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Role of a Guanine Nucleotide-Binding Protein in α_1-Adrenergic Receptor-Mediated Ca^{2+} Mobilization in DDT_1 MF-2 Cells (42596)

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Abstract. In this study the mechanisms involved in α_1-adrenergic receptor-mediated Ca^{2+} mobilization at the level of the plasma membrane were investigated. Stimulation of $^{45}Ca^{2+}$ efflux from saponin-permeabilized DDT_1 MF-2 cells was observed with the addition of either the α_1-adrenergic agonist phenylephrine and guanosine-5′-triphosphate or the nonhydrolyzable guanine nucleotide guanylyl-imidodiphosphate. In the presence of [^{32}P]NAD, pertussis toxin was found to catalyze ADP-ribosylation of a M_r = 40,500 (n = 8) peptide in membranes prepared from DDT_1 MF-2 cells, possibly the α-subunit of N_i. However, stimulation of unidirectional $^{45}Ca^{2+}$ efflux by phenylephrine was not affected by previous treatment of cells with 100 ng/ml pertussis toxin. These data suggest that the putative guanine nucleotide-binding protein which couples the α_1-adrenergic receptor to Ca^{2+} mobilization in DDT_1 MF-2 cells is not a pertussis toxin substrate and may possibly be an additional member of the guanine nucleotide binding protein family.

For some time, α_1-adrenergic agonists have been known to alter intracellular calcium distribution in a number of cell types (see (1) for a review). An early step in α_1-adrenergic action is stimulation of the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) by a Ca^{2+}-dependent phosphodiesterase which yields the second messengers, inositol-1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DG) which appear to act as intracellular second messengers (see (2) for a review). Direct observations have been made which suggest that a guanine nucleotide-binding protein may be involved in mediating α_1-adrenergic responses at the level of the plasma membrane (3, 4). Whether the protein which couples the α_1-adrenergic receptor to a Ca^{2+}-dependent phosphodiesterase in hepatocytes is functionally and structurally distinct from other known guanine nucleotide binding proteins as has been suggested by Blackmore *et al.* (3) is currently not clear.

In this study, we have investigated the possibility that the stimulatory effect of phenylephrine on undirectional $^{45}Ca^{2+}$ efflux from the DDT_1 MF-2 smooth muscle cell line is mediated by a guanine nucleotide-binding protein. Using saponin-permeabilized DDT_1 MF-2 cells, we have demonstrated that added guanine nucleotides are required for phenylephrine-stimulated $^{45}Ca^{2+}$ efflux. We also have demonstrated a specific substrate for ADP-ribosylation by pertussis toxin in DDT_1 MF-2 cell membranes. However, this peptide does not appear to be involved in α_1-adrenergic receptor-mediated calcium mobilization since treatment of DDT_1 MF-2 cells with pertussis toxin did not alter phenylephrine-stimulated $^{45}Ca^{2+}$ efflux.

Materials and Methods. *Chemicals used.* [^{32}P]NAD and $^{45}Ca^{2+}$ were purchased from NEN Research Products (Boston, MA) and ICN Radiochemicals (Irvine, CA), respectively. GTP and Gpp(NH)p were purchased from Boehringer Mannheim (Indianapolis, IN). Phenylephrine, saponin, NAD, ADP-ribose, thymidine, arginine, and dithiothreitol were purchased from Sigma Chemical Co. (St. Louis, MO). Pertussis toxin was obtained from List Laboratories, Inc. (Campbell, CA). SDS–PAGE reagents were purchased from Bio-Rad Laboratories (Emeryville, CA). Dulbecco's modified Eagle's medium and streptomycin were obtained from GIBCO Laboratories (Grand Island, NY). Fetal bovine serum and fungizone were purchased from Hyclone Laboratories (Logan, UT). RPMI 1640 medium was purchased from K.C. Biologicals (Lenexa, KS). All other chemicals used were reagent grade or better purchased from commercial sources.

