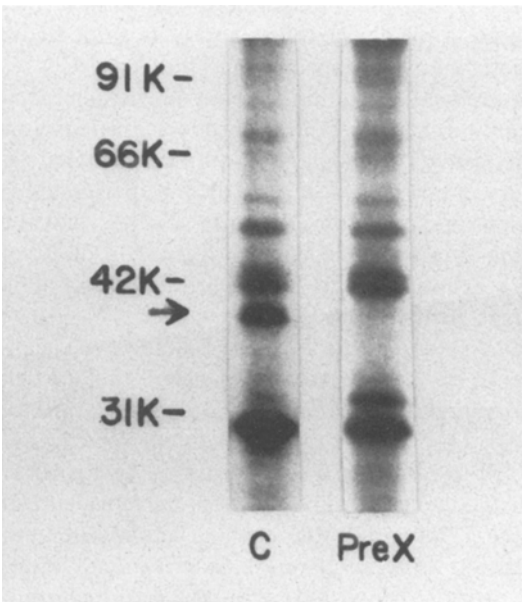

Figure 1. Autoradiogram of an SDS-polyacrylamide gel electrophoresis experiment demonstrating ADP-ribosylation of a 40 kDa (K) band (arrow) by pertussis toxin and inhibition of ADP-ribosylation by pertussis toxin pretreatment. Left lane: (C = control) a particulate preparation of neonatal rat ventricular myocytes incubated for 2 h with pertussis toxin in the presence of 32P-NAD (see methods for details). Right Lane: identical membrane preparation obtained from intact cells which had been pretreated (PRE X) with 0.5 mg/ml pertussis toxin overnight. This experiment was repeated two additional times with identical results.

Inositol phosphate formation (fmol/mg protein)

		Control	Pertussis toxin-treated
IP_1			
	Basal	714 ± 177	574 ± 110
	NE	1480 ± 393*	1109 ± 297*
IP_2			
	Basal	60 ± 8	50 ± 7.5
	NE	167 ± 43*	140 ± 50*
IP_3			
	Basal	45 ± 8	40 ± 6.5
	NE	71 ± 16*	62 ± 14*

Data are from 4 separate cultures and are shown as mean ± SE. IP_1 = inositol-1-phosphate; IP_2 = inositol bisphosphate; IP_3 = inositol trisphosphate. NE = incubation for 5 min with 1 μM (−)-norephinephrine. Pertussis toxin pretreatment of intact neonatal rat ventricular myocytes was overnight using 1.0 μg/ml. * = $p < 0.05$ vs basal levels. None of the differences shown between control and pertussis toxin-treated cells are statistically significant.

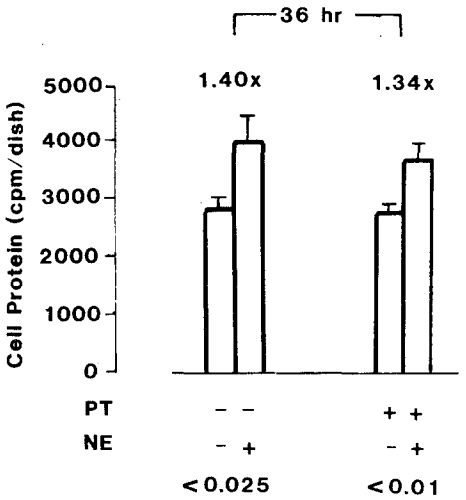

Figure 2. Cell protein content after incubation with 2 μM (−)-norepinephrine (NE) for 36 h. Pretreatment of the intact neonatal rat ventricular myocytes with pertussis toxin (PT) 1.0 μg/ml overnight had no effect on the ability of NE to augment protein content. Data for each bar are the mean and standard deviation from four separate culture dishes. This experiment was repeated one additional time with identical results.

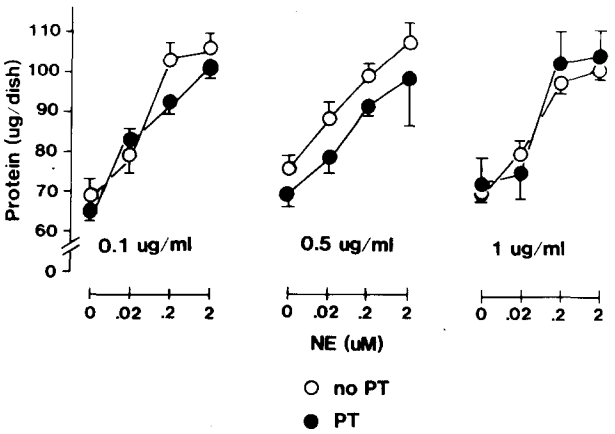

Figure 3. Cardiac myocytes were incubated with the indicated concentrations of (−)-norepinephrine (NE) for 72 h in the presence of either 0.1, 0.5 or 1 μg/ml of pertussis toxin (PT). PT had no effect on the ability of NE to enhance protein content. Shown are the results from a representative experiment. Each data point is the mean and standard deviation from four 35-mm culture dishes. Four similar experiments yielded identical results.

tagonist, at 1.0 and 0.1 μM inhibited IP_1 accumulation by only 52% and 27%, respectively (n = 2). These observations established that in neonatal rat ventricular myocytes the phosphatidylinositide response to (−)-norepinephrine stimulation is an α_1-adrenoceptor mediated response and are consistent with data reported by others[4, 5, 19].

