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CHARACTERIZATION OF β-ADRENORECEPTORS OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) ERYTHROCYTES

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Summary

Although many studies have characterized these receptors according to pharmacological criteria, this work represents only the second direct characterization of the rainbow trout β-adrenergic receptors. Radioligand binding assays using (±)-4-(3-*t*-butylamino-2-hydroxy-propoxy)-[5,7-^{3}H]benzimidazol-2-one ([^{3}H]CGP 12177) and 1-[4,6-propyl ^{3}H]dihydroalprenolol ([^{3}H]DHA) were conducted to determine equilibrium binding times, ligand–receptor dissociation constants (K_D) and binding capacities (B_{max}). Furthermore, we assessed the influence of erythrocyte handling, suspension medium and endogenous catecholamines on B_{max} and K_D. Maximal binding was obtained when erythrocytes were handled minimally and maintained suspended in plasma rather than physiological saline. Washing and resuspending the erythrocytes, as well as the transfer of the erythrocytes into saline, significantly impaired apparent radioligand affinity and receptor density. Endogenous catecholamines, at levels considered normal for non-stressed animals, did not interfere with the radioligand binding assays, and thus eliminated the need to wash and resuspend erythrocytes. Based on the binding characteristics after intentional lysis of erythrocytes, it is shown that the total receptor population of trout erythrocytes can be estimated by propranolol-displaceable DHA binding, the density of high-affinity surface receptors can best be determined by isoproterenol-displaceable CGP binding, and the number of receptors located within the erythrocytes can be calculated from the difference between the total receptor density and the number of isoproterenol-displaceable DHA binding sites. Each of these components must be considered when performing radioreceptor assays using these radioligands and this has significant implications for the interpretation of erythrocyte β-adrenoreceptor localization and mobilization.

Introduction

In response to a variety of stresses, including hypercapnia (Perry *et al.* 1989),

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hypoxia (Fievet *et al.* 1987) and exhaustive exercise (Primmett *et al.* 1986), trout experience significant elevations in circulating catecholamine levels. One specific result of this catecholamine surge is an internal alkalization of the erythrocytes (Nikinmaa, 1982, 1986; Primmett *et al.* 1986). The consensus of many studies is that the erythrocyte alkalization is caused by a catecholamine-mediated activation of an erythrocyte surface Na^+/H^+ antiporter (Baroin *et al.* 1984; Cossins and Richardson, 1985). Activation of the β-adrenoreceptors results in an increased activity of adenylate cyclase with a subsequent rise in intracellular cyclic AMP concentration. It is the elevation in the level of this intracellular second messenger that modifies the Na^+/H^+ antiporter, thus triggering H^+ extrusion and erythrocyte alkalization (Baroin *et al.* 1984; Cossins and Richardson, 1985; Borgese *et al.* 1986; Nikinmaa, 1986; Nikinmaa and Tufts, 1989). Maintenance or actual elevation of trout erythrocyte pH (pHi) in the face of reductions in whole-blood pH (pHe) is well-documented and is thought to minimize reductions in arterial blood oxygen content caused by the Bohr and Root effects (see reviews by Nikinmaa and Tufts, 1989; Perry and Wood, 1989)

β-Adrenoreceptor numbers and affinities have been determined in a variety of vertebrate erythrocytes, including frog (Lefkowitz *et al.* 1974; Wessels *et al.* 1979), turkey (Hoffman *et al.* 1979), rat (Kaiser *et al.* 1978) and chicken (Marsh and Sweeney, 1989). Only two previous studies have attempted to characterize the β-adrenoreceptors of teleost erythrocytes. Bennett and Rankin (1985) established the presence of β-adrenoreceptors on European eel (*Anguilla anguilla* L.) erythrocytes using direct pharmacological techniques. However, based on the selection of their radioligand ([^{3}H]DHA), the authors were not able to determine erythrocyte β-adrenoreceptor localization, or whether the detectable β-adrenoreceptors were functionally coupled or uncoupled. The only other characterization of β-adrenoreceptor populations on fish erythrocytes was reported in a study of rainbow trout [*Oncorhynchus mykiss* (Walbaum)] and carp (*Cyprinus carpio* Linnaeus) by Marttila and Nikinmaa (1988). These authors examined the binding of two β-adrenoreceptor antagonists, (±)-4-(3-*t*-butylamino-2-hydroxy-propoxy)-[5,7-^{3}H]benzimidazol-2-one ([^{3}H]CGP) and 1-[4,6-propyl ^{3}H]dihydroalprenolol ([^{3}H]DHA), to intact erythrocytes and concluded that the extent to which fish generated a β-adrenergic response was directly related to the apparent number of β-adrenergic receptor sites.

Given the physiological importance of β-adrenergic erythrocyte Na^+/H^+ exchange, it is surprising that the literature contains only two reports that describe briefly the presence and the characteristics of fish erythrocyte β-adrenoreceptors. Thus, it was the objective of this study to characterize in detail the β-adrenoreceptor population of trout erythrocytes. In addition to the direct determination of receptor population density and ligand–receptor dissociation constant, this study was also designed to identify factors that might alter receptor quantification, including erythrocyte handling, endogenous catecholamine concentration and choice of radioligand.

Materials and methods

Experimental animals

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] of either sex weighing between 150 and 266 g were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario) and transported to the University of Ottawa. Fish were held in large fibreglass tanks (Living Stream; Toledo, Ohio) supplied with flowing, aerated and dechlorinated City of Ottawa tapwater. Fish were acclimated to these conditions for at least 4 weeks prior to experimentation. Water temperature in the holding facility varied between 12 and 15 °C. Photoperiod was kept constant at 12 h light:12 h dark. Fish were fed daily with commercial trout pellets (Purina Trout Chow) and tanks were syphoned after feeding to eliminate the build-up of organic material.

Animal preparation

Fish were anaesthetized in a 1:10 000 (w/v) solution of ethyl *m*-aminobenzoate (MS-222, Sigma) adjusted to pH 7.5 with sodium bicarbonate. Fish were then placed onto an operating table that permitted continuous retrograde irrigation of the gills with either the anaesthetic solution or fresh water. Dorsal aortic cannulae were implanted following standard techniques (Soivio *et al.* 1975) using flexible polyethylene tubing (Clay-Adams PE-50, i.d. 0.580 mm, o.d. 0.965 mm).

