Identification of the Prostacyclin Receptor by Radiation Inactivation*

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Evidence has been obtained for a specific protein receptor for prostacyclin on cells of the NCB-20 somatic hybrid. A new stable prostacyclin analog, 5-[(E)-15,55,6R,7R]-7-hydroxy-6-[1(E)-3S,4RS]-3-hydroxy-4-methyl-1-octene-6-ylidene)bicyclo[3.3.0]-octan-3-ylidenepentanoic acid (Iloprost, ZK36374) activates adenylate cyclase of NCB-20 cell membranes to an extent similar to prostacyclin and with a comparable high affinity. The binding of \[^{3}H\]Iloprost to NCB-20 membranes was rapid with an association rate constant (k\(_{a}\)) of 2.01 \(\times\) 10\(^8\) M\(^{-1}\) s\(^{-1}\) at 20°C. The rate constant for the dissociation of the ligand-receptor complex (k\(_{d}\)) was 1.19 \(\times\) 10\(^{-9}\) s\(^{-1}\), giving a dissociation constant (k\(_{d}/k_{a}\)) of 5.9 nM. The equilibrium dissociation constant was 29.9 nM, and the membranes had a maximum binding capacity of 347 fmoles mg\(^{-1}\) protein.

Radiation inactivation has been employed to determine the molecular weights of the functional prostacyclin receptor and components of the adenylate cyclase system in the plasma membrane of the NCB-20 cells. Cell membranes were lyophilized prior to irradiation, which lead to the formation of high-molecular-weight aggregates. The aggregation was avoided, however, when membranes were prepared in an isotonic Tris-HCl buffer containing sucrose. Molecular weight values of 111,000 for the catalytic subunit of adenylate cyclase, 89,000 for the regulatory subunit, and 83,000 for the prostacyclin receptor were obtained. Loss of \[^{3}H\]Iloprost binding capacity after irradiation of lyophilized membranes yielded a molecular weight value (mean \(\pm\) S.E.) for the prostacyclin receptor of 82,800 \(\pm\) 12,900 \(n = 3\).

Prostacyclin (epoprostenol) is an unstable metabolite of PG\(_3\) endoperoxide (1) that activates adenylate cyclase (ATP pyrophosphate lyase (cyclizing) EC 4.6.1.1) in platelets (2, 3) and vascular smooth muscle (4). PG\(_3\) is a potent vasodilator (5-7) and inhibitor of platelet aggregation (1-3, 8). It is thought to have an important physiological role in the regulation of platelet aggregation after injury (9), and more speculative functions include protection against vascular disease (10). The expression of a single population of PG\(_3\) receptors has been demonstrated in NCB-20 cells (11), and adenylate cyclase activity in NCB-20 homogenates is increased 10- to 15-fold by PG\(_3\) (12). The NCB-20 cell line (13) was derived by fusion of N18TG2 mouse neuroblastoma cells (14) with fetal hamster brain cells.

It has not been possible to solubilize the PG\(_3\) receptor without loss of the capacity of the receptor to bind PG\(_3\). No molecular weight determinations have been made for the receptor, and its structure is unknown. The technique of radiation inactivation has been employed here to determine the molecular weight of the functional PG\(_3\) receptor within the plasma membrane of NCB-20 cells. This technique (for reviews see Refs. 15 and 16) has the advantage that membrane-bound receptors and enzymes need not be purified in order to determine their molecular weight. NCB-20 membranes were lyophilized and irradiated in the beam of a linear accelerator in order to examine the components of the PG\(_3\)-stimulated adenylate cyclase system.

Previous reports of radioligand binding to high affinity PG\(_3\) receptors have described experiments with \[^{3}H\]9PG\(_3\) (17, 18) and \[^{3}H\]11\(\beta\)-PG\(_3\) (11, 19), neither of which radioligands are currently available from the usual commercial sources. The synthesis of \[^{3}H\]11\(\beta\)-PG\(_3\) (20) is an exacting task, and the instability of the compound makes it unsuitable in many ways for radioligand binding. A new and stable PG\(_3\) analog, 5-[(E)-15,55,6R,7R]-7-hydroxy-6-[1(E)-3S,4RS]-3-hydroxy-4-methyl-1-octene-6-ylidene)bicyclo[3.3.0]-octan-3-ylidenepentanoic acid (Iloprost, ZK36374), has been employed in these studies to characterize and determine the molecular weight of the PG\(_3\) receptor.

