Table 2. Association and dissociation kinetics for [$^3$H]NECA binding to $A_2$ receptors in the absence and presence of 700 nM PACPX

| Association conditions | $K_{obs}$ ($min^{-1}$) | $K_{+1}$ ($min^{-1}$ $nM^{-1}$) | Dissociation conditions | $T_{\frac{1}{2}}$ (min) | $K_{-1}$ ($min^{-1}$) |
|---|---|---|---|---|---|
| (a) [$^3$H]NECA | 0.053 ± 0.007 | 0.0045 | 5 μM 2-CADO | 20.2 ± 2.7 | 0.035 ± 0.004 |
| (b) [$^3$H]NECA + 700 nM PACPX | 0.047 ± 0.003 | 0.0035 | 5 μM 2-CADO | 21.3 ± 2.9 | 0.033 ± 0.004 |
| (c) [$^3$H]NECA | | | 5 μM 2-CADO + 700 nM PACPX | 21.2, 27.8 | 0.033, 0.025 |

Results are mean ± SD for 3–5 separate observations except in condition c where the results from two separate observations are given. Conditions a and b represent association and dissociation parameters from experiments in which 4 nM [$^3$H]NECA was incubated, in the presence and absence of 700 nM PACPX, with striatal membranes in the presence of 50 nM CPA to label $A_2$ receptors. Condition c represents the dissociation parameters of 4 nM [$^3$H]NECA when dissociation was initiated by the addition of 5 μM 2-CADO and 700 nM PACPX. Kinetic parameters were determined as described by Burt [19] where $K_{+1}$ = association rate, $K_{-1}$ = dissociation rate. $K_{obs}$ = observed association rate, and $T_{\frac{1}{2}}$ = dissociation half-life.

xanthines with adenosine receptors and gain further information as to the steric requirements for high affinity binding and for the reasons related to the pronounced interspecies differences previously reported [16, 20]. That xanthine interactions may not always be described in terms of a simple competitive interaction is suggested by the finding [22] that chronic caffeine treatment can attenuate *in vitro* responses to the xanthine, as measured by the ability of the xanthine to increase transmitter release, but not those associated with adenosine agonist effects.

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## Alpha$_1$-adrenergic receptor photoaffinity labeling in intact cells

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The molecular characterization of the $\alpha_1$-adrenergic receptor has been the focus of intense research for the past several years [1]. Photoaffinity labeling and purification of the receptor from rat hepatic membranes suggest that the major polypeptide representing the hormone binding subunit has a molecular weight in the range of 75,000–80,000 daltons [2–5]. The $\alpha_1$-adrenergic receptor in non-hepatic tissues appears to have a similar subunit molecular weight based on photoaffinity labeling of the receptor in membrane preparations [6–8]. To date, however, there have been no

reports in which photoaffinity labeling of the native $\alpha_1$-adrenergic receptor has been demonstrated, using either intact cells or whole tissues. In this report, we use the photoaffinity analog of prazosin, 2-[4-(4-azido-3-iodobenzoyl)piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline ([$^{125}$I]CP65,526), to identify and compare the $\alpha_1$-adrenergic receptor in intact DDT$_1$ MF-2 smooth muscle cells and smooth muscle cell membranes. Our data suggest that, in intact cells, the $\alpha_1$-adrenergic receptor may be heterogeneous with two subunits of 79,000 and 75,000 daltons.

*Materials and methods*

*Materials.* DDT$_1$ MF-2 smooth muscle cells were grown in culture to confluency as previously described [9]. Carrier-free Na[$^{125}$I] was purchased from Amersham, Inc. [$^3$H]Prazosin (82 Ci/mmol) was obtained from New England Nuclear, Inc. [$^{125}$I]CP65,526 (sp. act. 2175 Ci/mmol) was prepared by radioiodination of the prazosin analog 2-[4-(4-azido-3-iodobenzoyl)piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline (CP63,155) and purified as previously described [4]. Bovine pancreatic deoxyribonuclease I (DNase) and all other chemicals were obtained from the Sigma Chemical Co. Cell culture reagents were purchased from Biofluids, Inc. Low molecular weight proteins (14,000–95,000 daltons) for electrophoresis calibration were obtained from Pharmacia.