After surgery, fish were placed in black Perspex boxes (volume 3 l) supplied with continuously flowing, dechlorinated and aerated water. Fish were allowed at least 48 h to recover from surgery before experiments commenced. Dorsal aortic cannulae were flushed at least once daily with 0.2–0.3 ml of heparinized (10 i.u. ml^{-1} ammonium heparin) Cortland saline (Wolf, 1963).

Experimental protocol

Binding assay

The characterization of trout erythrocyte β-adrenoreceptors was accomplished by radioreceptor assay techniques (Titeler, 1981) using both a hydrophobic ligand, [^{3}H]DHA (specific activity 2.59–4.07 TBq $mmol^{-1}$; Amersham) and a hydrophilic ligand, [^{3}H]CGP 12177 (specific activity 1.26–1.70 TBq $mmol^{-1}$; Amersham) in a manner similar to that of Bennett and Rankin (1985) and Marttila and Nikinmaa (1988). Briefly, blood samples were taken from several animals and centrifuged for about 10 s at 12 000 *g*; the plasma was discarded and the erythrocytes were resuspended in Cortland saline (pH 7.8). The centrifugation and resuspension was repeated three times within 20 min to wash the cells. The washed cells were diluted to 20 % haematocrit in the saline for [^{3}H]DHA binding experiments and to 40 % haematocrit for [^{3}H]CGP binding experiments. The final erythrocyte suspensions were contained within heparinized (125 i.u. ml^{-1}) tonometer flasks placed on ice. Erythrocyte suspensions were frequently swirled prior to and during radioreceptor assays to prevent settling of the erythrocytes. The time course for [^{3}H]DHA and [^{3}H]CGP binding was determined using erythrocytes incubated with varying

concentrations of either radioligand (1, 10 and 100 nmol l^{-1} [^{3}H]DHA; 1 10 and 40 nmol l^{-1} [^{3}H]CGP) for increasing durations (2, 4, 8, 16, 32 and 64 min). All incubations were carried out in triplicate, at room temperature (19°C) in 10 mm×75 mm borosilicate culture tubes (Fisher).

Binding was initiated by adding 40 μl of suspended erythrocytes to 360 μl of Cortland saline into which the radioligand had been added in combination with either 10 μmol l^{-1} (±)-propranolol (Sigma) or 200 μmol l^{-1} (−)-isoproterenol (Sigma), or alone. Both propranolol, a β-adrenoreceptor antagonist, and isoproterenol, a β-adrenoreceptor agonist, compete with the radioligands for receptor binding. However, while propranolol competes with DHA and CGP for both apparent high- and low-affinity receptor sites, isoproterenol competes only for high-affinity receptor sites (André *et al.* 1981). The number of erythrocytes added to the incubation solution was determined by diluting 10 μl of erythrocyte suspension in 10 ml of saline then counting the erythrocytes present using a haemocytometer and taking into account the dilution and volume of erythrocyte suspension used. Additional samples were taken at this time for the determination of blood haemoglobin concentration ([Hb]) and haematocrit (Hct).

Incubations were terminated by repeated washings (four times) with 5 ml of ice-cold Cortland saline after the erythrocytes had been transferred to borosilicate filters (no. 32, Mandel Scientific) using a cell membrane harvester (Brandel 24R). This procedure lysed the erythrocytes. The filters were placed in glass liquid scintillation vials followed by the addition of 8 ml of fluor (ACS II, Amersham). Samples were then counted on an LKB Rackbeta (model 1214) with all counts corrected for quenching using an external standard technique. The maximal number of propranolol- or isoproterenol-displaceable binding sites (B_{max}, in disints min^{-1} $cell^{-1}$) at the chosen radioligand concentration was assessed to determine the time for receptor saturation.

The maximal number of propranolol- or isoproterenol-displaceable binding sites and their apparent dissociation constants (K_D) were determined using Scatchard plot analysis (Scatchard, 1949). Receptor density (or maximal number of specific binding sites) was then converted to and expressed on a receptor per erythrocyte basis by multiplying the maximal number of specific receptor sites (disints min^{-1} $cell^{-1}$) by the radioligand specific activity and Avogadro's number. These incubations were conducted as for the time course incubations except that the erythrocytes were exposed to increasing concentrations of either DHA (0–100 nmol l^{-1}) or CGP (0–40 nmol l^{-1}) in the absence or presence of propranolol and isoproterenol for a fixed incubation time based on the outcome of the time course experiments.

Receptor integrity

The elaborate and time-consuming procedure of erythrocyte washing and resuspension was performed to remove potentially elevated levels of endogenous catecholamines and other plasma factors, such as protein, that could interfere with the outcome of the binding assays. However, it was also possible that this handling

of the erythrocytes might have a direct effect on the numbers of erythrocyte receptors and their affinity for the radioligands. Therefore, to determine the impact of erythrocyte washing on the β-adrenoreceptor population of trout erythrocytes, the binding experiments were repeated with two significant modifications. Blood was sampled and pooled from a variety of fish as detailed previously; however, not all of this blood was repeatedly washed and resuspended in saline. One sample was placed directly into a chilled, heparinized tonometer flask (referred to as unwashed or plasma), while a second sample was centrifuged and handled as if the erythrocytes were to be washed and resuspended. These erythrocytes, referred to as 'sham'-washed, were resuspended in their own plasma following each centrifugation. The remainder of the blood was treated as previously outlined in the binding assay protocol and is referred to as washed erythrocytes. β-Adrenoreceptor binding assays were completed on all three treatment groups (washed, plasma and sham) concurrently, as outlined above.

Interference with the assay by endogenous catecholamines

To determine whether endogenous catecholamines interfere with the binding assay, blood was removed from additional animals and allowed to sit on ice for several hours in order to generate catecholamine-reduced whole blood. Samples of this blood were then placed in separate heparinized tonometer flasks. Specific concentrations of noradrenaline or adrenaline were added to the various flasks to yield a range of nominal concentrations of each catecholamine (0.1, 1.0, 10.0 nmol l^{-1}). The blood was mixed gently for 10 min, and the binding assays were then initiated. Any interference by either catecholamine would be observed as a reduction in the number of isoproterenol-displaceable binding sites at 2, 20 and 40 nmol l^{-1} [^{3}H]CGP.

