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Role of $G_{\alpha q}$ or $G_{\alpha o}$ Proteins in α_1-Adrenoceptor Subtype-Mediated Responses in Fischer 344 Rat Aorta

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SUMMARY

Previous studies showed that α-adrenoceptor (AR) stimulation with norepinephrine is more potent at eliciting contraction in aortas from 1-month-old Fischer 344 rats than from older rats and that this response is mediated by α_{1b}- and α_{1d}-AR subtypes in 1-month-old rats. We examined the G proteins responsible for α_1-AR-mediated contractile response and inositol phosphate accumulation in the aortas of 1-month-old Fischer 344 rats. Pertussis toxin (PTX) treatment (2.5 μg/ml for 4 hr) of aortic rings partially inhibited phenylephrine (PHE)-stimulated contraction and inositol phosphate accumulation, suggesting the involvement of PTX-sensitive and -insensitive G proteins. Specific antisera directed against $G_{\alpha q}$ and $G_{\alpha o}$ but not $G_{\alpha s}$ and $G_{\alpha i}$ precipitated specific α_1-AR binding sites labeled with 2-[β-(4-hydroxy-3-[^{125}I]iodophenyl)ethylaminomethyl]tetralone. The number of 2-[β-(4-hydroxy-3-[^{125}I]iodophenyl)ethylaminomethyl]tetralone binding sites precipitated by G_α proteins was increased by activating membrane α_1-ARs with PHE. Moreover, PHE stimulated the palmitoylation of $G_{\alpha q}$ and $G_{\alpha o}$, and this response was blocked by the α_1-AR antagonist prazosin. Characterization of the α_1-AR subtypes that couple to G proteins indicates that although aortic α_{1a}-, α_{1b}-, and α_{1d}-ARs were associated with $G_{\alpha q}$, α_{1b}-AR was also linked to $G_{\alpha o}$. These results suggest that α_1-ARs mediate the contractile response in rat aorta by coupling to both G_q protein and the PTX-sensitive G_o protein.

Three α_1-AR subtypes (α_{1a}, α_{1b}, and α_{1d}) have been cloned, and mRNA for each has been detected in the rat aorta (1, 2). The specific role of each α_1-AR subtype in regulating vascular smooth muscle function has not been completely established. When overexpressed in cultured cells, each of the subtypes has been shown to be capable of eliciting characteristic α_1-adrenergic responses, including activation of PLC and increased intracellular calcium (3, 4). Several studies have shown that α_1-ARs can couple to G_q and activate phospholipase C, resulting in production of inositol trisphosphate and release of intracellular calcium (5). However, it has been shown that α_1-AR-mediated contractile responses in vascular smooth muscle are partially inhibited by PTX treatment, suggesting the involvement of both PTX-sensitive and -insensitive G proteins in the contractile response (6–8). Thus, in vascular smooth muscle, it is not clear what combination of endogenous α_1-AR subtypes and G proteins is responsible for activating PLC and eliciting the contractile response.

In previous studies, we showed that although aortas from 1-month-old Fischer 344 rats express α_{1a}-, α_{1b}-, and α_{1d}-ARs, the contractile response to NE is mediated by stimulation of the α_{1b}- and α_{1d}-ARs (9, 10). NE produced a more potent contractile response in these aortas compared with aortas of older rats, in which the expression and functional role of the α_{1a}-AR increase (9, 11). The determination of the coupling of α_1-AR subtypes to specific G proteins will facilitate understanding of the functional roles of α_1-AR subtypes in the aorta; therefore, the aim of the current study was to define α_1-AR/G protein coupling in aortic membranes. This was achieved by examining the sensitivity of α_1-AR-mediated responses to PTX treatment; assessing α_1-ARs that coimmunoprecipitated with G_α proteins using specific antisera directed against G_α subunits, G_α proteins that coimmunoprecipitated with α_1-AR subtypes using specific antisera directed against α_1-AR subtypes; and examining α_1-AR-stim-

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ABBREVIATIONS: AR, adrenoceptor; PHE, phenylephrine; NE, norepinephrine; IP, inositol phosphate; PTX, pertussis toxin; PLC, phospholipase C; [^{125}I]HEAT, 2-[β-(4-hydroxy-3-[^{125}I]iodophenyl)ethylaminomethyl]tetralone; PSS, physiological salt solution; PBS, phosphate-buffered saline; TBS, Tween-20 containing phosphate-buffered saline; SDS, sodium dodecyl sulfate; ECL, enhanced chemiluminescence; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ANOVA, analysis of variance.

ulated palmitoylation of α subunits or receptor-stimulated changes in coprecipitation of α_1-AR binding sites with G_α subunits in aortic membranes.

Experimental Procedures

Animals. One-month-old male Fischer 344 rats were obtained from National Center for Toxicological Research (Jefferson, AR), where they are bred and maintained under the auspices of the National Institute on Aging. On receipt at our institution, animals were maintained for 1–2 weeks under barrier conditions comparable to those under which they were raised.