Cell culture and membrane preparation. DDT_1 MF-2 cells were grown as previously

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described (5). The culture medium used was a 50:50 blend of Dulbecco's modified Eagle's medium and RPMI 1640 medium supplemented with 10 m*M* Hepes, 1% fungizone, 1% penicillin–streptomycin, and 5% fetal bovine serum. The calcium concentration of the culture medium was 1.19 m*M*. Partially purified membranes were obtained by differential centrifugation (6) and were stored at −80°C prior to use. Protein concentration was determined by the method of Bradford (7) using bovine serum albumin as the standard.

[^{32}P] *ADP ribosylation.* Pertussis toxin was activated by incubation in 50 m*M* glycine, 10 m*M* Tris (pH 7.4), 50 m*M* dithiothreitol, 0.2 m*M* EDTA, and 0.6 m*M* NaN_3 for 10 min at 30°C. ADP ribosylation assays were performed essentially as described by Kaslow *et al.* (8). Partially purified membranes (1.5 mg protein) were centrifuged at 30,000*g* for 20 min and resuspended at a concentration of 1 mg/ml in 15 m*M* potassium phosphate (pH 7.4), 20 m*M* thymidine, 5 m*M* ADP-ribose, 20 m*M* arginine, and 100 U/ml aprotinin. Assay tubes (total volume = 100 μl) contained 0.1 mg membrane protein, 100 μ*M* GTP, 10 μ*M* NAD (1 μCi [^{32}P]NAD), and pertussis toxin at concentrations indicated in the figure legends. The incubation was carried out for 20 min at 37°C at which time 100 μl of 20% trichloroacetic acid (TCA) was added. The assay tubes were centrifuged at 1000*g* for 10 min, and the pellets were resuspended in 200 μl 10% TCA. Following centrifugation at 1000*g* for 10 min, the pellets were resuspended in 75 μl of SDS sample buffer (9).

SDS–PAGE and autoradiography. Samples in SDS sample buffer were heated to 100°C for 2 min prior to electrophoresis on 5–15% gradient pore gels (9). Gels were stained with 0.25% Coomassie brilliant blue R250 for visualization of protein. For autoradiography, the gels were dried for 1.5 hr under vacuum, juxtaposed to Kodak AR X-OMAT film, and autoradiographed in cassettes with Cronex intensifying screens for 7 to 14 days at −70°C. Densitometric tracings of autoradiographs were made with an E-C Model 910 transmission densitometer. A light aperture of 0.1 × 3 mm, a wavelength of 540 nm, and a scanning speed of 1.1 in./min were used.

Unidirectional calcium efflux. DDT_1 MF-2 cells were subcultured into 12-well culture dishes at a density of 500,000 cells/well. Twenty-four hours later the culture medium was replaced with fresh medium which was supplemented with $^{45}Ca^{2+}$. The concentration of $^{45}Ca^{2+}$ varied from experiment to experiment, but ranged from 3 to 5 μCi/ml. Following a 3-hr incubation and immediately prior to the start of desaturation, the cells were washed using a modification of a procedure described by Sanui and Rubin (10) to remove cell surface-associated cations from cultured cells. Briefly, the culture medium was aspirated and the cells were washed once with 250 m*M* sucrose, 5 m*M* EGTA, 5 m*M* Hepes (pH 7.0), followed by four washes with 250 m*M* sucrose, 5 m*M* Hepes (pH 7.0). Wash buffers were maintained at 4°C. Immediately following the washing step, 400 μl of culture medium without $^{45}Ca^{2+}$ which contained either the drugs to be tested or the vehicle for the drugs was added to each well which started unidirectional $^{45}Ca^{2+}$ efflux. Sampling of the culture medium occurred at 1, 2, 3, 5, 7.5, 10, 15, and 20 min. At each time point, aliquots of 200 μl were taken for scintillation counting; the remaining culture medium was immediately aspirated and was replaced with 400 μl of culture medium without $^{45}Ca^{2+}$ which contained the drugs. After the 20-min time point, the cells were solubilized in 0.2 *N* NaOH, and the protein concentration and radioactivity present were determined.