*DDT$_1$ MF-2 cell and membrane preparation.* DDT$_1$ MF-2 cells were harvested by centrifugation at 1200 rpm for 10 min and then were washed twice in 50 ml volumes of buffer A (150 mM NaCl, 15 mM $MgCl_2$, and 10 mM Tris–HCl, 2.5 mM EGTA*; pH 7.4). The final cell pellets were resuspended in 10–15 ml of buffer A, and cell counts were measured with a hemocytometer. Cell viability was determined by trypan blue exclusion. Only preparations that demonstrated greater than 90% viability were used in an assay. Plasma membranes from DDT$_1$ MF-2 cells were prepared as described by Cornett and Norris [9].

*Whole cell and membrane binding assay.* Equilibrium saturation binding experiments were done by incubating $5 \times 10^5$ DDT$_1$ MF-2 cells or 100 $\mu$g of cell membranes with increasing concentrations of [$^3$H]prazosin (0.1 to 6.0 nM) in the absence or presence of 1.0 $\mu$M prazosin for 30 min at 23°. Final assay volumes were 150–300 $\mu$l, and all assays were done in triplicate. The binding assays were terminated by placing the assay tubes in ice water for 10 min followed by the addition of 4 ml of ice-cold wash buffer (100 mM Tris–HCl, pH 7.4) and vacuum filtration through S&S glass fiber filters. The filters were washed with an additional $3 \times 4$ ml buffer and counted for trapped radioactivity in 10 ml of scintillation cocktail.

*Photoaffinity labeling of $\alpha_1$-adrenergic receptors.* DDT$_1$ MF-2 membranes (50–200 $\mu$g) in 100 $\mu$l buffer B (100 mM Tris–HCl, 5 mM EDTA, 1 mM $MgCl_2$, pH 7.4) were incubated with 25 $\mu$l of [$^{125}$I]CP65,526 (0.3 nM) and 25 $\mu$l of buffer or competing ligand for 45 min at 23° in the dark. The samples were photolyzed for 15 min with a hand held, long wave ultraviolet lamp (Ultraviolet Lamp, Inc.). Following photolysis, 1 ml of ice-cold buffer B containing 1 mM glutathione was added to each sample. The samples were centrifuged for 5 min in an Eppendorf Microfuge, and the supernatant fractions were discarded. The membrane pellets were resuspended in 100 $\mu$l of high sucrose quench buffer (30% sucrose, 0.45 M DTT, 6% SDS, 60 mM EDTA, and bromphenol blue) and allowed to solubilize overnight in the dark before SDS–PAGE analyses on 10% polyacrylamide gels [10].

Intact smooth muscle cells ($1 \times 10^6$ cells) in 400 $\mu$l buffer A were incubated with 50 $\mu$l [$^{125}$I]CP65,526 (0.3 nM final) and 50 $\mu$l of buffer or competing ligand for 10 min at 23° in the dark. The samples were photolyzed for 15 min with a long wave ultraviolet lamp, then 500 $\mu$l of ice-cold buffer A containing 1 mM glutathione was added, and the cells were pelleted for 1 min in an Eppendorf Microfuge. The cell pellets were lysed by resuspending in 15 $\mu$l of 10 mM $MgCl_2$ containing 10 $\mu$g of DNase followed directly by addition of 1 ml of distilled water with or without protease inhibitors [1 mM PMSF, 5 mM EGTA, 1 mM bacitracin, 10 $\mu$g/ml leupeptin, 10 $\mu$g/ml chymostatin, 20 $\mu$g/ml antipain, and 10 $\mu$g/ml soybean trypsin inhibitor (SBTI)]. The samples were incubated for 30 min at 37°, and the material from the lysed cells was pelleted by centrifugation for 5 min in the microfuge. The pelleted material was resuspended in 100 $\mu$l of high sucrose quench buffer and solubilized overnight at 23° before SDS–PAGE analysis. The 10% acrylamide gels were dried on a BioRad gel drier, and autoradiography was performed by exposing Kodak XAR-5 film on the dried gels at −70° for 3–9 days. Treating the whole cell lysates with DNase was necessary because nuclear material was found to interfere with SDS–PAGE. No difference in the photolabeling profile for membranes was observed when treated under the same conditions.