**MATERIALS AND METHODS**

*Cell Culture—*Cells of the NCB-20 neuronal hybrid cell line were cultured in Dulbecco's modified Eagle's medium (Gibco Europe) containing either 10% (v/v) newborn calf serum (Gibco Europe) or 5% (v/v) newborn calf serum and 5% (v/v) fetal calf serum (Flow Laboratories). In addition the medium was supplemented with 1 \(\mu\)M aminophenol (Sigma London Chemical Co. Ltd.), 100 \(\mu\)M hypoxanthine (Sigma London Chemical Co. Ltd.), and 16 \(\mu\)M thymidine (Sigma London Chemical Co. Ltd.). The cells were maintained at 37°C in a humidified atmosphere of 10% CO\(_2\) in air and were harvested by agitation in Ca\(^{2+}\)- and Mg\(^{2+}\)-free Dulbecco's phosphate-buffered saline (Gibco Europe). Cell pellets were frozen at \(-80^\circ\)C until required.

*Preparation of Cell Membranes—*NCB-20 cells were suspended in 25 mM Tris-HCl buffer, pH 8.5, containing 0.29 M sucrose, and homogenized at 4°C with a tightly fitting Dounce homogenizer. Homogenates containing approximately 7.0 mg/ml of protein were used immediately or frozen at \(-80^\circ\)C until required. To prepare membranes, cell homogenates were centrifuged at 500 \(\times\) g for 20 min to remove undisrupted cells and nuclei. The membranes remained in suspension and were pelleted by centrifugation at 100,000 \(\times\) g for 20 min at 4°C. The membranes were washed 3 times by suspension in 50 mM Tris-HCl buffer, pH 8.5, and centrifugation was at 100,000 \(\times\) g. Finally the membranes were resuspended in 25 mM Tris-HCl buffer, pH 8.5, containing 0.29 M sucrose before lyophilization and irradiation, or for adenylate cyclase experiments. Membranes were resuspended in 50 mM Tris-HCl buffer, pH 7.4, for experiments to deter-
brane through GF/C glass fiber discs (Whatman). The filters were washed three times with the same ice-cold buffer and then dried. Water or buffer followed by lyophilization. Acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) activity was measured in NCB-containing approximately 1.5 mg of protein per tube and frozen at -80 °C prior to lyophilization. Three marker enzymes were used as molecular weight standards. Lysozyme (muramidase, EC 3.2.1.17) was measured (29) by the addition of 50 µl of 1.5 mg/ml NCB-20 membranes which had been prepared and lyophilized in 25 mM 20 membranes through GF/C glass fiber discs (Whatman). The filters were washed three times with the same ice-cold buffer and then dried under an infrared lamp for 1 h. The filters were counted in 10 ml of Tris-HCl buffer, pH 8.5, containing 0.29 M sucrose.

Activation of adenylate cyclase by prostaglandins is mediated by single receptor populations. The δ values (maximum enzyme activation) is given by the x intercept and was similar for all 3 prostaglandins (Iloprost = 15.7, PGF2α = 18.5, carbachol = 15.8 pmol of cAMP min⁻¹ mg⁻¹ protein). The results are shown with vertical lines indicating S.E. of triplicate determinations. The data are presented as an Eadie-Hofstee plot (B). Regression lines are shown with r values of 0.998, 0.984, and 0.985, respectively.

Activation of Adenylate Cyclase—A saturating concentration of Iloprost activated adenylate cyclase to an extent similar to PGI2, and its stable analog carbachol (Fig. 1). Each of the prostaglandins yielded linear Eadie–Hofstee plots (Fig. 1B) suggesting that activation of adenylate cyclase by these prostaglandins is mediated by single receptor populations. The δ values (maximum enzyme activation) is given by the x intercept and was similar for all 3 prostaglandins (Iloprost = 15.7, PGF2α = 18.5, carbachol = 15.8 pmol of cAMP min⁻¹ mg⁻¹ protein). The results are shown with vertical lines indicating S.E. of triplicate determinations. The data are presented as an Eadie–Hofstee plot (B). Regression lines are shown with r values of 0.998, 0.984, and 0.985, respectively.
mg⁻¹ protein). The $K_{in}$ values (concentration of prostaglandin required for half-maximum enzyme activation) were given by $-1/slope$ (Iloprost = 80.7 nM, PG1₂ = 61.4 nM, and carbacyclin = 175.4 nM).