Contraction. Rats were killed through decapitation, and the aorta was removed and placed in ice-cold PSS composed of 120 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 25 mM $NaCO_3$, 1.8 mM $CaCl_2$, 11 mM glucose, and 0.024 mM EDTA. Vessels were cleansed of fat and connective tissue and cut into 3-mm-wide rings. Aortic ring segments were mounted at 37° in 15-ml organ baths using stainless steel hooks connected by fine gold chain at the bottom to a stationary glass rod attached to the bath and at the top to a Grass Instruments (Quincy, MA) model FT0.03 force-displacement transducer and bubbled continuously with 95% O_2/5% CO_2. Responses were recorded on a Grass model 7 polygraph. Rings were equilibrated for 1 hr at a previously optimal resting tension of 1.5 g. Concentration-response curves were determined through cumulative increases in the concentration of agonist. Rings then were washed extensively by several changes of PSS over 1–2 hr until tension stabilized at the precontraction level. For experiments with PTX, rings were incubated with the toxin for 4 hr in PSS. Then, the rings were washed for 1 hr with several changes of PSS, and cumulative concentration-response curves to PHE were obtained.

IP accumulation. The method used for measuring [^{3}H]inositol metabolism was described previously (12, 13). Aortic rings were prepared as described above and then preincubated in oxygenated buffer composed of 122 mM NaCl, 4.9 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 3.6 mM $NaCO_3$, 1.3 mM $CaCl_2$, 11 mM glucose, and 30 mM HEPES, pH 7.4, at 37° for 1 hr. Subsequently, artery segments were incubated for 1.5 hr in buffer containing 20 μCi/ml of *myo*-[^{3}H]inositol (17 Ci/mmol; New England Nuclear Research Products, Boston, MA) under the same conditions. Labeled artery segments were washed four times and placed inot individual tubes containing buffer with 10 mM LiCl (total assay volume, 300 μl). Treatment with PTX was as described above. Incubation with agonist was for 60 min with oxygenation at 15-min intervals and was stopped by the addition of 300 μl of ice-cold 15% trichloroacetic acid and then left on ice for 15 min. The tubes were centrifuged (1500 $\times$ *g* for 10 min), and aliquots (350 μl) of supernatant were added to 125 μl of 10 mM EDTA in 1.5-ml microcentrifuge tubes, followed by 500 μl of 1:1 Freon/tri-*n*-octylamine. The samples were vortexed and left to stand for 10 min before centrifugation (12,000 $\times$ *g* for 10 min), and 350 μl of upper aqueous phase was taken for analysis of IPs. Samples were loaded onto Dowex-1(X8) ion exchange columns (formate form, 100–200 mesh, 1 ml). The columns were washed initially with 16 ml of *myo*-[^{3}H]inositol (5 mM). Then, IPs were eluted with 4 ml of 0.1 M formic acid/1 M ammonium formate. Radioactivity was measured by liquid scintillation counting.

Preparation of aortic membrane. Rats were killed by decapitation, and the aorta was removed; placed in 20 mM NaH_2PO_4-Na_2HPO_4 buffer (pH 7.6) containing 154 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 20 μg/ml aprotinin, and 25 μg/ml pepstatin; cleansed of fat and connective tissue; homogenized using a glass-to-glass homogenizer; and centrifuged at 500 $\times$ *g* for 10 min at 4°. The supernatant was centrifuged at 100,000 $\times$ *g* for 60 min at 4°. The resulting pellet was resuspended, rehomogenized, and then recentrifuged under the same conditions. The final pellet was resuspended in PBS. Protein content was measured according to the method of Bradford (14).

Solubilization of aortic membrane. Aortic membranes were solubilized by modification of a previously described procedure (15). Briefly, aortic membranes were prepared as described above. They were then solubilized by gentle end-over-end shaking for 60 min at 4° in PBS containing 1.5% digitonin, 0.5 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 20 μg/ml aprotinin, and 25 μg/ml pepstatin. The sample was centrifuged at 100,000 $\times$ *g* for 60 min at 4°, and the supernatant was used for the soluble fraction of the membrane. The pellet was also collected for determination of the insoluble α_1-ARs and G protein α subunits remaining in the membranes. The solubilized α_1-ARs were detected by measuring [^{125}I]HEAT binding. The samples were incubated with a 300–400 pM concentration of [^{125}I]HEAT for 60 min at 37°. Reactions were terminated by rapid filtration using a Brandel (Montreal, Quebec, Canada) cell harvester and Whatman (Clifton, NJ) DE81 filters to trap the solubilized proteins. Filters were washed four times with 4 ml of ice-cold PBS. The filter-bound radioactivity was determined in a Beckman Instruments (Palo Alto, CA) γ-counter. Nonspecific binding was defined as binding in the presence of 0.1 μM prazosin or 1 mM NE with identical results. Assays were conducted in duplicate. After solubilization of aortic membranes, 20–30% of the initial α_1-AR binding was detected in soluble fraction.

Immunoprecipitation of G protein α subunits and α_1-AR subtypes. Solubilized G protein α subunits were immunoprecipitated as described previously (15, 16). Soluble membrane protein (10–15 fmol of α_1-AR) was incubated with an appropriate dilution of G_α-specific antiserum overnight in a rotatory shaker at 4°C. Nonimmune serum at the same dilution was used as a control. Appropriate dilution was determined when no further immunoprecipitation was observed at a higher concentration of the antiserum (maximum concentration, 1:50). Then, 100 μl of a 1:1 suspension of protein A/Sepharose beads (CL-4B: Sigma Chemical, St. Louis, MO), prewashed three times and diluted in PBS, was added to the samples and incubated overnight in a rotary shaker at 4°C. The samples were centrifuged at 10,000 $\times$ *g* for 3 min; the supernatant was collected to measure remaining α1-ARs; and the pellet was resuspended in PBS and recentrifuged as described above. The immunoprecipitate was resuspended in PBS, and α_1-ARs were detected by measuring [^{125}I]HEAT binding as described above. In some samples, the immunoprecipitate was separated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membrane, and immunoblotted with anti-G_α antisera to confirm the identity of the precipitated G protein α subunits. In several experiments, the immunoprecipitate was incubated with 0.1 mM guanosine-5′-(β,γ-imido)triphosphate for 60 min at 25° and then centrifuged at 10,000 $\times$ *g* for 3 min. The pellet was resuspended in PBS, and α_1-ARs in the immunoprecipitate were determined using the radioligand binding assay described above.