*Data analysis.* Radioligand binding data were evaluated with the mass-action based, weighted, nonlinear curve-fitting procedure LIGAND [11], on a Vax Digital computer system. Molecular weights of the photolabeled peptides were determined by linear regression analysis based on the mobility of protein standards electrophoresed simultaneously. The ability of prazosin and epinephrine to inhibit photoaffinity labeling of the $\alpha_1$-adrenergic receptor subunits in the autoradiographs was evaluated as follows. Autoradiographs of the polyacrylamide gels demonstrating the various peptides labeled with [$^{125}$I]CP65,526 in the absence or presence of prazosin or various concentrations of epinephrine were scanned using an LKB 22202 Laser Densitometer. The relative intensity of the individual bands was calculated from the integrated relative areas obtained using a Hewlett–Packard Integrator. Labeling in the absence of competing ligand was used to determine "total binding" and that in the presence of phentolamine ($10^{-5}$ M) was used to determine "non-specific binding". Labeling in the presence of competing ligands, normalized for "specific binding" (total − non-specific), was then used to evaluate specific "binding". Under the photolabeling conditions described above, 5–10% of the label was incorporated in the receptor bands based on the densiometric analysis of the autoradiographs.

*Results and discussion*

[$^3$H]Prazosin bound specifically and with high affinity to an apparently homogeneous population of $\alpha_1$-adrenergic receptors on intact DDT$_1$ MF-2 cells. The dissociation binding constant ($K_D$) of the receptor for [$^3$H]prazosin, calculated by Scatchard analyses of saturation binding experiments, was 0.14 ± 0.01 nM (N = 3) with a corresponding $B_{max}$ of 49,000 ± 3330 sites per cell (data not shown). A similar $K_D$ was calculated for [$^3$H]prazosin binding to $\alpha_1$-adrenergic receptors in DDT$_1$ membranes (0.12 ± 0.02 nM; N = 3).

Demonstration of [$^{125}$I]CP65,526 photoaffinity labeling of $\alpha_1$-adrenergic receptors in DDT$_1$ MF-2 membrane preparations (A) or whole DDT$_1$ MF-2 cells (B) is presented in Fig. 1. Specific labeling of two proteins bands with subunit molecular weights of approximately 75,000–80,000 daltons and 35,000–40,000 daltons was observed in membrane tissue preparations. Specificity of labeling was demonstrated by the absence of [$^{125}$I]CP65,526-labeled bands when DDT$_1$ MF-2 membranes were coincubated with 1 $\mu$M unlabeled prazosin before photolysis. The $\beta$-adrenergic receptor antagonist alprenolol (1 $\mu$M) did not inhibit labeling of either protein band. The 30,000–40,000 dalton protein

* Abbreviations: EGTA, ethyleneglycolbis (aminoethylether)tetra-acetate; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; and PMSF, phenylmethylsulfonyl fluoride.

