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EVIDENCE FOR HIGH AFFINITY PROSTACYCLIN BINDING SITES IN VASCULAR TISSUE: RADIOLIGAND STUDIES WITH A CHEMICALLY STABLE ANALOGUE*

WERNER RÜCKER and KARSTEN SCHRÖR†
Pharmakologisches Institut der Universität Köln, Gleueler Str. 24, 5000 Köln 41, West Germany

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Abstract—Prostacyclin-specific binding sites are described in the muscularis of pig aorta using [^{3}H]ZK 36374, a chemically stable prostacyclin analogue, as radioligand. Under standard incubation conditions [300 μg membrane protein in 350 μl Tris buffer (50 mmoles/l., pH 7.4) containing 3 mM Ca^{2+} at 37° for 10 min] both association and dissociation were complete within 30 sec, thus not allowing the determination of association or dissociation rate constants. The Scatchard plot was upward convex, whereas the Hill plot was linear, having a slope of 1.9. The equilibrium dissociation constant (K_D) was 22.4 nmoles/l. and the specific binding was saturated at 360 fmoles [^{3}H]ZK 36374/mg protein. The reversibility of binding was demonstrated by displacement of bound ligand with ZK 36374, its 5-(*Z*)-stereoisomer (ZK 36375), PGI_2 and PGE_1, but not with $PGF_{2\alpha}$. The data suggest high affinity binding sites for ZK 36374 in the smooth muscle cells of pig aorta for which PGI_2 may be the physiological ligand. They also demonstrate a possible co-operativity with two molecules binding simultaneously to two interacting sites.

There are a number of data suggesting that the various effects of prostaglandins are receptor-mediated. This was first shown for rat adipocytes and later confirmed for other tissues, including thyroid, corpus luteum, erythrocytes and platelets [1]. However, using prostacyclin (PGI_2), specific binding sites were so far only demonstrated on platelets [2, 3], neuronal hybrid cells [4] and lung tissue [5]. A major problem in studying this compound is its instability in aqueous medium at pH 7.4 because of rapid hydrolysis into a biological inactive degradation product, 6-oxo-prostaglandin $F_{1\alpha}$.

ZK 36374 (Fig. 1) is a chemically stable prostacyclin analogue. The compound was found *in vitro* to be equipotent to PGI_2 with respect to antiaggregatory and vasodilatory activities [6, 7]. Therefore, this derivative offered the unique possibility to study PGI_2 binding by using the radio-labelled compound as a ligand. This technique was successfully used previously by others to demonstrate PGI_2 binding sites on whole vessel preparations [8].

In the present study we have investigated the binding characteristics of ZK 36374 to membranes derived from the muscularis of pig aorta.

MATERIALS AND METHODS

Substances. [7-^{3}H]ZK 36374 (sp. act. 7.7–8.5 Ci/mmole), unlabelled ZK 36374, its 5-(*Z*)-stereoisomer ZK 36375, prostaglandin E_1 (PGE_1) and PGI_2

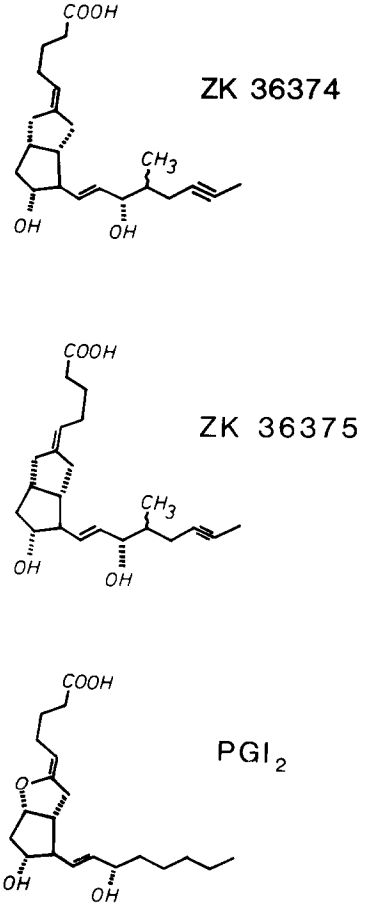

Fig. 1. Chemical structures of ZK 36374, ZK 36375 and prostacyclin.