In a separate experiment, aortic membranes (400 μg) were solubilized by and incubated with antisera directed against the α_{1a}-, α_{1b}-, or α_{1d}-ARs (1:250 dilution) for 3 hr followed by a 60-min incubation with 100 μl of a 10% suspension of protein A, bearing *Staphylococcus aureus* cells (Pansorbin cells; Calbiochem, San Diego, CA). Initial characterization of these antibodies has been reported previously (17). After centrifugation and washing, the immunoprecipitates were solubilized in sample preparation buffer (62.5 mM Tris·HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.1% bromphenol blue), and proteins were separated by 10% SDS-polyacrylamide gel electrophoresis, transferred electrophoretically to a nitrocellulose membrane, and immunoblotted with polyclonal antibody against G_α proteins (1:2000 in 0.1% Tween-20 in PBS). In some samples, the selectivity of the antisera directed against α_{1a}-, α_{1b}-, or α_{1d}-ARs, and the effectiveness of the immunoprecipitation was tested. The immunoprecipitates were separated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with anti-α_{1a}-, -α_{1b}-, or -α_{1d}-AR antisera to test the identity and the amount of the precipitated α_1-AR subtype.

Immunoblots. Aortic membranes, solubilized membranes, or membrane immunoprecipitates were subjected to 10% SDS-poly-

acrylamide gel electrophoresis (18) and then transferred electrophoretically to nitrocellulose. Immunoblotting was performed using antisera for α subunits of G proteins [RM/1 ($G_{\alpha s}$), AS/7 ($G_{\alpha i}$), GC/2 ($G_{\alpha o}$), QL ($G_{\alpha q/11}$); dilution 1:2000; New Nuclear England Research Products] and ECL as described previously (15, 19). Briefly, nitrocellulose membranes were incubated overnight at 4° in PBS containing 3% bovine serum albumin and 8% nonfat dry milk. Blots were washed several times with 0.1% TBS and then incubated with antisera at room temperature for 1–2 hr with shaking. Blots were then washed four times (10 min each) with 0.1% TBS and then incubated with 1:10,000 dilutions of horseradish peroxidase-labeled donkey anti-rabbit IgG or rabbit anti-goat (α_{1a}-AR) in 0.1% TBS for 1 hr at room temperature. Blots were washed once with 0.3% TBS for 30 min followed by four 5-min washes with 0.1% TBS and then incubated with ECL Western blotting reagent (SuperSignal substrate, Western Blotting; Pierce, Rockford, IL) for 4 min and exposed to X-ray film for 15–45 sec.

Agonist-induced palmitoylation of G_α proteins. Aortas were homogenized and centrifuged (500 $\times$ *g* for 10 min at 4°) as described above except with HEPES buffer containing 25 mM HEPES, pH 7.4, and 2 mM EGTA. The supernatant was centrifuged at 100,000 $\times$ *g* for 60 min at 4°. The resulting pellet was resuspended, rehomogenized, and then recentrifuged under the same conditions. The final pellet was resuspended in oxygenated Krebs-HEPES buffer containing 25 mM HEPES, pH 7.4, 154 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 0.2% 2-mercaptoethanol, 25 μg/ml leupeptin, 25 μg/ml pepstatin A, 0.01unit/ml soybean trypsin inhibitor, and 0.05 mM phenylmethylsulfonyl fluoride and used as the crude membrane preparation. The assay mixture (250 μl) containing 200 μg of membrane protein, 800 μCi/ml [9,10-^{3}H]palmitic acid (specific activity, 50 Ci/mmol; New Nuclear England Research Products) was incubated at 37° for 10 min followed by an additional 5-min incubation with either buffer or agonist. To test the specificity of this receptor mediated response, membranes were incubated with a selective α_1-AR antagonist for 5 min before the addition of the agonist. The reaction was terminated by dilution with 750 μl of ice-cold Krebs-HEPES containing 1 mM EGTA, mixed, placed on ice, and immediately centrifuged at 16,000 $\times$ *g* for 30 min in microcentrifuge. The pellets were solubilized in 1.5 ml of Krebs-HEPES buffer containing 1.5% digitonin, and soluble membrane proteins were immunoprecipitated using antisera directed against the G_α proteins as described above. The radioactivity in the immunoprecipitate was measured by liquid scintillation counting. The radioactivity precipitated by the normal rabbit serum was considered background and subtracted from all values.

Data analysis. Differences were determined by ANOVA and *post hoc* analysis for multiple comparisons. A value of $p < 0.05$ was considered significant.