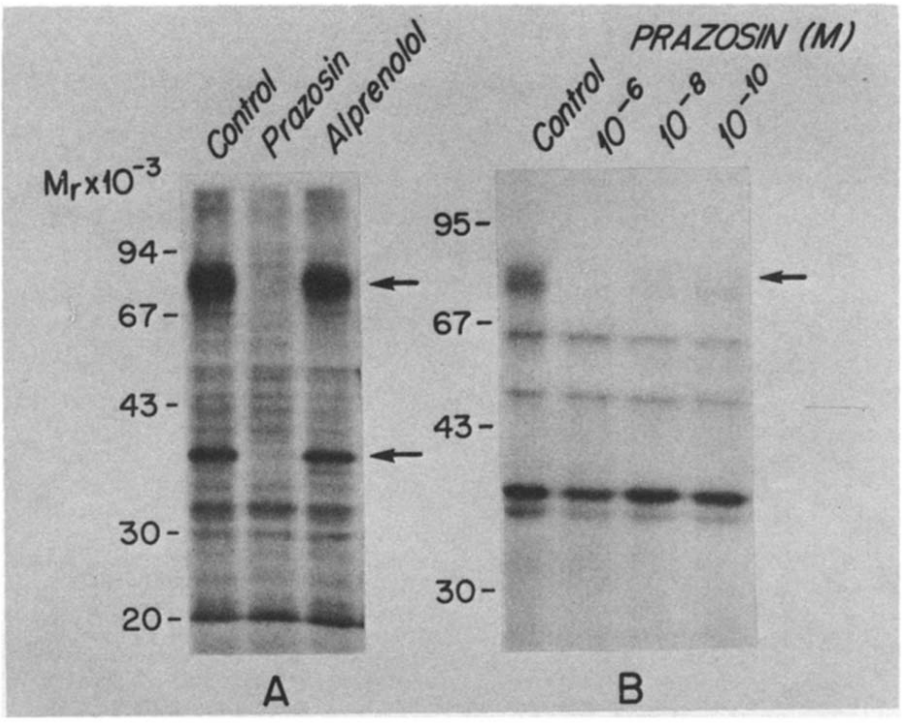


Fig. 1. Photoaffinity labeling of $\alpha_1$-adrenergic receptors in $DDT_1$ MF-2 membranes (A) and intact cells (B) with [$^{125}$I]CP65,526. $DDT_1$ MF-2 membranes were incubated with radioligand and either buffer, prazosin (1 $\mu$M) or alprenolol (1 $\mu$M), photolyzed, and then analyzed by SDS–PAGE as described in Materials and Methods. $DDT_1$ MF-2 cells were incubated with radioligand and either buffer or increasing concentrations of prazosin as indicated. The arrows refer to labeled bands between 75,000–80,000 $M_r$ and 35,000–40,000 $M_r$, which are specifically inhibited by prazosin but not by alprenolol. Molecular weights of known protein standards, electrophoresed simultaneously, are shown.

species was always apparent in membranes despite the use of multiple protease inhibitors of varying specificities (see Materials and Methods). The inhibition of [$^{125}$I]CP65,526 photoaffinity labeling of this peptide could be demonstrated only with prazosin and not with phentolamine or epinephrine (not shown), suggesting that it is not a proteolytic fragment of the receptor. It is of interest to note that a protein band of similar molecular weight purified from $DDT_1$ MF-2 membranes has been shown to be phosphorylated by the phorbol ester PMA [12].

SDS–PAGE analyses of $DDT_1$ MF-2 whole cell photoaffinity labeling with [$^{125}$I]CP65,526 demonstrate that two distinct bands having subunit molecular weights of 79,000 ± 1900 daltons and 75,000 ± 1800 daltons (N = 7) were labeled (Fig. 1B). The labeling of both bands was blocked dose dependently by prazosin. The non-selective $\alpha$-adrenergic receptor antagonist phentolamine (1 $\mu$M) also inhibited [$^{125}$I]CP65,526 receptor labeling; however, the $\beta$-adrenergic receptor antagonist propranolol (1 $\mu$M) did not (data not shown). Interestingly, there was no photoaffinity labeling of a 35,000–40,000 dalton protein species in $DDT_1$ MF-2 whole cells as was observed in membrane preparations (Fig. 1A).

The photoaffinity labeling of two bands between 75,000 and 80,000 daltons on intact $DDT_1$ MF-2 cells was supported by analyses of autoradiograms using laser densitometry (Fig. 2). A biphasic densitometry tracing was observed for labeled receptor on intact cells (top) compared to a monophasic (Gaussian) curve, which was observed for labeled receptor in membranes (bottom). Inhibition of photolabeling by prazosin (1 $\mu$M) is also shown in the tracings.