**Binding of [³H]Iloprost to NCB-20 Membranes**—The binding at equilibrium of selected concentrations of [³H]Iloprost to NCB-20 membranes is shown in Fig. 2. Specific binding was saturable, and the Scatchard plot (Fig. 2B) showed that $B_{max}$ (maximum binding capacity of the membranes) was 347 fmol mg⁻¹ protein, and the equilibrium dissociation constant ($K_d$) was 29.9 nM.

The rate of association of 15 nM [³H]Iloprost to the PG1₂ receptor of the NCB-20 membranes was monitored at 20 °C (Fig. 3). At this concentration of ligand, nonspecific binding was 15% of total binding. A pseudo first-order plot of the data (Fig. 3B) had a slope (observed rate constant, $k_{obs}$) of 4.2 $\times$ $10^{-3}$ s⁻¹. The forward rate constant ($k_+$) was calculated to be 2.01 $\times$ $10^6$ M⁻¹ s⁻¹, from $k_+ = (k_{in} - k_{-1})/\text{[ligand]}$, where $k_-$ is the first-order rate constant for the dissociation of the ligand-receptor complex. The value of $k_-$ was determined by measurement of the rate of dissociation of the ligand from the receptor after addition of an excess (final concentration = 4 μM) of cold ligand (Fig. 4). A semi-log plot of the data (Fig. 4B) revealed a half-time ($t_{1/2}$) of 584.5 s, from which $k_{-1}$ was calculated to be 1.19 $\times$ $10^{-3}$ s⁻¹. The dissociation constant ($k_{-1}/k_+$) was calculated to be 5.9 nM.

The binding of [³H]Iloprost to NCB-20 membranes was inhibited by PGI₂, carbacyclin, PGE₂, and, to a lesser extent, other prostaglandins (Table I). The inhibition constants ($K_i$) for each of the prostaglandins were determined from the equation $K_i = IC_{50}/1 + ([PG]/K_d)$ where the IC₅₀ was the concentration of prostaglandin required to inhibit specific [³H]Iloprost binding by 50% and $K_d$ was the equilibrium dissociation constant of [³H]Iloprost determined previously.

**Radiation Inactivation—N-Acetyl-β-D-glucosaminidase and lysozyme were irradiated under two conditions. When the enzymes were dissolved in 50 mM Tris-HCl buffer, pH 8.5,
and lyophilized, the molecular weights determined by radiation inactivation were: lysozyme 16,000 \((r = 0.976)\) and N-acetyl-\(\beta\)-d-glucosaminidase 84,000 \((r = 0.997)\). In contrast, when the enzymes were dissolved in water and lyophilized the molecular weights obtained were much larger (lysozyme = 106,000 \((r = 0.987)\) and N-acetyl-\(\beta\)-d-glucosaminidase = 151,000 \((r = 0.995)\)).

Because of these results, membranes from the NCB-20 cells were suspended in 50 mM Tris-HCl buffer, pH 8.5, and lyophilized. After reconstitution the membranes retained 88.0% of their basal and PGIp-stimulated adenylate cyclase activity and 100% of their acetylcholinesterase activity. The molecular weight of the catalytic \((C)\) subunit of adenylate cyclase was obtained by measurement of the loss of basal enzyme activity with \(\text{Mn}^{2+}\) (5 mM) as cation. The molecular weight of the catalytic and regulatory \((N)\) complex (CN) was found from measurement of the loss of fluoride (10 mM)-stimulated adenylate cyclase activity with \(\text{Mg}^{2+}\) (5 mM) as cation. The molecular weight of the catalytic, regulatory, and receptor \((R)\) complex (CNR) was obtained from measurement of the loss of carbacyclin-stimulated adenylate cyclase activity \((10^{-4}\text{ M carbacyclin}) in the presence of GTP or GppNHp (10^{-4}\text{ M}) with \(\text{Mg}^{2+}\) as cation (Table II). The experiments yielded high molecular weights which were not in agreement with previous reports. Under these conditions, two target sizes were obtained for \(C\) of 166,000 and 842,000: CN yielded a molecular weight of 430,000 and CNR was 904,000. Similar results were obtained when membranes were prepared and lyophilized in a high salt buffer (50 mM Tris-HCl buffer, pH 8.5, containing 100 mM NaCl).