Materials. For these studies, pargyline HCl, soybean trypsin inhibitor, and the buffer reagents were purchased from Sigma. The chemicals used for IP isolation and determination were purchased from Fisher Scientific (Pittsburgh, PA). PHE was purchased from Research Biochemicals (Natick, MA). Normal rabbit serum and Pansorbin were purchased from Calbiochem. Prazosin HCl was generously supplied by Pfizer (New York, NY). [9,10-^{3}H]Palmitic acid (50 Ci/mmol), [^{125}I]HEAT (2200 Ci/mmol), and the antisera to $G_{\alpha s}$ (RM/1), $G_{\alpha i(1,2)}$ (AS/7), $G_{\alpha o}$ (GC/2), and $G_{\alpha q/11}$ (QL) were purchased from New Nuclear England Research Products. Antisera to α_{1b}- and α_{1d}-ARs were produced by one of the authors (R.D.B.). Antiserum to α_{1a} was purchased from Santa Cruz Biochemicals (Santa Cruz, CA). Horseradish peroxidase-labeled donkey anti-rabbit IgG or rabbit anti-goat (SuperSignal substrate, Western Blotting; Pierce).

Results

Effect of PTX on contraction and IP accumulation. PTX treatment maximally inhibited PHE-induced contractile response at a concentration of 2.5 μg/ml (Fig. 1). Higher PTX concentrations did not cause further inhibition in the responses to PHE, and KCl-induced contraction was not altered by PTX treatment (data not shown). PHE-induced IP accumulation was also inhibited (52 $\pm$ 8%) by 2.5 μg/ml PTX treatment (Fig. 2).

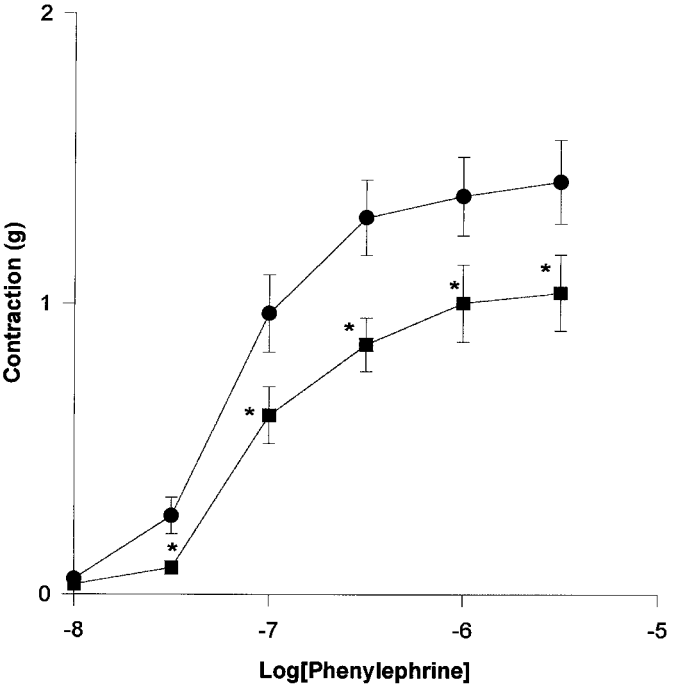

Fig. 1. PHE-induced contraction of aortic ring segments in 1-month-old Fischer 344 rats before (●) and after (■) treatment with PTX (2.5 μg/ml, 4 hr at 37°). Data represent mean $\pm$ standard error of determinations obtained from five or six animals. A significant reduction in PHE concentration-response curve was obtained after PTX treatment as determined by ANOVA followed by Newman-Keuls test for multiple comparisons (*, $p < 0.05$).

Coupling of G proteins and α_1-ARs. The possibility that α_1-ARs directly couple to G proteins was tested by coimmunoprecipitation of α_1-ARs with anti-G_α protein antibodies. We have previously shown by immunoblot analysis the presence of single bands for $G_{\alpha o}$ (39 kDa), $G_{\alpha i}$ (41 kDa), and $G_{\alpha q}$ (42 kDa) and two bands for $G_{\alpha s}$ (45 and 52 kDa) in aortic membranes (15, 19). The results summarized in Fig. 3 demonstrate that antisera against $G_{\alpha q}$ and $G_{\alpha o}$ but not $G_{\alpha s}$ or $G_{\alpha i}$, at dilutions of 1:200, precipitated specific α_1-AR binding sites labeled by the selective α_1-AR ligand [^{125}I]HEAT. To confirm the specificity of the immunoprecipitation, the precipitates obtained using $G_{\alpha q}$ and $G_{\alpha o}$ antisera were subjected to immunoblot analyses. $G_{\alpha q}$ and $G_{\alpha o}$ antisera selectively precipitated the respective proteins (data not shown). The coupling of G_α proteins to α_1-AR subtypes α_{1a}, α_{1b}, and α_{1d} was investigated by monitoring the G_α proteins that were coimmunoprecipitated with specific antisera directed against the α_1-AR subtypes. Immunoprecipitates of the α_1-AR subtypes derived from 400 μg of solubilized aortic membranes were separated on SDS-polyacrylamide gels and blotted with antibodies against the G_α proteins. Fig. 4, A and B, illustrates that α_{1a}-, α_{1b}-, and α_{1d}-AR antibodies coimmunoprecipitated $G_{\alpha q}$ protein, whereas the α_{1b}-AR antibody also immunoprecipitated $G_{\alpha o}$ protein. Densitometric analysis of the results indicate that ~4.5–6% of membrane $G_{\alpha q}$ was found to be associated with α_{1a}-, α_{1b}-, and α_{1d}-ARs, whereas only 2.8% of $G_{\alpha o}$ was