* Part of these results was presented to the Fifth International Conference on Prostaglandins, Florence, May 1982.

† Address for correspondence: Dr. Karsten Schrör, Pharmakologisches Institut der Universität Köln, Gleueler Str. 24, 5000 Köln 41, West Germany.

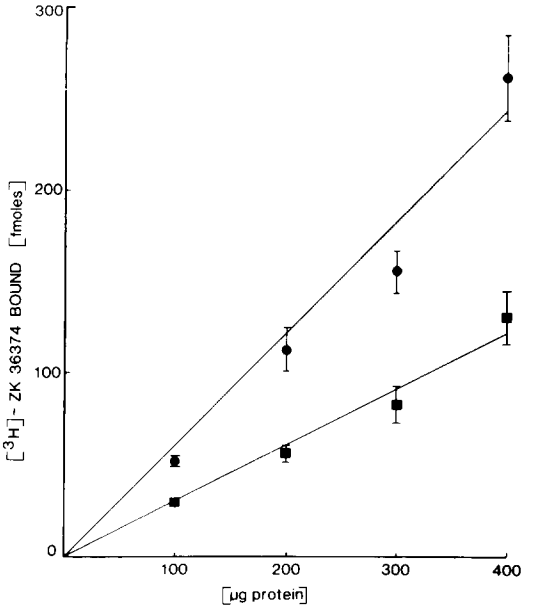

Fig. 2. Binding of [^{3}H]ZK 36374 (30 nmoles/l.) to pig aorta membranes in the absence (●) or presence (■) of 0.1 mmoles/l. ZK 36374: dependency on the protein content. Incubations were done in 50 mmoles/l. Tris buffer, pH 7.4, plus 3 mmoles/l. $CaCl_2$ at 37°. Each value is the mean ± S.E. of three determinations.

were a gift from Schering AG (Berlin/Bergkamen, West Germany). The chemical structures of the ZK compounds and PGI_2 are indicated in Fig. 1. The radiochemical purity of [^{3}H]ZK 36374 was >98% as determined by TLC (solvent system: methylene chloride–isopropanol, 9:1, v/v). Prostaglandin $F_{2\alpha}$ was obtained from Upjohn (Kalamazoo, U.S.A.). All other chemicals were purchased from available sources at analytical grade purity.

Membrane preparations. Fresh thoracic pig aortas were suspended in ice-cold oxygenated (95% O_2, 5% CO_2) Krebs–Henseleit buffer, dissected free from adventitia and intima and cut into small pieces. Microsomal membranes were isolated at 4° according to Dutta and Mustafa [9]. The media pieces were briefly homogenized by an Ultra-Turrax (IKA-Werk, Staufen, West Germany) for 20 sec in 8 vols. (w/v) of 5 mmoles/l. Tris buffer (pH 7.4) supplemented with 0.25 moles/l. sucrose. The homogenate was filtered through four layers of gauze cloth and centrifuged at 1000 *g* for 10 min to remove the cell debris. The supernatant was centrifuged again at 10,000 *g* for 10 min. From this supernatant the microsomal fraction was prepared by centrifugation at 105,000 *g* for 4 hr. The pellet was washed once with 50 mmoles/l. Tris buffer (pH 7.4) and finally resuspended in the same medium. According to Dutta and Mustafa [9], this fraction exhibits the highest sp.act. of 5'-nucleotidase, a marker enzyme for plasma membranes. The protein concentration was determined according to Lowry *et al.* [10] and adjusted to 3–5 mg/ml. Membranes were stored at −18° for up to 1 week without loss of specific binding capacity.