β-Adrenoreceptor compartmentalization

It has been suggested that, owing to their lipophilic tendencies, [^{3}H]DHA and propranolol are more readily able to penetrate the cell membrane, and thus bind to receptors located primarily within the erythrocyte, than [^{3}H]CGP or isoproterenol. This could be used to investigate receptor compartmentalization and mobilization if it were shown to be an adequate interpretation of the binding data. To investigate this, binding assays were conducted using both whole blood (plasma erythrocytes) and whole blood in which the erythrocytes had been sonicated to remove the cell membrane, which we predicted would eliminate the differences between [^{3}H]CGP and [^{3}H]DHA binding. Erythrocytes were lysed using a pencil sonicator (Micro Ultrasonic Cell Disruptor, Kontes). As outlined in the binding assay protocol, samples of the sonicated whole blood were taken to determine erythrocyte Hb and protein concentrations and Hct, to ensure that the blood was adequately sonicated prior to completion of the binding assay.

Analytical procedures

Plasma catecholamine concentrations were measured by HPLC (Waters) using

electrochemical detection (Woodward, 1982) on a 200 μl plasma sample. Whole-blood or erythrocyte suspension haematocrit (Hct) was determined using heparinized microcapillary tubes spun for 10 min at 12 000 *g*; haemoglobin content was measured by a commercial spectophotometric assay (Sigma), and protein concentrations according to the procedure of Lowry *et al.* (1951).

Statistical analysis

All experiments were performed in triplicate with differences between mean values assessed by analysis of variance (ANOVA) followed by Fisher's LSD for multiple comparison, at a 95 % level of confidence, using a commercial statistical software package (Statview 512$^+$). All values have been reported as mean±S.E. (*N*)

Results

Saturation of specific [^{3}H]DHA and [^{3}H]CGP binding

Initial experiments were performed to establish a time at which both radiolabels reached an equilibrium with the erythrocyte receptors. Several concentrations of [^{3}H]CGP and [^{3}H]DHA were used. As illustrated in Fig. 1, [^{3}H]DHA reached equilibrium within 10 min (Fig. 1A), while [^{3}H]CGP consistently required just over 30 min to reach equilibrium at all concentrations (Fig. 1B). Thus, to ensure that all radioreceptor assays were conducted under equilibrium conditions, subsequent incubations were terminated after 45 min at room temperature.

Working with erythrocyte proteins trapped on borosilicate membranes was a concern with respect to reproducibility and efficiency of counting. In preliminary

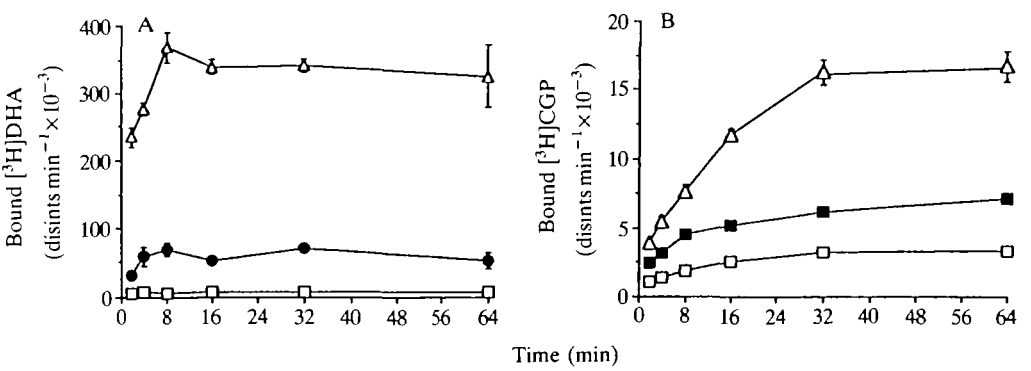

Fig. 1. Time course of [^{3}H]DHA (A) and [^{3}H]CGP (B) binding to trout erythrocytes. Erythrocytes were incubated for increasing durations in specific concentrations of [^{3}H]DHA (□, 1 nmol l^{-1}; ●, 10 nmol l^{-1}; △, 100 nmol l^{-1}) and [^{3}H]CGP (□, 1 nmol l^{-1}; ■, 10 nmol l^{-1}; △, 40 nmol l^{-1}) to establish the equilibrium time for the radioligand saturation kinetic experiments. Samples were prepared in triplicate. Values are mean±S.E.

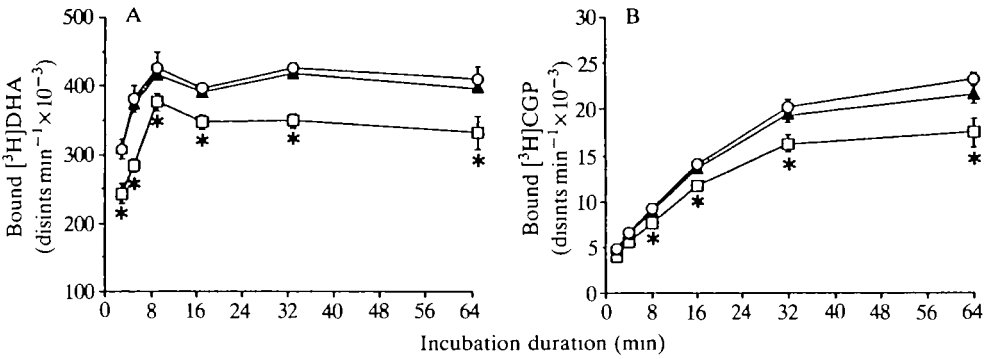

Fig. 2. Optimization of the counting of filter-bound membranes using liquid scintillation techniques. Samples obtained from radioligand time course experiments ([^{3}H]DHA, A; [^{3}H]CGP, B) were counted immediately (□) and after samples had been allowed to settle and mix with the scintillation cocktail for 24 h (▲) and 48 h (○). * indicates statistically significant differences when counts were compared to those in the 48 h samples. Samples were prepared in triplicate. Values are mean±1 S.E.

experiments it was noted that the distins min^{-1} of both [^{3}H]CGP and [^{3}H]DHA obtained from the borosilicate filter-bound erythrocyte proteins increased with the time that the filters stayed in the counting cocktail prior to counting (Fig. 2). Counts obtained immediately after the isolation of the membrane fragments for both radioligands were significantly lower than those measured after the filters had been in the fluor for 24 h (Fig. 2), presumably because of inadequate penetration of the scintillation cocktail into the borosilicate filters. However, no additional significant gain in radioactivity was obtained by allowing the samples to settle for a further 24 h.