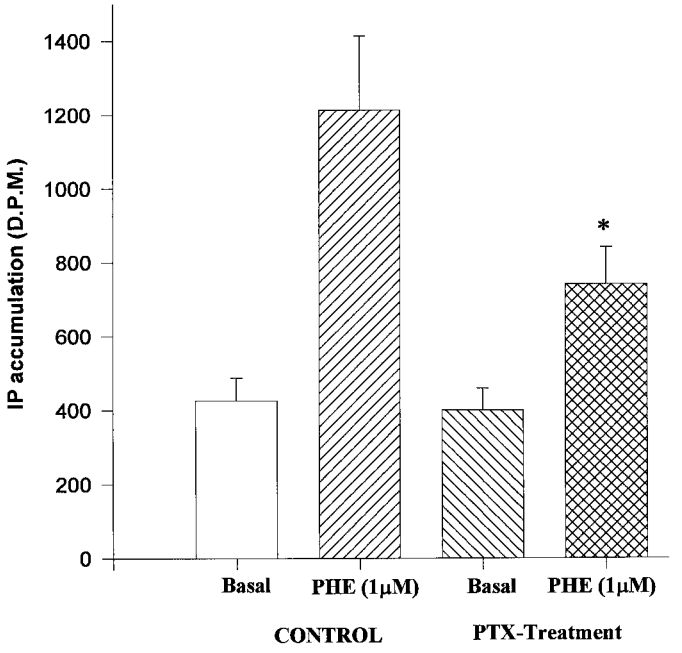

Fig. 2. PHE-stimulated IP accumulation in aortic ring segments from 1-month-old aorta before and after treatment with PTX (2.5 μg/ml, 4 hr at 37°). Aortic rings were labeled with *myo*-[^{3}H]inositol and subjected to incubation with agonist ,and IPs were separated by ion exchange chromatography. Data represent mean ± standard error of determinations obtained from five or six animals. A significant reduction in PHE-activated IP accumulation was obtained after PTX treatment as determined by ANOVA followed by Newman-Keuls test for multiple comparisons (*, $p < 0.05$).

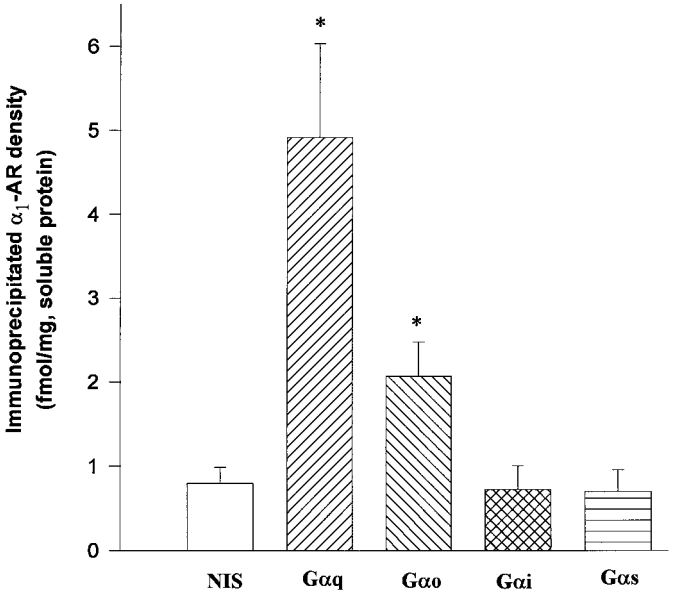

Fig. 3. Immunoprecipitation of α_1-ARs by antisera to $G_{\alpha s}$, $G_{\alpha o}$, $G_{\alpha q}$, and $G_{\alpha i}$ and by nonimmune serum (*NIS*) in solubilized aortic membranes from 1-month-old rat. Soluble membrane proteins were incubated with antisera directed against the G_α proteins, and immuncomplexes were precipitated with protein A/Sepharose beads. The α_1-ARs were detected by measuring [^{125}I]HEAT binding in the precipitate. Each value represents the mean ± standard error of six or seven individual experiments. *, $p < 0.05$, significant difference by ANOVA followed by Newman-Keuls test for multiple comparisons.