Binding assay. If not otherwise indicated, 300 µg membrane protein was incubated in 350 µl 50 mmoles/l. Tris buffer (pH 7.4) containing 3 mmoles/l. $CaCl_2$ and 30 nmoles/l. [^{3}H]ZK 36374 at 37° for 10 min. In time-course experiments incubation was done in a batch containing constituents in the same concentrations. A 300 µl aliquot of the incubation mixture was filtered through Whatman GF/B glass fibre filters (25 mm diameter, Whatman, U.K.) and washed twice with 5 ml ice-cold 50 mM Tris buffer (pH 7.4) to separate bound and free [^{3}H]ZK 36374. Filtration and washing were performed in less than 8 sec. The filters were immediately transferred to liquid scintillation vials, and 10 ml of scintillation fluid (Quickscint, Zinsser,

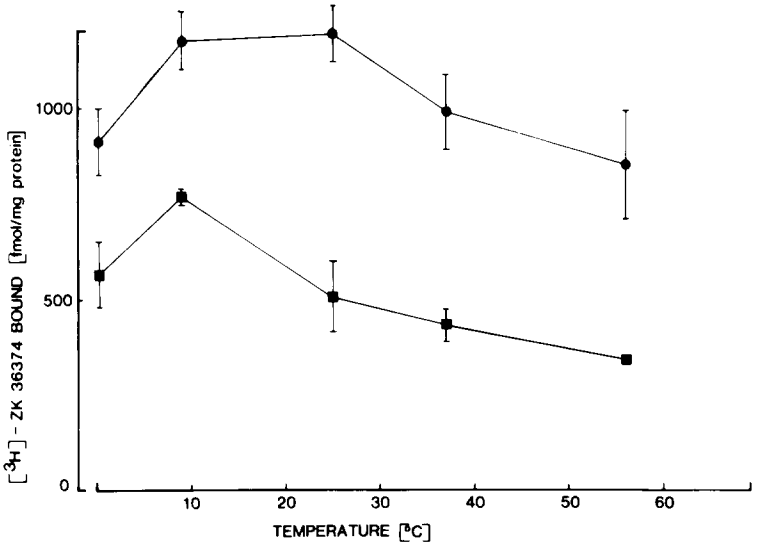

Fig. 3. Temperature dependency of total (●) and nonspecific (■) binding of 30 nmoles/l. [^{3}H]ZK 36374 to pig aorta membranes. Incubations were done in 50 mmoles/l. Tris buffer, pH 7.4, plus 1 mmole/l. $CaCl_2$ for 1 hr. Values are the mean ± S.E. of three determinations.

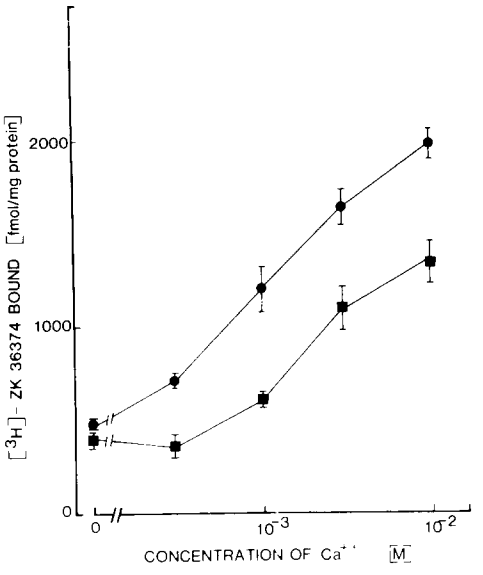

Fig. 4. Ca^{2+} dependency of [^{3}H]ZK 36374 binding (30 nmoles/l.) to pig aorta membranes in the absence (●) or presence (■) of 0.1 mmole/l. ZK 36374. Incubations were done in 50 mmoles/l. Tris buffer, pH 7.4, for 1 hr at 37°. Each value is the mean ± S.E. of three determinations.