We next determined whether neonatal rat ventricular myocytes contain an ADP-ribosylatable substrate for pertussis toxin. Figure 1 shows an autoradiogram of an SDS polyacrylamide gel electrophoresis experiment demonstrating ADP-ribosylation of a 40 kDa band by pertussis toxin and inhibition of ADP-ribosylation by pertussis toxin pretreatment. These observations indicate that overnight incubation of intact neonatal rat ventricular myocytes with 0.5 μg/ml of pertussis toxin completely ADP-ribosylates all the available pertussis toxin substrate in this cell culture system. The table shows the results of a 5-min stimulation by 1 μM (−)-norepinephrine on generation of inositol phosphates in the presence and the absence of pretreatment with 1.0 μg/ml pertussis toxin overnight. Although (−)-norepinephrine stimulation consistently increased inositol phosphate production significantly compared to basal values, the data indicate that pertussis toxin pretreatment had no effect on the production of either IP_1, IP_2, or IP_3.

To determine the effects of treatment with pertussis toxin on cell growth, neonatal ventricular myocytes grown in serum-free medium were exposed to 2.0 μM (−)-norepinephrine for 36 h. As can be seen in figure 2, cell protein content measured isotopically increased by 40% in the control cells and 34% in the cells pretreated with pertussis toxin (P = NS), indicating that there was no effect of this agent on (−)-norepinephrine-induced hypertrophy. The data shown in figure 3 demonstrate that the dose-response relationship of augmented myocardial cell protein content measured spectrophotometrically in response to increasing concentrations of (−)-norepinephrine for 72 h was unaffected by concurrent treatment with pertussis toxin ranging from 0.1 to 1.0 μg/ml.

The present experiments are the first to examine the potential role of a pertussis toxin sensitive substrate in α_1-adrenoceptor-mediated myocardial cell growth. Our observations demonstrate that in neonatal rat ventricular myocytes G protein(s) ADP-ribosylated by pertussis toxin do not play a role in two α_1-adrenoceptor-mediated processes, phosphatidylinositide turnover or induction of hypertrophy. Previous studies of α_1-adrenergic stimulation of phosphatidylinositide hydrolysis in cardiac tissue have yielded conflicting results. Thus Brown et al.[4] and Steinberg et al.[20] were unable to demonstrate IP_3 accumulation after (−)-norepinephrine stimulation in adult and neonatal rat cardiomyocytes, respectively. By

contrast, others have shown accumulation of all three inositol phosphates in isolated, perfused rat hearts, isolated rat left atria or rat papillary muscles[5-7,21]. To our knowledge, our observations are the first to demonstrate the production of IP_3 in cultured neonatal rat ventricular myocytes using column chromatographic techniques.

The role of a pertussis toxin substrate in transducing α_1-adrenoceptor-mediated phosphatidylinosotide hydrolysis in myocardial tissue has been controversial. Recently Steinberg et al.[19,20] reported in a rat ventricular myocyte culture system that α_1-adrenergic responses are linked to a pertussis toxin substrate both after innervation and following stimulation with (−)-norepinephrine. Although these investigators were unable to demonstrate the presence of IP_3 using Dowex column chromotography they reported an average 50% reduction in α-agonist-stimulated IP_1 formation after pertussis toxin pretreatment. By contrast, in a preliminary report the same investigators noted that (−)-norepinephrine-stimulated IP_3 production measured by HPLC was insensitive to pertussis toxin pretreatment[22]. Bohm et al.[23] could not demonstrate an effect of pertussis toxin on the positive inotropic effect of α_1-adrenoceptor agonism using (−)-phenylephrine in isolated rat left atria and Schmitz et al.[6] have shown in a similar preparation that pertussis toxin does not inhibit the α_1-adrenoceptor-mediated hydrolysis of phosphatidylinositides in the heart as measured by IP_1, IP_2, or IP_3 production. Our data in neonatal rat ventricular myocytes are consistent with these latter observations. Our data are also consistent with the absence of pertussis toxin effects on (−)-norepinephrine-stimulated inositol phosphate accumulation in FRTL5 thyroid cells[24], in canine kidney and BC3H1 smooth muscle cells[25], and (−)-phenylephrine-stimulated breakdown of PIP_2 in hamster brown adipocytes[26].

The lack of effect of pertussis toxin on phosphatidylinositide turnover does not necessarily imply that a G protein may not be involved in phospholipase C-mediated events stimulated by α_1-adrenoceptor-mediated mechanisms. For example, in murine exocrine pancreatic cells muscarinic-cholinergic receptor activation of phospholipase C and stimulation of IP_3 formation is guanine-nucleotide dependent, but the specific G protein transducing this signal is not inhibited by pretreatment with either pertussis toxin or cholera toxin[11]. Recently a cDNA clone for a GTP binding protein α subunit that lacks an ADP-ribosylation site for pertussis toxin has been identified[12]. In chick heart cells permeablized by treatment with saponin and cultured overnight in the presence of ^{3}H-inositol, Jones et al.[13] demonstrated that the nonhydrolyzable analog of GTP, GTP γ S, stimulated formation of IP_1, IP_2, and IP_3. These observations suggest that a G protein interacts with phospholipase C present in cardiac cells and thereby transduces receptor binding into phospholipase C activation.

Previous observations from our laboratory have indicated that long (48 h) exposure of neonatal rat ventricular myocytes grown in serum-free medium to (−)-norepinephrine does not cause α_1-adrenoceptor down-regulation or desensitization of the subsequent response to (−)-norepinephrine as measured by IP_1 production (27). Recent experiments have also indicated that the same is true of IP_2 and IP_3 formation (manuscript in preparation). These observations, taken together with the data presented above, are consistent with a role for phospholipase C-mediated membrane phosphatidylinositide hydrolysis acting through a pertussis toxin insensitive G protein in the regulation of myocardial cell hypertrophy. These observations also are consistent with the possibility that IP_3, through its effect on calcium mobilization, may be important in activating the molecular signals that could result in reprogramming of cardiac gene expression during myocardial hypertrophy.

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