The curves showing the total binding of either radioligand were composite, containing specific, saturable components and a non-specific component (Fig. 3). The saturation of the specific binding sites was obtained using the β-adrenoreceptor agonist isoproterenol and antagonist propranolol. As is evident in Fig. 3A, no apparent difference was observed in the binding of [^{3}H]CGP in the presence of either isoproterenol or propranolol. The saturation of the isoproterenol- and propranolol-displaceable binding sites occurred at low free CGP concentration, with this specific binding representing at least 40 % of the total CGP binding. The binding of DHA and the inhibition of DHA binding by either isoproterenol or propranolol occurred at a 10-fold greater concentration of DHA than was accomplished with CGP. Furthermore, unlike the specific CGP binding, the abilities of propranolol and isoproterenol to displace DHA from erythrocyte binding sites were significantly different (Fig. 3B). Although the propranolol-displaceable DHA binding represented approximately 90 % of the total DHA binding, the isoproterenol-displaceable DHA binding represented half the number of propranolol-displaceable site at the same concentration of DHA.

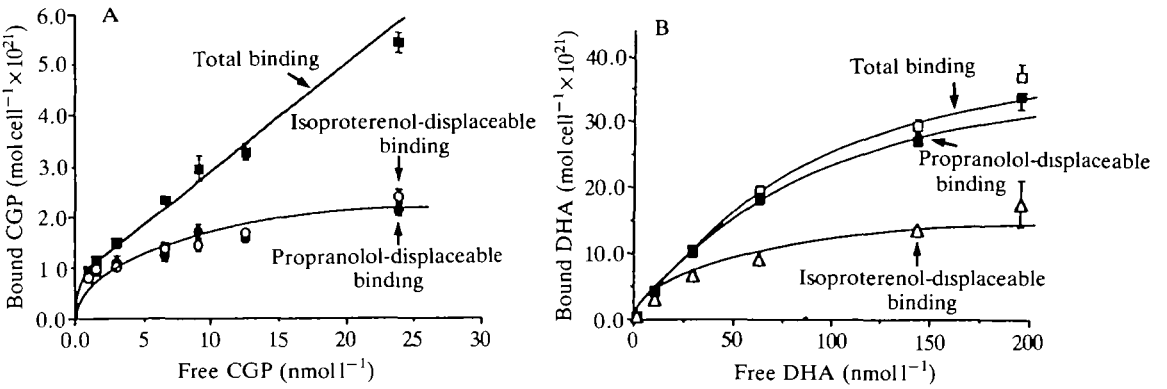

Fig. 3. An example of [^{3}H]CGP (A) and [^{3}H]DHA (B) binding to the erythrocytes of one rainbow trout. Erythrocytes were incubated in the presence of a radioligand alone, or in combination with either isoproterenol (10 μmol l^{-1}) or propranolol (200 μmol l^{-1}), for 45 min. Radioligand binding in the absence of antagonist represents total erythrocyte radioligand binding, whereas specific binding was obtained from the difference between the total radioligand binding and the radioligand binding in the presence of either isoproterenol or propranolol. Samples were prepared in triplicate. Values are mean ±1 S.E.

Linearization of the specific binding components of the CGP and DHA saturation curves emphasized the similarities and difference in the binding of these radioligands to trout erythrocytes (Fig. 4). Scatchard plot analysis (Fig. 4A) of the specific CGP binding shown in Fig. 3 revealed a single homogeneous population of receptors with the maximal number of binding sites (B_{max}) being 1681±74 per erythrocyte (N=3) and a dissociation constant (K_D) of 6.49±0.23 nmol l^{-1} (N=3) measured in the presence of 10 μmol l^{-1} isoproterenol. No significant difference was found in either receptor density or dissociation constant for CGP binding in the presence of 200 μmol l^{-1} propranolol (B_{max}=1461±82 receptors per erythrocyte, N=3; K_D=4.69±0.21 nmol l^{-1}, N=3) compared to these parameters determined using isoproterenol. The analysis of the specific DHA binding similarly revealed a single population of receptors; however, the use of both isoproterenol and propranolol clearly identified two distinct erythrocyte binding sites based on the maximal numbers of binding sites and on K_D values (Fig. 4B). The binding of DHA in the presence of propranolol identified 33 495±1384 receptors per erythrocyte (B_{max}, N=3) with a K_D value of 130.0±9.5 nmol l^{-1} (N=3), whereas this radioligand reveals nearly 60 % fewer binding sites (13 391±1084 receptors per erythrocytes) possessing a K_D value of 75.8±3.79 nmol l^{-1} (N=3) when in the presence of isoproterenol.

Receptor integrity

The receptor density (B_{max}) and binding affinity (K_D) of trout erythrocytes were significantly altered by handling and resuspension of the erythrocytes (Fig. 5). The handling of the erythrocytes (i.e. centrifugation and resuspension; sham) resulte