Experiments were designed to investigate further the effects of salt concentration and solute molarity on the target sizes of \(C\), \(CN\), and \(CNR\). When membranes were suspended in 25 mM Tris-HCl buffer, pH 8.5, containing 0.29 M sucrose and lyophilized, irradiation yielded much smaller molecular weights (Table II), all of which were single target sizes derived from simple exponential decay curves. The molecular weight of acetylcholinesterase found by irradiation of NCB-20 membranes under these conditions was 62,000 \((r = 0.997)\).

The binding was measured of \([3\text{H}]\text{Iloprost} to irradiated NCB-20 membranes prepared and lyophilized in the Tris-HCl buffer containing sucrose. In three separate experiments simple exponential decay curves were obtained, yielding a molecular weight value \((mean \pm S.E.) of 82,800 \pm 12,900. The results of one of these experiments are shown in Fig. 5.

**TABLE II**

| Substrate and ligands | Membranes prepared in 50 mM Tris-HCl, pH 8.5 | Membranes prepared in 25 mM Tris-HCl, pH 8.5, containing 0.29 M sucrose |
|-----------------------|---------------------------------------------|----------------------------------------------------------|
| \(\text{Mn}^{2+}\text{ATP}\) | 196,000 + 842,000 | 112,000 \((r = 0.981)\) |
| \(\text{Mg}^{2+}\text{ATP}, \text{F}^-\) | 430,000 \((r = 0.956)\) | 200,000 \((r = 0.972)\) |
| \(\text{Mg}^{2+}\text{ATP}, \text{carbacyclin, and GTP or GppNHp}\) | 904,000 \((r = 0.959)\) | 283,000 \((r = 0.980)\) |

**FIG. 5.** Radiation inactivation of the prostacyclin receptor. Lyophilized membranes were irradiated in triplicate at 4 °C and the surviving specific binding of 25 nM \([3\text{H}]\text{Iloprost} was measured in duplicate. \(B_0\) was the surviving specific binding of \([3\text{H}]\text{Iloprost, and B_o the original binding (r = 0.958) . Mean results are presented of triplicate samples (vertical lines indicate S.E.). Mrad, megarad.**

**DISCUSSION**

Direct evidence of a protein receptor for PGIp on NCB-20 cells has been presented. The receptor is sensitive to trypsin, and incubation of the membranes with 0.5 mg ml\(^{-1}\) trypsin for 20 min at 30 °C reduces specific binding of \([3\text{H}]\text{Iloprost to 24% of control values (data not presented). Iloprost is a novel ligand which activates adenylate cyclase to an extent similar to the natural ligand PGIp and another synthetic PGIp agonist, carbacyclin. Iloprost has previously been shown to mimic the biological activity of PGIp in its action on platelets and bovine coronary arteries (33-35). \([3\text{H}]\text{Iloprost binds to specific PGIp receptors on platelets (36), bovine coronary artery (37), and porcine aorta (38). The report of binding to porcine aorta differed from this and other studies using \([3\text{H}]\text{Iloprost or \([3\text{H}]\text{PGIp, (11, 17-19) in that the data presented were interpreted as indicative of positive cooperativity. \([3\text{H}]\text{Iloprost has been employed in these studies and is well suited as a radioligand because of its relative stability compared to PGIp and its low nonspecific binding. The maximum binding capacity of \([3\text{H}]\text{Iloprost in NCB-20 membranes is similar to the maximum \([3\text{H}]\text{PGIp binding (11), and the rank order of potency of selected prostaglandins in displacing bound \([3\text{H}]\text{Iloprost (Table I) and \([3\text{H}]\text{PGIp is identical (11). These results correlate well with the order of potency of each prostaglandin for adenylate cyclase activation (12).**