Frankfurt, West Germany) was added. Before counting, the vials were kept at 4° for at least 6 hr to allow the filters to become translucent. Non-specific binding was determined in the presence of 100 μmoles/l. of unlabelled ZK 36374. Under these conditions, non-specific binding of 30 nmoles/l. [^{3}H]ZK 36374 was about 50% of the total radioactivity bound. Non-specific adsorption to the filters was about 0.3% of added radioactivity. [^{3}H]ZK 36374 was stable in the incubation mixture as determined by radiochromatography.

RESULTS AND DISCUSSION

Binding of [^{3}H]ZK 36374 was dependent on protein content (Fig. 2), having a broad temperature optimum (Fig. 3) and required bivalent cations, i.e. Ca^{2+} (Fig. 4) or Mg^{2+} (not shown) in a mmolar concentration range. In time-course experiments, specific binding of 10 and 30 nmoles/l. [^{3}H]ZK 36374 was complete within 30 sec (Fig. 5). Dissociation of [^{3}H]ZK 36374 binding, induced by a 50-fold dilution with the incubation buffer at 37° was also completed within this time (Fig. 6). The same fast dissociation of the specific binding was found in competition experiments with 100 μmoles/l. unlabelled ZK 36374 (not shown). Due to the high velocity of these reactions, no association or dissociation rate constants could be determined. Similar fast kinetics have been described by others with media membrane preparations from dog carotid arteries with [^{3}H]adenosine as a ligand [11] and for platelet membranes with [^{3}H]PGE_1 as a ligand [12].

Saturation experiments were done with increasing amounts of undiluted [^{3}H]ZK 36374. Plotting the concentration of the ligand against the specific binding per mg membrane protein, the resultant curve showed a sigmoid shape with one inflection point. The Scatchard plot was upward convex, whereas the Hill plot was linear with a slope of 1.9 (Fig. 7).

The calculated apparent equilibrium dissociation constant (K_D) was 22.4 nmoles/l., the specific binding was saturated by 360 fmoles [^{3}H]ZK 36374/mg membrane protein (B_{max} = 360 fmoles/mg protein). These data suggest a high affinity binding site, showing positive co-operativity with two molecules binding to one site.

The values for K_D and B_{max} compare favourably with values described for the high affinity binding sites for [^{3}H]PGI_2 on platelets, neuronal hybrid cells and lung tissue (Table 1). In contrast to our results, a positive co-operativity was not demonstrated in any of these other studies.

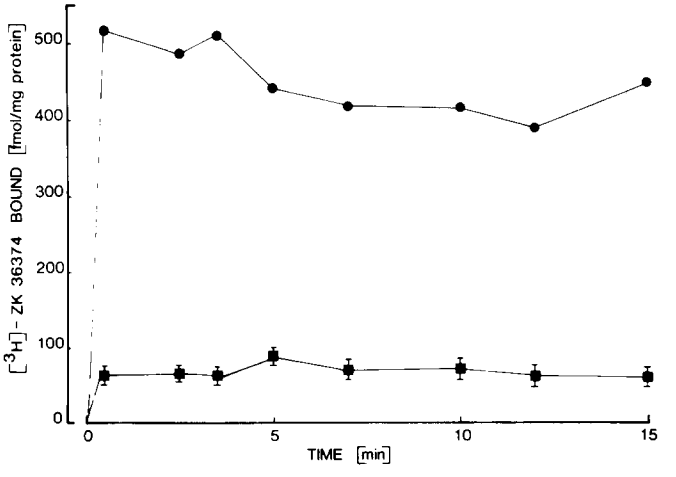

Fig. 5. Time-course of specific binding of [^{3}H]ZK 36374 to pig aorta membranes. Incubations were done in 50 mmoles/l. Tris buffer, pH 7.4, plus 3 mmoles/l. $CaCl_2$ at 37° in the presence of 10 nmoles/l. [^{3}H]ZK 36374 (■) or 30 nmoles/l. [^{3}H]ZK 36374 (●). Values for 10 nmoles/l. [^{3}H]ZK 36374 are the mean ± S.E. of six determinations. Values for 30 nmoles/l. [^{3}H]ZK 36374 are the mean of two determinations.