In experiments with saponin-permeabilized cells, prior to the addition of cells, 10 μg/ml poly-L-lysine in Dulbecco's phosphate-buffered saline (pH 7.4) was added to each well for 1 hr at 37°C. This step prevented cell detachment in the presence of saponin. The solution was discarded and DDT_1 MF-2 cells were subcultured at a density of 700,000 cells/well. Twenty-four hours later the culture medium was replaced with 800 μl fresh culture medium supplemented with $^{45}Ca^{2+}$ (3–5 μCi/ml). Following a 3-hr incubation, the cells were washed as described above. A procedure similar to that previously described (11) was used to permeabilize DDT_1 MF-2 cells. Briefly, following the last wash, 400 μl of 110 m*M* KCl, 10 m*M* NaCl, 1.0 m*M* KH_2PO_4, 5.0 m*M* KH_2CO_3, 20 m*M* Hepes, pH 7.2, 0.3 m*M* $MgCl_2$, 20 m*M* creatine phosphate, 3.0 m*M* MgATP, creatine kinase at 10 units/ml, and saponin at 10 μg/ml (HiK buffer) were added

to each well. After 5 min at 37°C, a 200-μl medium sample was taken from each well, and the remaining HiK buffer was aspirated and replaced with HiK buffer which contained the drugs to be tested. Additional 200-μl samples were taken as described above. After the 20-min time point, the cells were solubilized in 0.2 *N* NaOH, and the protein concentration and radioactivity of the cell digests were determined.

Statistics. Data are reported as the means ± the standard error. Statistical analyses used included paired Student's *t* test, one-way analysis of variance, and Dunnet's test for multiple comparisons.

Results. Experiments were carried out in which the effect of saponin on unidirectional $^{45}Ca^{2+}$ efflux was examined. The results from a representative experiment shown in Fig. 1 indicate that saponin at 10 μg/ml had no appreciable effect on unidirectional $^{45}Ca^{2+}$ efflux from DDT_1 MF-2 cells compared to control cells. Within 5 min of treatment with saponin at 10 μg/ml, greater than 80% of the cells became permeable to trypan blue (data not shown). At higher concentrations of saponin, significant numbers of cells detached and became free floating (data not shown).

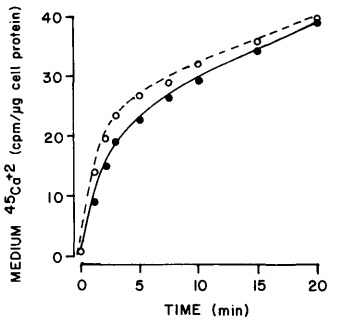

FIG. 1. Effect of saponin on unidirectional $^{45}Ca^{2+}$ efflux from DDT_1 MF-2 cells. DDT_1 MF-2 cells were cultured and equilibrated with $^{45}Ca^{2+}$ as described for saponin-permeabilized cells in the text. The $^{45}Ca^{2+}$ containing media was replaced with HiK buffer which included either 10 μg/ml saponin (closed circles) or no addition (open circles), and unidirectional $^{45}Ca^{2+}$ efflux was carried out as described in the text. The 5-min equilibration period normally used with saponin-permeabilized cells was omitted in this experiment such that sampling of the media began immediately after the washing step. Each point represents data obtained from duplicate wells. This experiment was repeated three times with identical results.

The effect of phenylephrine and GTP on unidirectional $^{45}Ca^{2+}$ efflux in saponin-permeabilized cells is shown in Fig. 2. A significant enhancement of $^{45}Ca^{2+}$ efflux was only observed in the combined presence of phenylephrine and GTP. Furthermore, the small stimulatory effect of phenylephrine and GTP occurred at the earliest time point (1 min); no additional stimulation was observed, unlike the results seen when the nonhydrolyzable guanine nucleotide Gpp(NH)p was used (see Fig. 3). Phenylephrine at a concentration which stimulated $^{45}Ca^{2+}$ efflux from intact DDT_1 MF-2 cells (see Fig. 4) had no effect on $^{45}Ca^{2+}$ efflux from saponin-permeabilized cells. The addition of 100 μM GTP had a small stimulatory effect on $^{45}Ca^{2+}$ efflux; however, this effect was not statistically significant ($P > 0.05$) (Fig. 2, bottom panel).