To date, no reports have been published of successful solubilization of the PGIp receptor with retained capacity of the receptor to bind PGIp. However, previous studies have suggested the presence of high-molecular-weight receptors for other prostaglandins. Rat liver plasma membranes have been prelabeled with \([3\text{H}]\text{PGE}_2, and solubilized in Triton X-100, indicating a molecular weight of 105,000 by gel filtration of the putative ligand-receptor complex (39). Similarly the PGF\(_{2\alpha}\) receptor of bovine corpora lutea has been prelabeled and solubilized (40) indicating a molecular weight of 107,000. In these studies, however, it was not possible to verify the presence of the solubilized receptor with certainty, as the capacity for ligand binding was lost. In addition, prostaglandins generally have high values for nonspecific binding, and hence prelabeling of the membranes may yield radioligand
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coupled to proteins other than the “specific” membrane receptors.

The physiochemical characteristics of many receptors have been examined after subspalling, for example the opiate (41), insulin (42), and α- and β-adrenergic (43, 44) receptors. Similar results have not been achieved with any prostaglandin receptor, which prompted the present study to examine the PGI₁ receptor in its native membrane environment by the technique of radiation inactivation. Preliminary results with marker enzymes indicated a need for buffering during lyophilization to avoid what seemed to be aggregation of the enzymes. The molecular weights obtained for the enzymes lyophilized in water were greater than the values reported previously, based on hydrodynamic and radiation methods (45-47). For this reason NCB-20 membranes were prepared for lyophilization and irradiation by suspension in Tris-HCl buffer. It has been suggested (48), in the case of acetylcholinesterase, that Tris may minimize the spread of electrons from one polypeptide chain to another during irradiation. Under these conditions the target size obtained would be that of the smallest functional unit. However, the irradiation of NCB-20 membranes in Tris-HCl buffer reported here yielded molecular weights of C and CN much greater than those determined from hydrodynamic studies (Table II).

Measurements by conventional methods of the GTP-sensitive catalytic subunit (CN) have yielded molecular weights between 160,000 and 220,000 from rat renal medulla (49), canine cerebral cortex (50), mature rat testis (51), and S49 lymphoma cells (52). In some of these reports smaller molecular weight subunits have also been described. A component of the regulatory subunit (N) was first identified as a 42,000 polypeptide labeled by GTP and cholera toxin (53). Subsequent measurement of the molecular weight of the complete N subunit yielded a value of approximately 130,000 in erythrocyte membranes (54) and S49 lymphoma cells (55). In radiation inactivation studies, C has been assigned a molecular weight of 150,000 in frozen and lyophilized hepatic membranes (56). The CN complex has yielded molecular weights of 230,000 in canine cerebral cortex (50), mature rat testis (51), and S49 lymphoma cells (52). In some of these reports smaller molecular weight of C from membranes prepared and lyophilized in Tris-HCl buffer reported here yielded molecular weights of 83,000 derived from the adenylate cyclase data (calculated as the difference between the molecular weights of CN and CN). The molecular weight of the PGI₁ receptor was also determined by measurement of the decline of [3H]Iloprost binding with increasing radiation doses (Fig. 5). Membranes were again prepared and lyophilized in Tris-HCl buffer containing sucrose. The target size (mean ± S.E.) was calculated to be 82,800 ± 12,900 (n = 3). This was in good agreement with the molecular weight of 83,000 derived from the adenylate cyclase data (calculated as the difference between the molecular weights of CN and CN).

All experiments in this study were performed on lyophilized samples which avoided the necessity of a temperature correction factor. In the presence of sucrose, the molecular weight values obtained for acetylcholinesterase, C, and N corresponded closely to results obtained by irradiation of frozen membranes or by hydrodynamic methods. In conclusion, the molecular weight of the PGI₁ receptor obtained by monitoring the loss of the [3H]Iloprost binding capacity correlated well with the result derived from the adenylate cyclase data. Furthermore, the results obtained for the molecular weights of acetylcholinesterase (a membrane-bound enzyme) and for the regulatory and catalytic subunits of adenylate cyclase were similar to those reported in the literature. These points all serve to increase the confidence with which the molecular weight of the PGI₁ receptor is presented. However, it should be noted that conditions that yielded the lowest molecular weights were selected as the preferred experimental method, and the remote possibility still exists that the high-molecular-weight aggregates may be the functional or active structures within the membrane.

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