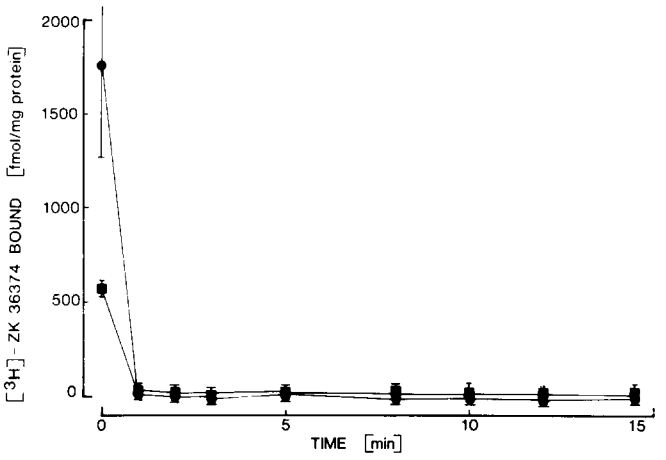

Fig. 6. Dissociation of total binding of [^{3}H]ZK 36374 to pig aorta membranes induced by 50-fold dilution. Preincubation was done in 50 mmoles/l. Tris buffer, pH 7.4, for 10 min at 37° plus $CaCl_2$. Each value is the mean ± S.E. of three determinations. (●) 75 nmoles/l. [^{3}H]ZK 36374, (■) 25 nmoles/l. [^{3}H]ZK 36374.

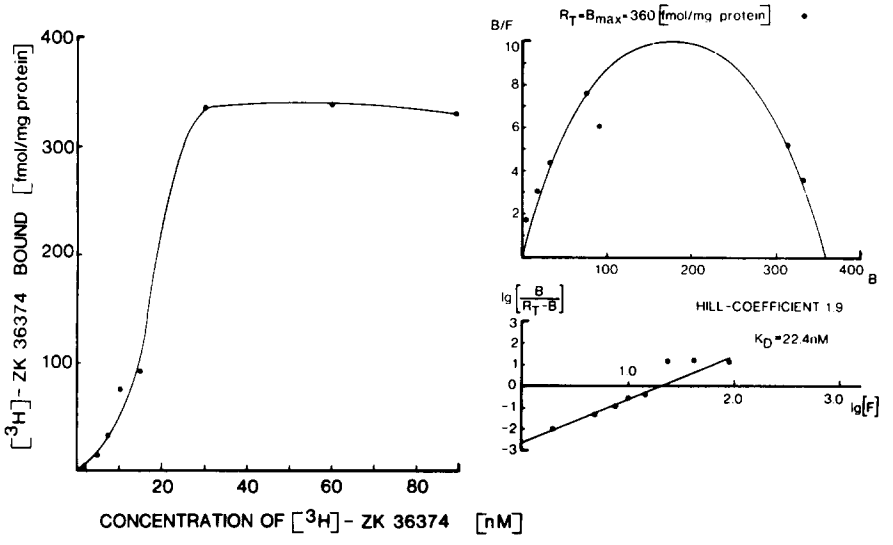

Fig. 7. Saturation of specific [^{3}H]ZK 36374 binding to pig aorta membranes. Incubations were done in 50 mmoles/l. Tris buffer, pH 7.4, plus 3 mmoles/l. $CaCl_2$ for 10 min at 37°. Each value is the mean of six determinations. Left graph, direct plot; upper right graph, Scatchard plot; lower right graph, Hill plot.