The effect of the nonhydrolyzable guanine nucleotide Gpp(NH)p on unidirectional $^{45}Ca^{2+}$ efflux from saponin-permeabilized cells is shown in Fig. 3. Gpp(NH)p stimulated $^{45}Ca^{2+}$ efflux in a dose-dependent manner. Furthermore, the stimulatory effect of Gpp(NH)p was persistent in contrast to the observed transitory effect of phenylephrine and GTP.

Incubation of membranes prepared from DDT_1 MF-2 cells with [^{32}P]NAD under the conditions described resulted in labeling of several peptides as identified by SDS–PAGE followed by autoradiography (Fig. 4, lane D). However, with the addition of pertussis toxin, labeling of two additional peptides was observed (Fig. 4, lanes A–C). From eight separate experiments carried out with different membrane preparations, the apparent molecular weight of the major peptide labeled in the presence of pertussis toxin was 40,500 ± 400 (n = 8). Minor labeling of a peptide with an approximate molecular weight of 30,000 was also observed in autoradiograms that were exposed for longer periods of time (Fig. 4, lanes A–C).

Experiments were carried out in order to determine whether the pertussis toxin substrate was involved in α_1-adrenergic receptor-mediated $^{45}Ca^{2+}$ unidirectional efflux. In these experiments, the DDT_1 MF-2 cells were not saponin-permeabilized prior to unidirectional $^{45}Ca^{2+}$ efflux. Phenylephrine significantly ($P < 0.05$) increased the $^{45}Ca^{2+}$ efflux rate from

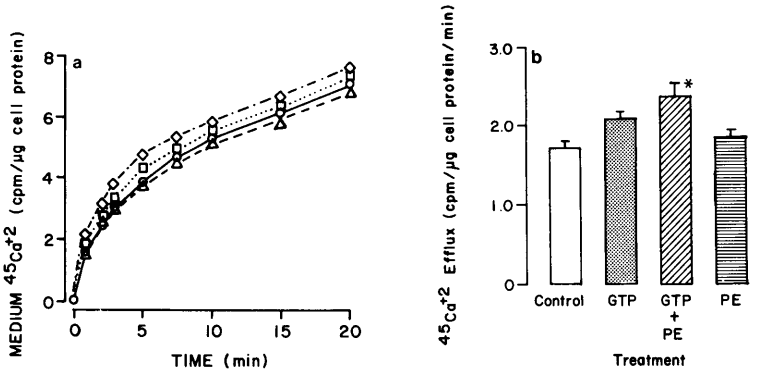

FIG. 2. Effect of phenylephrine and GTP on unidirectional $^{45}Ca^{2+}$ efflux from saponin-permeabilized DDT_1 MF-2 cells. DDT_1 MF-2 cells were cultured and equilibrated with $^{45}Ca^{+2}$ as described in the text. Left panel: Cumulative effect of phenylephrine and GTP. During unidirectional $^{45}Ca^{2+}$ efflux, the HiK buffer contained either vehicle (circles), 100 μM GTP (squares), 100 μM GTP and 10 μM phenylephrine (diamonds), or 10 μM phenylephrine (triangles). Each bar represents the mean of three wells from a single experiment. Right panel: Effect of phenylephrine and GTP during the first minute of unidirectional $^{45}Ca^{2+}$ efflux. The HiK buffer contained either vehicle (control), 100 μM GTP (GTP), 100 μM GTP and 10 μM phenylephrine (GTP + PE), or 10 μM phenylephrine (PE). Each bar represents the mean ± SE of six wells from two experiments. The asterisk indicates a significant ($P < 0.05$) difference from control by ANOVA and Dunnet's test.