The $\alpha_1$-adrenergic receptor agonist epinephrine blocked labeling of both bands in a stereoselective manner. Figure 3 demonstrates that the labeling of both receptor bands in whole cells was inhibited by (−)epinephrine (lanes 4 and 5) to a greater degree than by (+)-epinephrine (lanes 6 and 7). This effect is more clearly shown in Table 1, which demonstrates inhibition of receptor labeling by prazosin and epinephrine based on laser densitometric analysis of the lanes in Fig. 3. At each concentration of epinephrine tested ($10^{-4}$ M, $10^{-6}$ M), labeling of both bands in the presence of the (−)stereoisomer was decreased compared to the labeling in the presence of the (+)stereoisomer. Dose-dependent inhibition of the labeling of both bands by prazosin was also evident.

Several groups have reported the labeling of multiple peptides of the $\alpha_1$-adrenergic receptor in hepatic membrane preparations. Kunos *et al.* [2] identified a major 80,000 dalton band as well as a minor 58,000 subunit. The labeling of three subunits having molecular weights of 80,000, 68,000, and 58,000 daltons was subsequently demonstrated by Seidman *et al.* [4]. Leeb-Lundberg *et al.* [3] have demonstrated labeling of the $\alpha_1$-adrenergic receptor in the variety of mammalian tissues. The receptor subunit molecular weight for the major protein species labeled in their studies ranged from 78,000 to 85,000 daltons. Minor specifically labeled peptides of lower molecular weight (35,000–40,000 daltons) were observed in membranes from rat liver and spleen as well as in rabbit spleen. Terman and Insel [8] reported labeling of the $\alpha_1$-adrenergic receptor in membranes prepared from rat heart ventricle and rat heart ventricular myocytes and observed that, in rat ventricular membranes, two bands of 77,000 and 68,000 daltons were