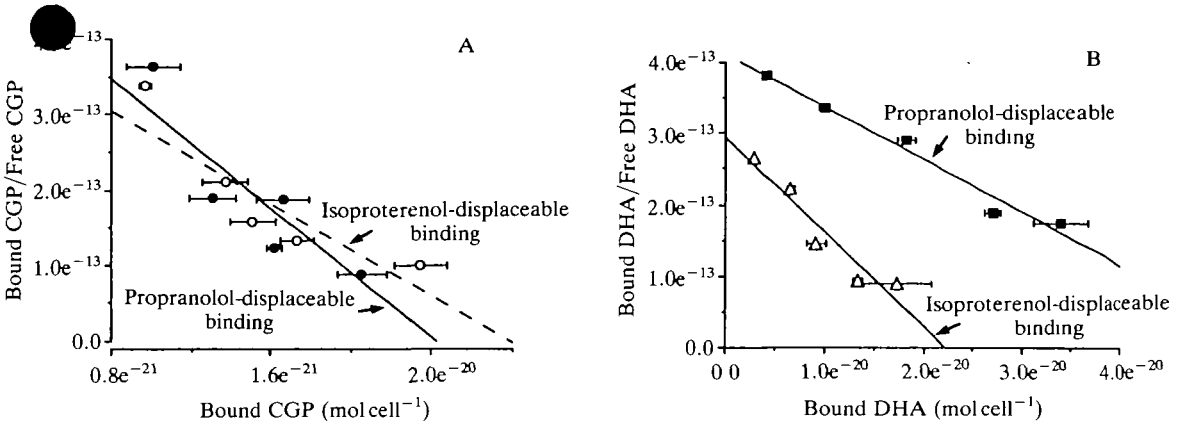

Fig. 4. Scatchard plots for isoproterenol- and propranolol-displaceable trout erythrocyte radioligand binding. (A) CGP Scatchard plot. Data taken from Fig. 3A. ○, isoproterenol-displaceable binding (dotted line; $y=2.15e^{-9}-1.54e^{8}x$, $r^2=0.781$;. $K_D=6.49\,nmol\,l^{-1}$, $B_{max}=1681$ receptors per cell); ●, propranolol-displaceable binding ($y=5.17e^{-13}-2.13e^{8}x$, $r^2=0.651$; $K_D=4.69\,nmol\,l^{-1}$, $B_{max}=1461$ receptors per cell). (B) DHA Scatchard plot. Data taken from Fig. 3B. ■, propranolol-displaceable binding ($y=4.11e^{-13}-7.40e^{6}x$, $r^2=0.972$; $K_D=130.0\,nmol\,l^{-1}$, $B_{max}=33\,495$ receptors per cell); △, isoproterenol-displaceable binding ($y=2.93e^{-13}-1.32e^{7}x$, $r^2=0.911$; $K_D=75.8\,nmol\,l^{-1}$, $B_{max}=13\,391$ receptors per cell). Values are mean±1 S.E., $N=3$.

in a 30% and a 77% increase in the K_D value for the propranolol- and isoproterenol-displaceable DHA binding sites, respectively (Fig. 5A). The same procedure, with the exception that the rinsing and resuspension of the erythrocytes was completed with, and in, Cortland saline (washed), resulted in a further 27% increase in the propranolol-displaceable DHA–receptor dissociation constant and a 122% increase in K_D value for the isoproterenol-displaceable DHA binding. Similar significant increases in the dissociation constant were noted when these experiments were completed using the alternative radioligand, CGP (Fig. 5C). However, it appeared that the centrifugation and resuspension alone resulted in significant increases in the K_D values for propranolol-displaceable (122%) and isoproterenol-displaceable (476%) CGP binding. No further effect on K_D was associated with washing and resuspension of the erythrocytes in saline, as the CGP K_D values were not significantly different from the sham treatment values.

The handling of the erythrocytes (i.e. centrifugation and resuspension; sham) was also associated with a 25% and an 11% reduction in the density of propranolol- and isoproterenol-displaceable DHA binding sites, respectively (Fig. 5B). The washing and resuspension of the erythrocytes in saline resulted in a further reduction in density of the two DHA receptor types of 70% (propranolol-displaceable) and 78% (isoproterenol-displaceable). The CGP binding sites underwent significant reductions in number associated with the sham and wash treatments (Fig. 5D), with the use of saline again having little or no effect beyond

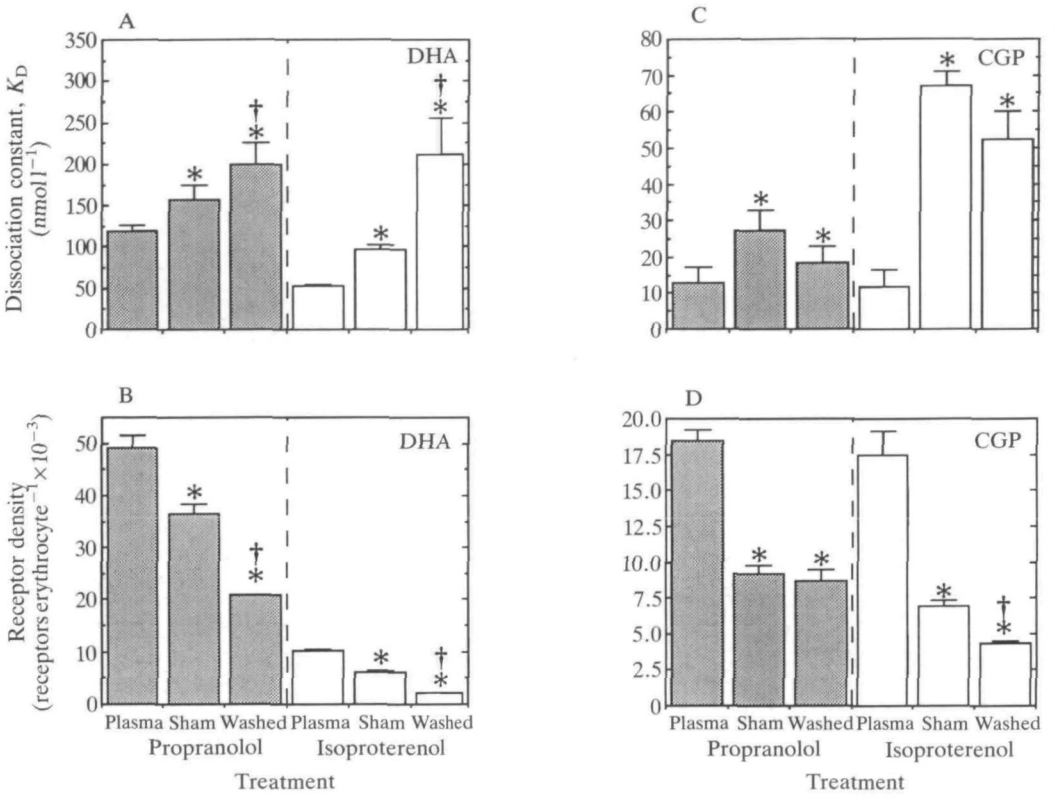

Fig. 5. The influence of handling and erythrocyte resuspension on erythrocyte ligand affinity and β-adrenoreceptor density. Erythrocyte β-adrenoreceptor dissociation constants (A,C) and densities (B,D) were measured on cells maintained in plasma with no additional treatment following removal from donor fish (Plasma), cells centrifuged and resuspended in the samples of their own plasma (Sham) and cells centrifuged and resuspended in Cortland saline (Washed) using both [^{3}H]DHA (A,B) and [^{3}H]CGP (C,D). Shaded histograms represent propranolol-displaceable radioligand binding, open histograms represent isoproterenol-displaceable radioligand binding. * and † indicate values significantly different from plasma and sham values, respectively. Bars represent±1 S.E., N=3.