DDT_1 MF-2 cells (Fig. 5). Similarly, phenylephrine significantly ($P < 0.05$) increased the $^{45}Ca^{2+}$ efflux rate from DDT_1 MF-2 cells that had been treated with 100 ng/ml pertussis toxin for 24 hr (Fig. 5). However, pertussis toxin treatment did not affect either the basal $^{45}Ca^{2+}$ efflux rate or the phenylephrine-stimulated $^{45}Ca^{2+}$ efflux rate compared to control cells (Fig. 5).

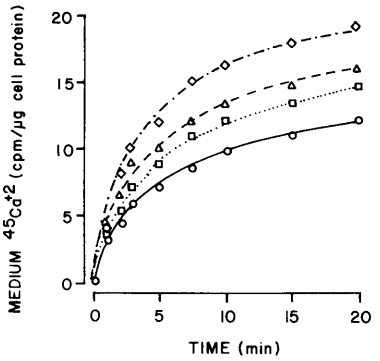

FIG. 3. Effect of Gpp(NH)p on unidirectional $^{45}Ca^{2+}$ efflux from saponin-permeabilized DDT_1 MF-2 cells. DDT_1 MF-2 cells were cultured and equilibrated with $^{45}Ca^{2+}$ as described for saponin-permeabilized cells in the text. During unidirectional $^{45}Ca^{2+}$ efflux, the HiK buffer contained vehicle (circles), 1.0 μM Gpp(NH)p (squares), 10.0 μM Gpp(NH)p (triangles), and 100.0 μM Gpp(NH)p (diamonds). Each point represents data obtained from duplicate wells. This experiment was repeated three times with identical results.

Twenty-four-hour pretreatment of DDT_1 MF-2 cells with pertussis toxin subsequently resulted in a dose-dependent inhibition of pertussis toxin-catalyzed ADP ribosylation of the M_r = 40,500 peptide in membranes prepared from treated cells (Fig. 6). Addition of pertussis toxin at a concentration of 100 ng/ml, the dose that had no effect on phenylephrine-stimulated $^{45}Ca^{2+}$ efflux (Fig. 5), resulted in greater than 95% reduction in ADP ribosylation of the M_r = 40,500 peptide.

Discussion. The DDT_1 MF-2 cell line was derived from a leiomyosarcoma of a Syrian hamster vas deferens (12). Previously, we demonstrated the presence of α_1-adrenergic receptors using radioligand assays (5, 13). Recent reports which indicate that phosphatidylinositol metabolism in DDT_1 MF-2 cells is increased by α_1-adrenergic agonists (14, 15) suggest that the DDT_1 MF-2 cell α_1-adrenergic receptor is physiologically functional. In this report, we have extended these findings by investigating the nature of the coupling of the α_1-adrenergic receptor to Ca^{2+} mobilization. To do this we used DDT_1 MF-2 cells that had

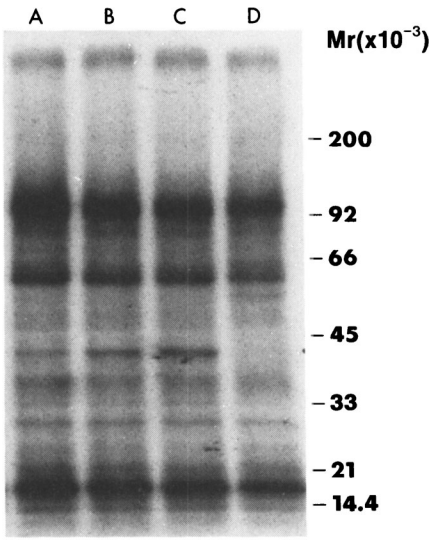

FIG. 4. ADP-ribosylation of DDT_1 MF-2 cell membranes. ADP-ribosylation were carried out as described in the text with the indicated amount of pertussis toxin in each assay tube given below. The positions of the molecular weight standards are indicated. Lane A, 1.5 μg pertussis toxin; lane B, 2.5 μg pertussis toxin; lane C, 3.75 μg pertussis toxin; lane D, no pertussis toxin.