Table 1. Comparison of PGI_2-binding sites in different tissues

Tissue preparation	Ligand	K_D (nmoles/l.)	B_{max} (fmoles/mg protein)	References
Particulate fractions of bovine platelets	[^{3}H]PGI_2	35.0	360	Schillinger and Prior [3]
Intact human platelets	[^{3}H]PGI_2	12.1	—	Siegl *et al.* [2]
Membranes from NCB-20 neuronal hybrid cells	[^{3}H]PGI_2	16.6	1280	Blair and MacDermot [4]
Membranes from guinea-pig lungs	[^{3}H]PGI_2	16.0	105	MacDermot *et al.* [5]
Membranes from whole bovine coronary arteries	[^{3}H]ZK 36374	21.0	40	Town *et al.* [8]
Membranes from the muscularis of pig aorta	[^{3}H]ZK 36374	22.4	360	This study

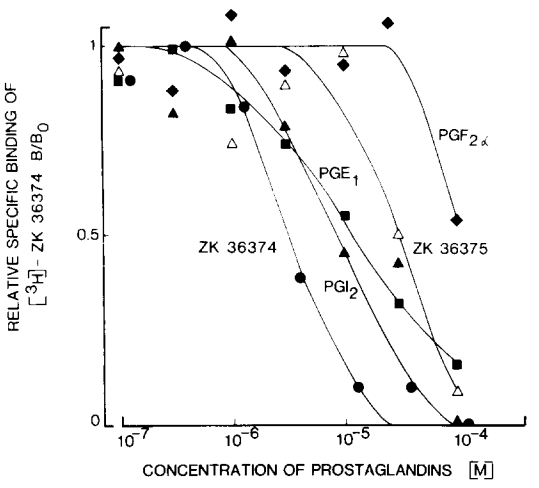

Fig. 8. Inhibition of specific binding of 30 nmoles/l. [^{3}H]ZK 36374 to pig aorta membranes by ZK 36374 (●), ZK 36375 (△). PGI_2 (▲), PGE_1 (■) and $PGF_{1\alpha}$ (◆). Incubations were done in 50 mmoles/l. Tris buffer, pH 7.4, plus 3 mmoles/l. $CaCl_2$ for 10 min (PGI_2 4 min) at 37° in the presence of 30 nmoles/l. [^{3}H]ZK 36374. Each value represents the mean of 3–6 determinations. *B*, [^{3}H]ZK 36374 bound in the presence of unlabelled prostanoids; B_0, [^{3}H]ZK 36374 bound in the absence of unlabelled prostanoids.

In competition experiments, unlabelled ZK 36374 exhibited an IC_{50} of 3.5 μmoles/l. The IC_{50} values for PGI_2, PGE_1 and the stereoisomer of ZK 36374, ZK 36375, were 8, 10 and 30 μmoles/l., respectively. ZK 36374, PGE_1 and ZK 36375 relaxed bovine coronary arteries [6, 13, 14], a specific feature of PGI_2-like compounds, in the same sequence of molar activity. In contrast, $PGF_{2\alpha}$, which contracts bovine coronary arteries [11] displaced [^{3}H]ZK 36374 to less than 50% even at a concentration of 100 μmoles/l. (Fig. 8). These data point to a specificity of the prostaglandin binding site, which apparently is associated with the biological actions of these prostanoids. It is also noteworthy that PGI_2, ZK 36374, PGE_1 and ZK 36375 inhibited the primary ADP-induced platelet aggregation in this sequence of molar activities ([13] and unpublished observations), whereas $PGF_{2\alpha}$ did not influence this process. Similar relative IC_{50}-values for ZK 36374, PGE_1 and $PGF_{2\alpha}$ were reported in binding studies on membranes from bovine coronary arteries using [^{3}H]ZK 36374 as ligand [8]. Unfortunately, the data of displacement experiments were only given in relative amounts setting ZK 36374 = 1. Therefore, no direct comparisons can be made with the absolute data indicated here.