that of the associated centrifugation and resuspension. Erythrocytes maintained in, and centrifuged and resuspended in, their own plasma (sham) were found to possess 54 % fewer propranolol-displaceable CGP binding sites and over 75 % fewer isoproterenol-displaceable binding sites than erythrocytes that did not experience the resuspension-associated handling (plasma). Rinsing and resuspension in saline had no additional impact on the number of propranolol-displaceable CGP binding sites, although it was found to result in a further significant reduction in the isoproterenol-displaceable CGP receptor density of 25 %.

Interference by endogenous catecholamines

Additions of either adrenaline or noradrenaline failed to alter significantly the

Table 1. *Interference by endogenous catecholamines with the binding of CGP to erythrocyte β-adrenoreceptors*

[^{3}H]CGP (nmol l^{-1})	[Adrenaline] (nmol l^{-1})			[Noradrenaline] (nmol l^{-1})		
	0.1	1.0	10	0.1	1.0	10
2	100 (3.5)	97 (6.0)	92.2 (7.5)	98.0 (5.1)	97.6 (3.3)	95.8 (5.0)
20	96.2 (5.2)	98.2 (6.0)	98.3 (7.0)	101.6 (6.7)	95.2 (4.3)	99.8 (5.3)
40	98.0 (5.1)	101.7 (5.6)	100.8 (6.8)	95.3 (5.2)	102.5 (5.8)	103.8 (6.5)

CGP binding was measured in the presence of 0.1 nmol l^{-1}, 1.0 nmol l^{-1} and 10 nmol l^{-1} adrenaline or noradrenaline.

The degree of binding was calculated as a percentage of the maximal isoproterenol-displaceable CGP binding measured in the absence of added catecholamine.

Numbers in parenthesis represent ±1 S.E.M. (N=3).

number of isoproterenol-displaceable CGP binding sites determined using our radioreceptor assay protocol (Table 1). At all concentrations of adrenaline and noradrenaline tested, the binding of CGP, in the presence of isoproterenol, was not found to be significantly different from the binding of CGP under these conditions in the absence of additional catecholamines.

Receptor compartmentalization

Sonication of the erythrocytes had a significant impact on the characteristics of the erythrocyte β-adrenoreceptor population (Fig. 6). Rupture of the erythrocyte membranes, as verified by measuring haematocrit (0.2%) and by counting cells (0), resulted in significant reductions in the dissociation constants of both propranolol- and isoproterenol-displaceable DHA binding of 88 and 69%, respectively (Fig. 6A). However, no significant alterations were observed in the measured K_D value of either the propranolol- or the isoproterenol-displaceable CGP binding.

Removal of the erythrocyte membrane led to a significant increase in the number of CGP receptors (Fig. 6B): 57% more propranolol-displaceable and 46% more isoproterenol-displaceable CGP receptors were found following the sonication of the plasma-suspended erythrocytes. Although the number of measurable CGP binding sites was found to increase following erythrocyte sonication, the numbers of the two DHA binding sites were significantly reduced (Fig. 6C). The density of the propranolol-displaceable DHA binding sites was reduced by approximately 80%, whereas the isoproterenol-displaceable DHA receptor population was reduced by over 74%. Furthermore, the magnitude of the difference between the number of propranolol-displaceable and isoproterenol-displaceable DHA binding sites, normally found to be of the order of 27000 receptors per erythrocyte, was significantly reduced to only approximately 3000 receptors per erythrocyte. In addition, sonication significantly reduced the ratio of