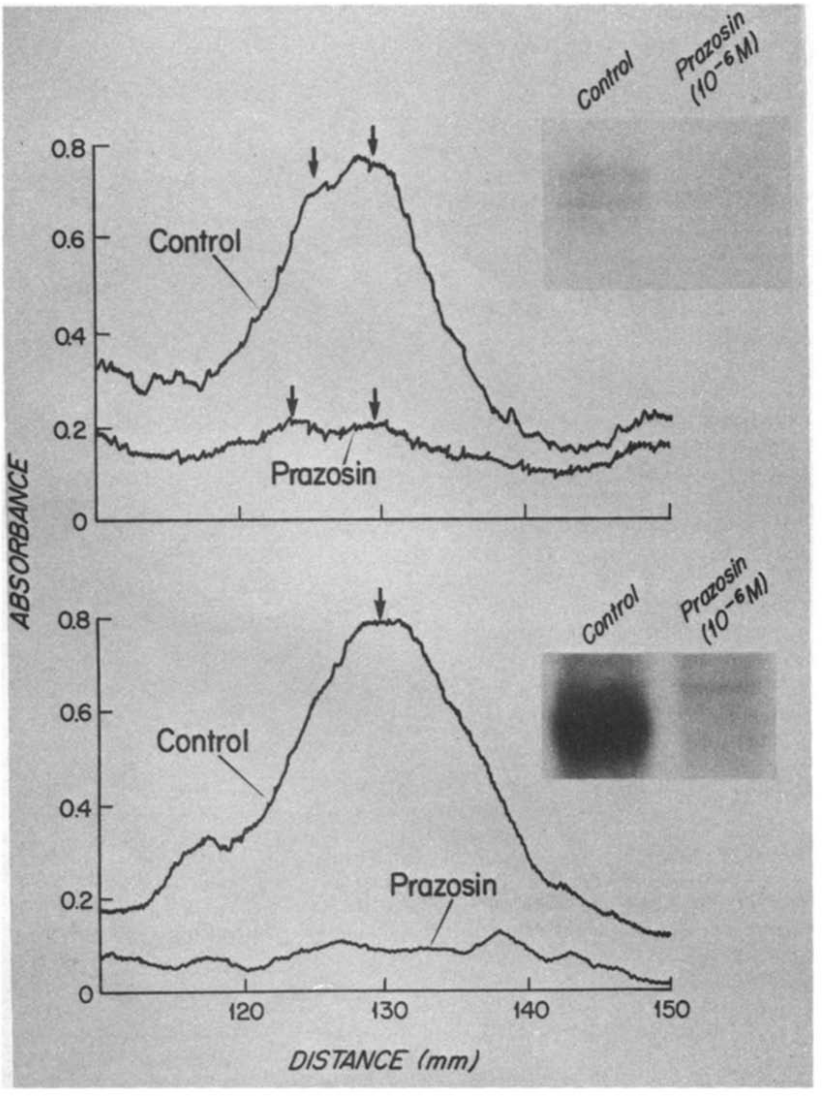


Fig. 2. Laser densitometric analysis of photolabeled $\alpha_1$-adrenergic receptor from whole $DDT_1$ MF-2 cells (top) or membranes (bottom). Representative autoradiograms of receptor labeling in cells and membranes were scanned using an LKB Scanning Laser Densitometer in the 70,000–85,000 dalton region. Control and prazosin (1 $\mu$M) lanes are shown in the insets of the figure. Densitometry tracings of each lane are presented and indicate the presence of two labeled peptides in whole cells (arrows), which were not labeled in the presence of prazosin. Only one band was observed in membrane preparations.

Table 1. Inhibition of $\alpha_1$-adrenergic receptor subunit photoaffinity labeling by [$^{125}$I]CP65,526 in intact $DDT_1$ MF-2 cells

| | Area under the curve | | [$^{125}$I]CP65,526 labeling (% control) | |
|---|---|---|---|---|
| | Band 1 | Band 2 | Band 1 | Band 2 |
| Control | 1.59 | 2.10 | 100 | 100 |
| Prazosin (1 $\mu$M) | 0.47 | 0.41 | 18 | 10 |
| Prazosin (0.01 $\mu$M) | 0.89 | 0.74 | 44 | 26 |
| (−)Epinephrine (100 $\mu$M) | 0.39 | 0.29 | 13 | 4 |
| (−)Epinephrine (1 $\mu$M) | 0.87 | 0.67 | 43 | 23 |
| (+)Epinephrine (100 $\mu$M) | 0.57 | 0.57 | 24 | 18 |
| (+)Epinephrine (1 $\mu$M) | 1.27 | 1.62 | 68 | 68 |

Each lane in Fig. 3 was analyzed using an LKB Scanning Laser Densitometer as described in Materials and Methods. Total binding is defined as the area under the curve (arbitrary units) for band 1 (79,000 daltons) and band 2 (75,000 daltons) respectively, in the absence of competing ligand (control). Phentolamine (10 $\mu$M) was used to define non-specific incorporation.