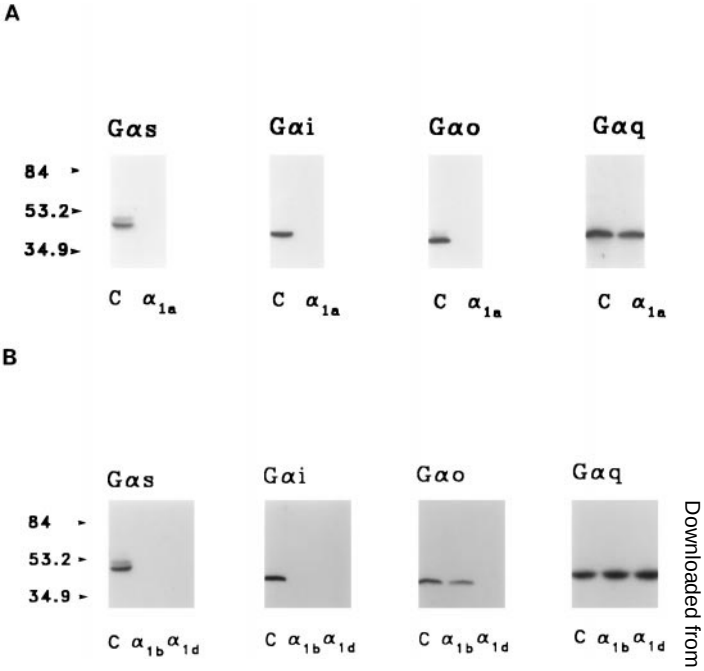

Fig. 4. Coimmunoprecipitation of α_1-AR subtypes with G_α proteins. Rat aortic membranes were solubilized and subjected to immunoprecipitation with anti-peptide antisera raised against α_{1a}-, α_{1b}-, or α_{1d}-AR (1:250 dilution). The immunocomplexes derived from 400 μg of solubilized aortic membranes were then solubilized and separated on 10% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, immunoblotted with specific G_α antisera (1:2000 dilution), and detected by ECL. $G_{\alpha q}$ was detected in α_{1a}-, α_{1b}-, and α_{1d}-AR immunoprecipitates (A and B), whereas $G_{\alpha o}$ was observed only in the α_{1b}-AR immunoprecipitate (B). *Lane C*, immunoblots of 25 μg of solubilized aortic membranes. The immunoblots shown are representatives of four individual experiments that yielded similar results.

linked to α_{1b}-AR. The specificity of the anti-receptor antisera and the effectiveness of the immunoprecipitation are presented in Fig. 5. The figure demonstrates that the antiserum for each of the receptor subtypes completely and selectively precipitated the respective protein. Furthermore, incubation of aortic membranes with 1 μM PHE was found to increase [^{125}I]HEAT binding in immunoprecipitates of $G_{\alpha q}$ and $G_{\alpha o}$

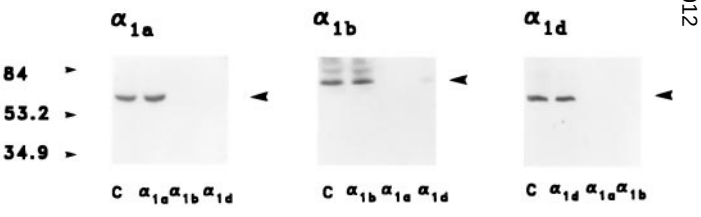

Fig. 5. Specificity of the antisera to the α_{1a}-, α_{1b}-, and α_{1d}-ARs. The specificity of each of the α_1-AR antisera was tested in rat aortic membranes. Rat aortic membranes were solubilized, and 400 μg of protein was subjected to immunoprecipitation with antisera raised against α_{1a}-, α_{1b}-, or α_{1d}-ARs (1:250 dilution). Aliquots of solubilized immunocomplexes representing 50 μg of the original solubilized membrane preparation were separated on 10% SDS-polyacrylamide gels; transferred onto nitrocellulose membranes; immunoblotted with specific α_{1a}-, α_{1b}-, or α_{1d}-AR antisera (0.25 μg/ml for α_{1a}, 1:1000 dilution for α_{1b} or α_{1d}); and detected by ECL. Data indicate there was no cross-reactivity among the three antisera tested. Furthermore, the data indicate that immunoprecipitation with each of the antiserum resulted in >90% recovery of the specific α_1-AR subtype compared with the signal obtained from 50 μg of solubilized aortic membranes (*lane C*). Immunoblots are representative of three individual experiments that yielded comparable results.

proteins by 370% and 350%, respectively (Fig. 6). These receptor stimulation-induced increases in receptor/G_{α} coupling were inhibited 70–80% by an equimolar concentration of the α_1-AR antagonist prazosin (Fig. 6). Thus, the data indicate that in aortic membranes, the α_{1a}- and α_{1d}-ARs are coupled to $G_{\alpha q}$ protein and the α_{1b}-AR subtype is linked to both $G_{\alpha q}$ and $G_{\alpha o}$ proteins.

Receptor-activated palmitoylation of G_{α} proteins. To further examine the coupling of α_1-AR to G_{α} proteins, PHE-stimulated palmitoylation of G_{α} proteins was assessed. Incubation of aortic membranes with PHE resulted in significant increases in [^{3}H]palmitoylation of $G_{\alpha q}$ and $G_{\alpha o}$ proteins (Fig. 7) without affecting $G_{\alpha s}$ and $G_{\alpha i}$ proteins. In addition, pretreatment of membranes with 1 μM of the α_1-AR antagonist prazosin blocked the PHE-stimulated incorporation of palmitic acid into $G_{\alpha q}$ and $G_{\alpha o}$ proteins by 76% and 73%, respectively (Fig. 7). These results therefore support the above data, which indicate that in aorta of 1-month-old Fischer 344 rats, α_1-ARs are coupled to both $G_{\alpha q}$ and $G_{\alpha o}$ proteins.

Discussion

Activation of PLC and increased intracellular Ca^{2+} are important elements of α_1-AR-mediated signaling, which result in the contractile response of vascular smooth muscle (20, 21). Despite considerable progress in elucidating the structure and signaling mechanisms of α_1-ARs, it is not clear which G protein or proteins are responsible for α_1-AR-mediated effects in blood vessels. Many G protein-coupled receptors were shown to activate PLC and increase the intracellular concentration of inositol-(1,4,5)-trisphosphate that eventually lead to vasoconstriction. Two α_1-AR-mediated pathways are known to activate PLC and to produce vascular contraction based on their sensitivity to PTX (5, 22). The α_{1a}, α_{1b}, and α_{1d} subtypes of the α_1-AR have been shown to activate PLC in transfected COS-7 cells via coupling to the PTX-insensitive G proteins $G_{q\alpha}/G_{11\alpha}$ (5). On the other hand, several studies have shown that PTX pretreatment only partially inhibits α_1-AR-mediated contraction in blood vessels (6, 7), implicating the existence of α_1-ARs that are linked to a PTX-sensitive G protein in vessels. $G_{\alpha o}$ protein was proposed to be that PTX-sensitive G protein based on the observation that α_{1b}-ARs, expressed in *Xenopus laevis* oocytes, use G_o protein in activating PLC-mediated Cl^- current (22, 23).