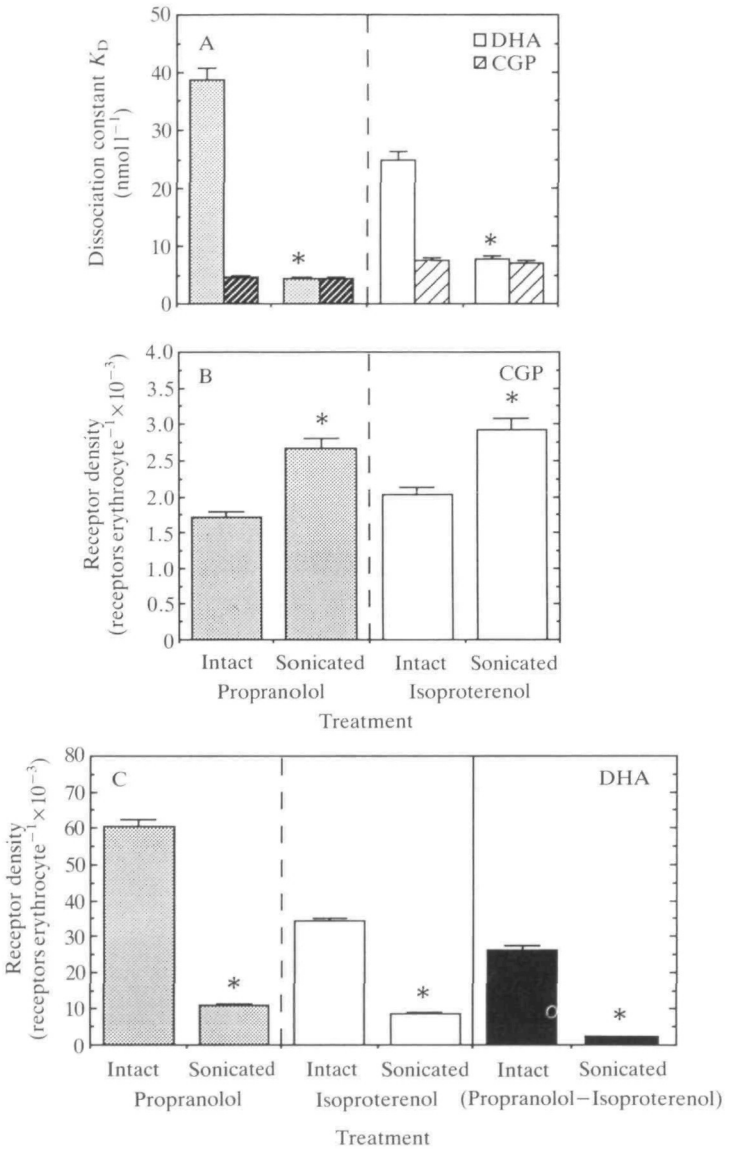

Fig. 6. β-Adrenoreceptor compartmentalization. β-Adrenoreceptor–radioligand dissociation constants (A; DHA, open histograms; CGP, striped histograms) and receptor density (B, CGP; C, DHA) were measured on trout erythrocytes prior to and following erythrocyte sonication. Shaded and shaded-striped histograms represent propranolol-displaceable radioligand binding. Open and striped histograms represent isoproterenol-displaceable radioligand binding. Filled histograms represent the number of internal β-adrenoreceptors and were calculated as the difference between the DHA propranolol-displaceable and isoproterenol-displaceable maximal binding capacites. * indicates a value significantly different from the intact value. Bars represent ±1 S.E., N=3.

specific DHA to specific CGP binding sites (Fig. 6B, C). Prior to sonication, the ratio of propranolol-displaceable DHA to propranolol-displaceable CGP erythrocyte binding sites was approximately 34:1, whereas the ratio of the number of isoproterenol-displaceable binding sites for these two tritiated ligands was nearly 15:1. Following the removal of the erythrocyte cell membrane, however, these ratios were reduced to only 4:1 and 3:1, respectively.

Discussion

β-Adrenoreceptors mediate the adrenergic regulation of trout erythrocyte volume and intracellular pH, which can affect the oxygen-carrying capabilities of erythrocytes (see reviews by Thomas and Motais, 1990; Perry and Wood, 1989). These receptors have been characterized by pharmacological criteria, but our study represents only the second direct characterization of rainbow trout erythrocyte β-adrenoreceptors. Bennett and Rankin (1985) first identified β-adrenoreceptors in teleost erythrocytes using radioligand binding techniques. Their protocol, however, involved [^{3}H]DHA in combination with the β-antagonist propranolol. In our study, propranolol-displaceable [^{3}H]DHA binding was significantly greater than isoproterenol-displaceable binding (Fig. 3B). Similar observations have been reported by Marttila and Nikinmaa (1988) and André *et al.* (1981). Based on the hydrophobic properties of both [^{3}H]DHA and propranolol, and the observation that most of the binding of [^{3}H]DHA could not be displaced by isoproterenol (β-agonist), André *et al.* (1981) suggested that the propranolol-displaceable ligand binding to turkey erythrocytes occurred at sites different from the functional β-adrenergic receptors. The study of Marttila and Nikinmaa (1988) and our data support this hypothesis for fish erythrocytes. The studies of André *et al.* (1981) and Marttila and Nikinmaa (1988), however, do not eliminate the possibility that propranolol binding displaces [^{3}H]DHA from both functionally uncoupled, or down-regulated, and functionally coupled erythrocyte β-adrenoreceptors located on the membrane surface. Even though the use of [^{3}H]DHA may provide information concerning the total number of eel erythrocyte β-adrenoreceptors, Bennett and Rankin (1985) failed specifically to isolate eel high-affinity erythrocyte surface receptors, which are considered to be the physiologically active catecholamine signal transducers.

The more recent study of Marttila and Nikinmaa (1988) examined the binding of two β-adrenoreceptor ligands ([^{3}H]DHA and [^{3}H]CGP) to intact erythrocytes from rainbow trout and carp (*Cyprinus carpio*). The rationale for using both ligands was that previous studies (Staehelin *et al.* 1983; Rademaker *et al.* 1985) found hydrophobic ligands (i.e. DHA) displayed greater apparent specific non-receptor binding and were taken up by intact cells. The internalization and greater non-receptor binding complicates the interpretation of saturation binding curves. Therefore, a hydrophilic ligand (CGP) was included by Marttila and Nikinmaa (1988) as it was not expected either to enter the intact cell or to bind to non-receptor sites, including the lipid domains of the cell membrane. Aspects of the

protocol of Marttila and Nikinmaa (1988) were adopted in our study to provide direct comparisons; certainly our findings are in general agreement with the trout erythrocyte β-adrenoreceptor characterization reported by this group. However, our investigation significantly improved upon that of Marttila and Nikinmaa (1988), both by determining the impact of resuspending erythrocytes in a saline-based buffer on the outcome of the radioligand binding assay and by demonstrating directly that differences in the specific displaceable ligand binding actually reflect the numbers of receptors located in different erythrocyte compartments.

The necessity to wash and resuspend erythrocytes repeatedly in a saline solution is based on two assumptions. First, by standardizing the erythrocyte haematocrit more consistent results are obtained. Obviously measurements of erythrocyte concentrations in the blood sample can be made (haematocrit, haemocytometer) and our animals were not treated in a fashion that might alter their haematocrit, so little variation in concentration was noted. Second, the influence of endogenous catecholamines on the radioreceptor assay was not known so, by removing the plasma, this unknown could be eliminated. Our findings suggest that this procedure is both unnecessary and detrimental. Additions of increasing concentrations of exogenous catecholamines to whole blood failed to interfere with the ability of [^{3}H]CGP to bind to specific sites in the presence of isoproterenol (Table 1). Furthermore, the removal of the plasma significantly modified the structure of the β-adrenoreceptors and reduced the actual number of receptors present within or on the erythrocytes. The conclusion that the structure of the β-adrenoreceptors is altered is based on the observation that the dissociation constants for both propranolol- and isoproterenol-displaceable DHA and CGP binding were significantly increased by repeated centrifugation and resuspension (Fig. 5A,C). As the binding affinity is inversely related to the dissociation constant, the affinity of the particular ligand for the β-adrenoreceptor is impaired. The fact that, at least with the DHA binding data, a further reduction in binding affinity was associated with the replacement of plasma with saline indicates that there must also be an effect of the resuspension medium. One could postulate that the β-adrenoreceptors require some plasma-borne factor(s) to maintain their structural integrity. Such was the conclusion reached by Sager and Jacobsen (1985) when they observed a significant reduction in human erythrocyte β-adrenoreceptors following washing and resuspension in a physiological saline.