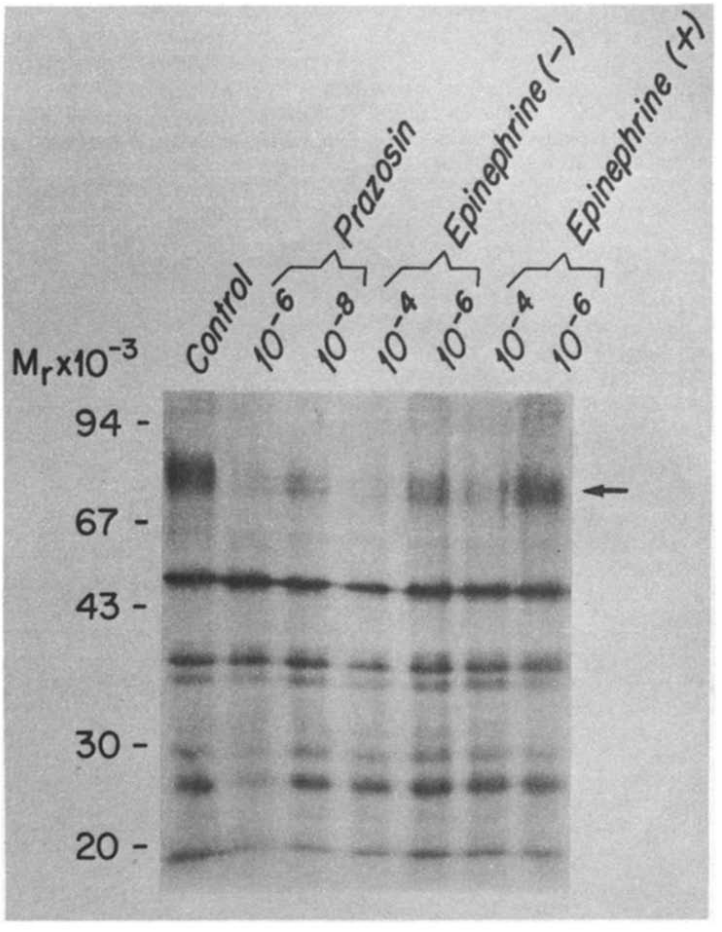


Fig. 3. Photoaffinity labeling of $\alpha_1$-adrenergic receptors on intact $DDT_1$ MF-2 cells with [$^{125}$I]CP65,526. $DDT_1$ MF-2 cells ($1 \times 10^6$ cells/lane) were incubated with radioligand and either buffer (control), prazosin (0.01 and 1 $\mu$M), (−)epinephrine (1 and 100 $\mu$M) or (+)epinephrine (1 and 100 $\mu$M), photolyzed, and then analyzed by SDS–PAGE as described in Materials and Methods. The closed arrow indicates the presence of two specifically labeled bands at a molecular weight of 79,000 daltons and 75,000 daltons. Molecular weights of known protein standards, electrophoresed stimultaneously, are shown.

specifically photolabeled. Only the 68,000 dalton peptide, however, was photoaffinity labeled in membranes prepared from collagenase-isolated myocytes. These authors speculated that proteolytic activity was responsible for the heterogeneity of photoaffinity labeling observed in cardiac tissue.

All of the preceding studies have been accomplished using membrane receptor preparations. To date there have been no studies in which photoaffinity labeling of the $\alpha_1$-adrenergic receptor has been described using intact cells or whole tissues. The utility of receptor photolabeling in intact cells is 2-fold. First, proteolysis, which can produce multiple peptide species, should be less significant since cell disruption occurs immediately prior to solubilization in SDS. Second, the entire receptor population should be more accurately represented when compared to membrane preparations where extensive isolation protocols are employed, potentially resulting in segregation of a particular subset of receptors.

Using this approach, we have compared the photoaffinity labeling patterns of the $\alpha_1$-adrenergic receptor in $DDT_1$ MF-2 smooth muscle cells and cell membranes. Our results suggest that the native $\alpha_1$-adrenergic receptor in whole $DDT_1$ MF-2 cells may be composed of multiple subunits of similar molecular weight. The heterogeneity observed in the $DDT_1$ MF-2 whole cell labeling of the $\alpha_1$-adrenergic receptor reported here is not likely to be due to proteolysis since inclusion of protease inhibitors in the DNase cell lysing solution used to treat the smooth muscle cell does not alter the whole cell labeling pattern observed when compared to experiments performed without protease inhibitors.

The difference in molecular weight between the receptor subunits may reflect different glycosylation states of the receptor [13] as has been demonstrated for the insulin receptor [14]. Alternatively, other forms of covalent modification, such as phosphorylation, may be responsible. In particular, if phosphorylation of the receptor results in two species, then dephosphorylation of the protein during the membrane isolation steps could result in the differences observed between photolabeling studies of intact cells and cell membranes. Studies aimed at addressing these possibilities are currently in progress.

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