The current study confirms the observation that PTX partially inhibits both α_1-AR-elicited contraction and IP accumulation in the aorta and suggests that both PTX-sensitive and -insensitive G proteins are involved in α_1-AR-mediated signal transduction in vascular smooth muscle. To identify these G proteins, we coimmunoprecipitated α_1-ARs with their associated G proteins in the rat aorta. Specific antiserum directed against $G_{\alpha q}$ protein coimmunoprecipitated α_1-ARs that are specifically labeled by [^{125}I]HEAT, suggesting a linkage between α_1-AR and $G_{\alpha q}$ protein. Antiserum specific for $G_{\alpha o}$ also coimmunoprecipitated [^{125}I]HEAT binding sites, indicating that endogenous α_1-ARs in aorta membranes also couple to $G_{\alpha o}$. These data are therefore in agreement with previous studies that indicated functional coupling between α_1-ARs and $G_{\alpha q}$ in transfected cells (5) and between α_{1b}-AR and $G_{\alpha o}$ in *X. laevis* oocytes that express these proteins (22). In the current study, functional relevance

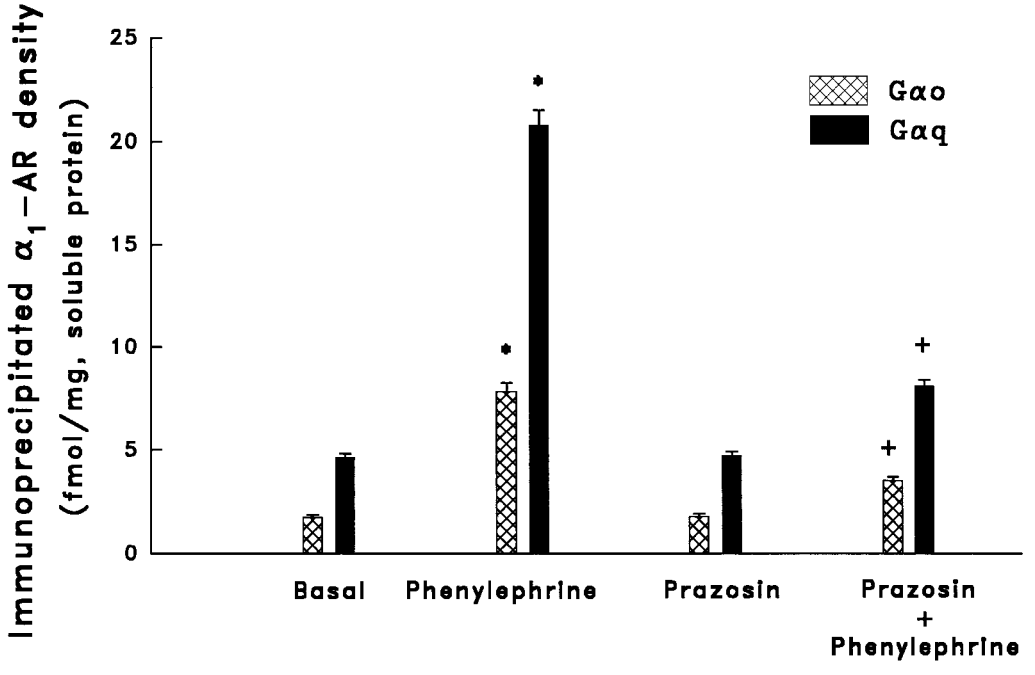

Fig. 6. Coupling of α_1-ARs to $G_{\alpha o}$ and $G_{\alpha q}$ in PHE-stimulated aortic membranes from 1-month-old rats. Aortic membranes were incubated with buffer or 1 μM PHE for 5 min. Tissues were solubilized, and membrane proteins were incubated with antisera directed against $G_{\alpha o}$ or $G_{\alpha q}$ proteins. Immuncomplexes were precipitated with protein A/Sepharose beads. In some experiments, aortic membranes were incubated with 1 μM concentrations of the selective α_1-AR antagonist prazosin for 5 min before the addition of buffer or PHE. The α_1-ARs in the immunoprecipitates were detected by measuring specific [^{125}I]HEAT binding. Value represent the mean ± standard error of five individual experiments. PHE stimulation caused 370% and 350% increases in coupling of $G_{\alpha q}$ and $G_{\alpha o}$ proteins to α_1-ARs. Prazosin inhibited the coupling of G_o and G_q to α_1-ARs by 70% and 80%, respectively. Statistical significance was determined by ANOVA followed by Newman-Keuls test for multiple comparisons. *, $p < 0.05$ compared with controls. +, $p < 0.05$ compared with PHE-induced response.