Another major interesting finding in this study was the evidence for positive co-operativity, i.e. simultaneous binding of two ligand molecules to the same binding site. In binding studies there are numerous chances to produce artefacts that may be misread as positive co-operativity. This includes degradation of the ligand, different affinities for labelled and unlabelled ligand, measurement before obtaining an equilibrium state, misclassification of bound and free ligand, contamination of the labelled compound, dimerization of the ligand and binding of the ligand to a component other than the receptor [15]. Most of these possible errors were ruled out. For example, the radioligand was more than 98% pure and stable in the incubation mixture, as proved by radiochromatography, and saturation was studied only with buffer-diluted labelled compound. The equilibrium was complete within 30 sec. In addition, a dimerization of [^{3}H]ZK 36374 seems unlikely, since Town *et al.* [8], using the same ligand, did not find any positive co-operativity in their binding studies with membranes from the whole bovine coronary artery. Moreover, using our different technique, we were able to reproduce the finding of Town *et al.* [8] (data not shown). Certainly, binding to a compartment different from the binding site could not be excluded. Finally, separation time was appreciably short and in the range of centrifugation methods. However, the possibility of a disturbed binding equilibrium due to the separation should not be ignored.

In conclusion, our data demonstrate specific and saturable high affinity binding sites for ZK 36374 in a muscular membrane preparation of pig aorta. It is assumed that the natural ligand of these binding sites is PGI_2. Moreover, evidence is presented for a positive co-operativity, i.e. binding of two ligand molecules to one receptor unit. In biological terms, this would implicate that the cellular reaction subsequent to binding is not well graduated but rather tends to be an all or none response. The biological meaning of this kind of binding warrants further investigation.

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REFERENCES

1. F. A. Kuehl, Jr., in *Hormone–Receptor Interaction: Molecular Aspects* (Ed. G. S. Levey), pp. 385–400. Marcel Dekker, New York (1976).
2. A. M. Siegl, J. B. Smith and M. J. Silver, *J. clin. Invest.* **63**, 215 (1979).
3. E. Schillinger and G. Prior, *Biochem. Pharmac.* **29**, 2297 (1980).
4. I. A. Blair and J. MacDermot, *Br. J. Pharmac.* **72**, 435 (1981).
5. J. MacDermot, P. J. Barnes, K. A. Waddell, C. T. Dollery and I. A. Blair, *Eur. J. Pharmac.* **75**, 127 (1981).
6. K. Schrör, H. Darius, R. Matzky and R. Ohlendorf, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **316**, 252 (1981).
7. J. Casals-Stenzel, M. Haberey, O. Loge, W. F. Losert, G. Mannesmann, B. Radüchel, E. Schillinger, W. Skuballa, M. H. Town and H. Vorbrüggen, *Acta Therap.* **6** (Suppl.), 37 (1980).
8. M.-H. Town, E. Schillinger, A. Speckenbach and G. Prior, *Prostaglandins* **24**, 61 (1982).
9. P. Dutta and S. J. Mustafa, *J. Pharmac. exp. Ther.* **214**, 496 (1980).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).

11. W. Schütz and G. Brugger, *Pharmacology* **24**, 26 (1982).
12. A. I. Schafer, B. Cooper, D. O'Hara and R. I. Handin, *J. biol. Chem.* **254**, 2914 (1979).
13. K. Schrör, H. Darius, R. Ohlendorf, R. Matzky and W. Klaus, *J. Cardiovasc. Pharmac.* **4**, 554 (1982).
14. P. S. Kulkarni, R. Roberts and R. Needleman, *Prostaglandins* **12**, 337 (1976).
15. J. M. Boenaems and J. E. Dumont, *J. Cyclic Nucleotide Res.* **1**, 123 (1975).