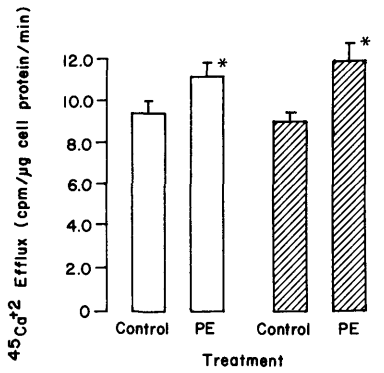

FIG. 5. Effect of pertussis toxin treatment on unidirectional $^{45}Ca^{2+}$ efflux. DDT_1 MF-2 cells were cultured for 24 hr in either the absence (open bars) or the presence (closed bars) of 100 ng/ml pertussis toxin. The cells were equilibrated with $^{45}Ca^{2+}$ as described in the text. During unidirectional $^{45}Ca^{2+}$ efflux, the media contained either vehicle (control) or 10 μM phenylephrine (PE). Each bar represents the mean ± SE of six wells from two experiments. Asterisks indicate significant ($P < 0.05$) difference from control by Student's paired t-test.

been treated with saponin which has been demonstrated to permeabilize the plasma membrane of numerous cell types (11, 16–18). Saponin interacts with cholesterol to form pores which makes the plasma membrane permeable to small molecules and ions (19). The integrity of membranes of organelles which contain only small amounts of cholesterol; for example, those of endoplasmic reticulum and mitochondria are not affected by saponin (19).

Several lines of evidence suggest that a guanine nucleotide-binding protein couples a variety of receptors, including the α_1-adrenergic receptor, to phosphatidylinositol hydrolysis and intracellular mobilization of calcium. Results from ligand binding studies demonstrate that receptors for numerous Ca^{2+}-mobilizing hormones exist in high- and low-affinity states which can be interconverted by guanine nucleotides. For example, effects of guanine nucleotides have been observed on the binding of agonists to the f–Met–Leu–Phe receptor of polymorphonuclear leukocytes and macrophages (20, 21), the thyrotropin-releasing hormone receptor of GH3 cells (22, 23), and the α_1-adrenergic receptor of vascular smooth muscle (24), cardiac myocytes (24), MDCK cells (25), and BC3H-1 cells (25). However, guanine nucleotide effects on agonist binding

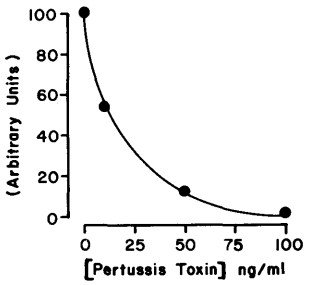

FIG. 6. Effect of pretreatment of DDT_1 MF-2 cells with pertussis toxin on subsequent ADP-ribosylation of M_r = 40,500 peptide. DDT_1 MF-2 cells were cultured for 24 hr in the presence of the indicated concentration of pertussis toxin. Cells were harvested and partially purified membranes prepared as described in the text. ADP-ribosylation assays were carried out as described in the text using 25 μg activated pertussis toxin per tube. Scanning densitometry of the autoradiograph was performed, and the area under the peak corresponding to the M_r = 40,500 peptide determined. The labeling intensity observed with membranes prepared from cells that had not been pretreated with pertussis toxin was arbitrarily set at 100. This experiment was performed twice with identical results.

to α_1-adrenergic receptors are not always observed (13, 26). This apparent disparity was addressed recently in a report that suggested that repeated washing of membranes and inclusion of EDTA and protease inhibitors during membrane preparation was necessary to observe guanine nucleotide shifts of agonist affinities for α_1-adrenergic receptors (25). More direct evidence in support of guanine nucleotide binding protein involvement in Ca^{2+} mobilization includes an observation that AlF_4-, known to modulate the activity of the guanine nucleotide binding proteins N_i, N_s and transducin, mimics the effects of Ca^{2+}-mobilizing hormones in hepatocytes (3). Finally, guanine nucleotides have been shown to stimulate hydrolysis of PIP_2 in rat liver plasma membranes (4) and membranes prepared from DDT_1 MF-2 cells (unpublished).