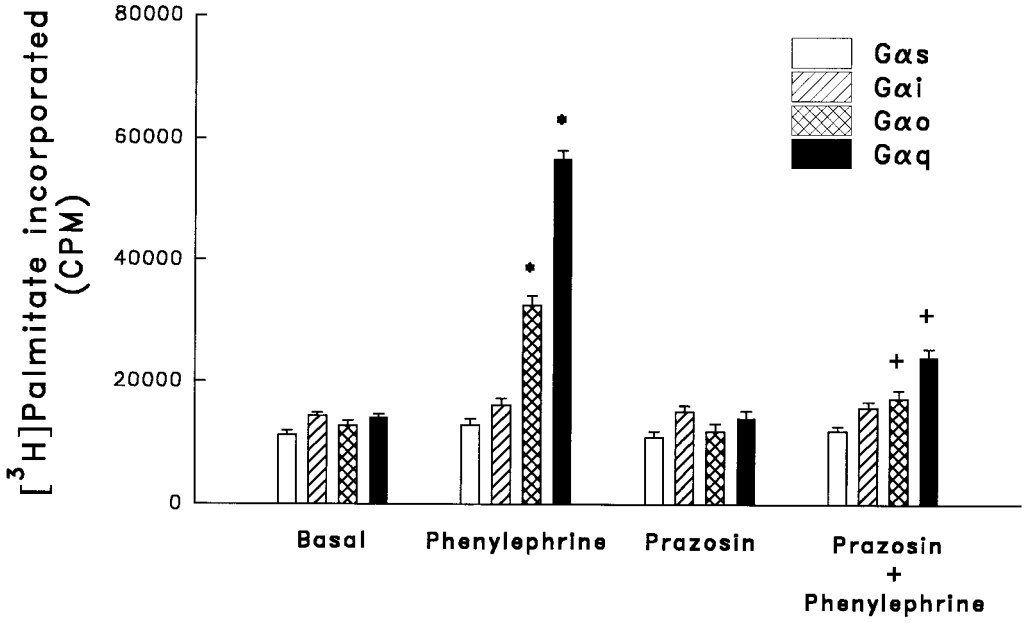

Fig. 7. Palmitoylation of $G_{\alpha q}$ and $G_{\alpha o}$ proteins in PHE-stimulated aortic membranes. Aortic membranes were incubated with 800 μCi/ml [9,10-^{3}H]palmitic acid, followed by stimulation with buffer or 1 μM PHE. In some experiments, aortic membranes were incubated with 1 μM concentration of the selective α_1-AR antagonist prazosin for 5 min before the addition of buffer or PHE. The membranes were solubilized and subjected to immunoprecipitation with antisera directed against a G protein α subunit (1:200 dilution). The radioactivity in the immunoprecipitates was measured. Data represent mean $\pm$ standard error of determinations obtained from four animals. PHE stimulation caused significant increases in the palmitoylation of $G_{\alpha q}$ and $G_{\alpha o}$ proteins as determined by ANOVA followed by Newman-Keuls test for multiple comparisons. $*$, $p < 0.05$ compared with controls. $+$, $p < 0.05$ compared with PHE-induced response.

of these linkages is also supported by the ability of the α_1-AR agonist PHE to increase the coupling of labeled receptors with both $G_{\alpha q}$ and $G_{\alpha o}$ proteins and to stimulate palmitoylation of $G_{\alpha q}$ and $G_{\alpha o}$ proteins in aortic membranes in a prazosin-sensitive manner. Palmitoylation of G protein α subunits is a reversible post-translational modification that is regulated by receptor stimulation (24–26). No linkage was detected between α_1-AR and G_s or G_i proteins using either coimmunoprecipitation or the palmitoylation approach. Thus, the data suggest that G_o, not G_i, is responsible for the PTX-sensitive responses to α_1-AR stimulation in aorta of the 1-month-old Fischer 344 rat.

It has been shown that rat aorta express three α_1-AR subtypes: α_{1a}-, α_{1b}-, and α_{1d}-ARs (1, 2). The expression level of these subtypes change with age, coincident with changes in the magnitude of aortic contraction (9, 10). This raises questions about possible differences in the functional roles of the α_1-AR subtypes. Although several studies have shown that the three subtypes are capable of stimulating the same signal transduction pathways when they are expressed in cultured cells (3, 4), data from the current study indicate that antisera directed against α_{1a}- and α_{1d}-ARs coimmunoprecipitated $G_{\alpha q}$ protein, whereas anti-α_{1b}-AR antiserum coimmunoprecipitated both $G_{\alpha q}$ and $G_{\alpha o}$ proteins. Furthermore, stimulation of α_1-AR with PHE elicited an increase in the coupling of α_1-ARs to both $G_{\alpha q}$ and $G_{\alpha o}$ proteins. Because G proteins determine the specificity and functional diversity of downstream intracellular effectors, identification of the interaction of a particular α_1-AR subtype with its G proteins represents an important step in unraveling the signal transduction cascades by which specific α_1-AR subtypes exert their effects.

In summary, the current results suggest that α_1-ARs are coupled to the PTX-insensitive $G_{\alpha q}$ and the PTX-sensitive $G_{\alpha o}$ in aorta derived from 1-month-old Fischer 344 rat. These G proteins therefore seem to couple to PLC and to mediate α_1-AR-stimulated contraction. It has been shown that the three subtypes of the α_1-AR activate PLC and cause increases in inositol trisphosphate level through $G_{\alpha q}/G_{\alpha 11}$ proteins (5). This and a previous study (22) demonstrate that α_{1b}-ARs stimulate PLC by coupling, in addition, to the PTX-sensitive $G_{\alpha o}$ protein. The ultimate definition of the coupling of α_1-AR subtypes with specific G_α proteins will help to understand the functional roles of the different α_1-AR subtypes. This study for the first time demonstrates the specific coupling between α_1-AR subtypes and G_α proteins in vascular smooth muscle membranes and implicates these G proteins in coupling of α_1-AR subtypes to the contractile response elicited by α_1-AR stimulation in 1-month-old Fischer 344 rat aorta.

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