To date the putative guanine nucleotide-binding protein that couples receptors for Ca^{2+}-mobilizing hormones to a Ca^{2+}-dependent phosphodiesterase remains unidentified. However, in neutrophils and polymorphonuclear leukocytes, some evidence suggests that N_i is the guanine nucleotide-binding protein that couples the f–Met–Leu–Phe receptor to PIP_2 metabolism. Results from several laboratories indicate that pertussis toxin inhibits the effects of the *N*-formyl peptides (27–29). Pertussis toxin is known to ADP ribosylate the α-subunits of three members of the guanine nucleotide-binding protein family, the M_r = 41,000 α-subunit of N_i, the M_r = 40,000 α-subunit of N_o, and the M_r = 39,000 subunit of transducin (30). In contrast to the activation of N_S by cholera toxin which results in sustained adenylate cyclase activity, pertussis toxin causes uncoupling of N proteins from receptor resulting in reduced signal transduction (30). Interestingly, Kikuchi *et al.* (31) have shown recently that f–Met–Leu–Phe stimulation of [^{3}H]inositol hydrolysis can be restored in differentiated human leukemic cells by reconstitution with either purified N_i or N_o. This finding raises the possibility that the guanine nucleotide-binding protein coupled to the f–Met–Leu–Phe receptor may not be N_i.

Similarly, the identity of the putative guanine nucleotide-binding protein which couples the α_1-adrenergic receptor to phosphatidylinositol breakdown is uncertain. Steinberg *et al.* (32) have reported that developmental acquisition of α_1-adrenergic response in cardiac myocytes is associated with the appearance of a substrate for pertussis toxin-stimulated ADP ribosylation, possibly N_i. Moreover, norepinephrine stimulation of inositol phosphate formation was found to be attenuated in myocytes pretreated with pertussis toxin (33). However, Schmitz *et al.* (34) have reported that treatment of rats with 150 μg/kg pertussis toxin had no effect on subsequent inositol phosphate production in heart. Furthermore, treatment with pertussis toxin has been shown to have no effect on α_1-adrenergic stimulation of either phosphatidylinositol metabolism or respiration of brown adipoctyes (35). These findings are similar to that which we obtained in our current study since treatment of DDT_1 MF-2 cells with pertussis toxin did not alter phenylephrine stimulation of unidirectional $^{45}Ca^{2+}$ efflux.

An important consideration in studies of this nature is the completeness of inactivation of the putative guanine nucleotide-binding protein by pertussis toxin treatment. In this regard, we observed no ADP ribosylation of the M_r = 40,500 peptide with [^{32}P]NAD in membranes prepared from DDT_1 MF-2 cells that had been exposed previously to 100 ng/ml pertussis toxin for a period of 24 hr. Of course, it is possible that ADP ribosylation of the α-subunit of the guanine nucleotide-binding protein coupled to the α_1-adrenergic receptor does not result in loss of activity in DDT_1 MF-2 cell membranes as is the case with N_i in other cell types (27–29).

Our data suggest that the substrate for pertussis toxin-stimulated ADP ribosylation in DDT_1 MF-2 cell membranes may not be involved in α_1-adrenergic receptor function. The identity of the ADP-ribosylated peptide and its role, if any, in DDT_1 MF-2 cell function remains unknown. Assuming that this peptide is the α-subunit of a guanine nucleotide-binding protein, either N_i or N_o, it is possible that it is coupled to another as yet unidentified receptor. Clearly, additional investigation is required.

In summary, our results suggest that α_1-adrenergic receptor-mediated Ca^{2+} mobilization in DDT_1 MF-2 cells occurs via a guanine nucleotide-binding protein. The identity of this coupling protein remains uncertain since pertussis toxin treatment of DDT_1 MF-2 cells did

not affect phenylephrine-stimulated unidirectional $^{45}Ca^{2+}$ efflux. Therefore, it is possible that the protein which couples the DDT_1 MF-2 α_1-adrenergic receptor to Ca^{2+} mobilization is an additional member of the guanine nucleotide-binding protein family.

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