It is the modification in receptor density that most clearly demonstrates the adverse effects of rinsing and resuspending erythrocytes. These data demonstrate that repeated erythrocyte washing is responsible for a massive reduction in the measurable receptor density and support our postulation that the removal of the plasma, or some plasma factor(s), directly results in the loss of β-adrenoreceptors (Fig. 5B,D). These findings have important implications with respect to assays of function/metabolism performed on washed and resuspended erythrocytes. If β-adrenoreceptors are the key to the physiological parameter of interest, this overhandling may cause a significant reduction in erythrocyte sensitivity and, therefore, lead to a significant underestimation of the true responsiveness of the

system. This conclusion from our study strongly indicates that the repeated rinsing and resuspending of erythrocytes in saline should be avoided when possible.

The second critical aspect of this investigation was the direct assessment of the differences in the K_D values and maximal binding capacities (B_{max}) obtained with the use of [^{3}H]DHA and [^{3}H]CGP with either propranolol or isoproterenol. Both DHA and propranolol are considered to be hydrophobic β-adrenoreceptor antagonists which penetrate the erythrocyte membrane as well as binding to receptors located at the erythrocyte surface. Propranolol also competes for both high- and low-affinity receptor sites with either radioligand (André *et al.* 1981). In contrast, isoproterenol, a hydrophilic β-adrenoreceptor agonist, competes only for high-affinity receptor sites (André *et al.* 1981). Propranolol-displaceable DHA binding, therefore, provides an indication of the total number of erythrocyte receptors, of either high or low affinity, inside and on the surface of the erythrocytes. As isoproterenol is expected to bind only to high-affinity receptors located at the erythrocyte membrane surface, the difference between the propranolol- and isoproterenol-displaceable DHA binding provides an indication of the number of β-adrenoreceptors specifically located within intact erythrocytes. Like isoproterenol, CGP, a hydrophilic β-adrenoreceptor antagonist, has been shown to bind to the external erythrocyte surface (André *et al.* 1981). Therefore, consistent with the interpretations of Marttila and Nikinmaa (1988), the lack of significant differences between propranolol- and isoproterenol-displaceable [^{3}H]CGP binding sites indicates that there exists a single population of β-adrenoreceptors at the erythrocyte surface characterized by a single affinity for [^{3}H]CGP. Thus, [^{3}H]CGP provides the best estimate of the number and the affinity of, presumably physiologically active, erythrocyte surface β-adrenoreceptors.

To test the validity of presumed interactions, trout erythrocytes were sonicated to remove the membrane barrier. If our assumptions are correct, the following outcomes on radioligand binding would be predicted: (i) a reduction in the difference between the extent of propranolol- and isoproterenol-displaceable DHA binding; (ii) an increase in the number of isoproterenol-displaceable CGP binding sites, possibly accompanied by an increase in the number of detectable propranolol-displaceable CGP binding sites; and (iii) an equivalency between DHA–receptor and CGP–receptor dissociation constants. Although we were unable to obtain the 'ideal' results, the removal of the erythrocyte membrane by sonication did result in significant modification to ligand–receptor K_D values and to receptor densities, consistent with the predicted results and, therefore, supports the use of [^{3}H]CGP and [^{3}H]DHA together with both propranolol and isoproterenol to establish the compartmentalization of erythrocyte β-adrenoreceptors.

The removal of the erythrocyte membrane resulted in the conversion of a receptor population containing a mixture of low- and high-affinity receptors to a single population of high-affinity receptors. This conclusion is based on two observations. First, the K_D values for propranolol- and isoproterenol-displaceable DHA binding were identical following erythrocyte sonication (Fig. 6A). Second,

differences between the receptor–ligand K_D values for CGP and DHA were eliminated following the sonication of the erythrocytes. CGP was able to bind to surface receptors and to those receptors formerly located within the erythrocytes, receptors that are normally beyond the reach of this hydrophilic β-adrenergic antagonist. The evidence for accessibility of CGP to the 'internal' receptor population is clearly demonstrated by the 1.5-fold increase in both the isoproterenol- and the propranolol-displaceable CGP receptor sites following erythrocyte sonication (Fig. 6B). Furthermore, the differences between the number of binding sites detected using DHA and CGP, in combination with either propranolol or isoproterenol, were significantly reduced following sonication (Fig. 6B, C). These two observations most strongly support the existence of at least two distinct receptor populations separated by the erythrocyte membrane, and the importance of using both radioligands (DHA and CGP) to characterize these β-adrenoreceptor pools. In addition, the combination of both radioligands reduces the likelihood of misinterpreting the DHA binding data. It has been proposed by others that the specific propranolol-displaceable DHA binding represents the interaction of this radioligand with lipid membrane domains (Staehelin *et al.* 1983; Rademaker *et al.* 1985), an interpretation that was unchallenged by Marttila and Nikinmaa (1988). However, as the lipophobic radioligand CGP is not likely to bind to these non-receptor sites, the binding of both radioligands to a similar number of sites following sonication supports our interpretation, and that of André *et al.* (1981), that there exist two β-adrenoreceptor pools and that use of CGP and DHA in concert with both propranolol or isoproterenol provides valuable information concerning the compartmentalization of these erythrocyte β-adrenoreceptors.

The reduction in the number of isoproterenol- and propranolol-displaceable DHA binding sites was not expected, and indicates that sonication destroys both the erythrocyte cell membranes and the β-adrenoreceptors themselves (Fig. 6C). However, as the difference between the propranolol-displaceable and the isoproterenol-displaceable DHA binding is reduced, the number of receptors located specifically within the erythrocytes is reduced. This reduction following sonication simply indicates that there was a reduction in the ability of isoproterenol and propranolol to bind to receptors of different locales.

Our interpretation of these data is supported by other studies. It is generally accepted, although not yet shown in fish erythrocytes, that at least two anatomical loci for β-adrenergic receptors exist in intact cells, and these show at least two functional states. Receptors may exist on the surface of cells in a high-affinity state for agonist, coupled to the stimulatory guanine nucleotide regulatory protein (G_S) and hence to the catalytic subunit of adenylate cyclase (Waldo *et al.* 1983). In mammalian systems, there is compelling evidence that β-adrenergic receptors, of low agonist affinity and uncoupled from adenylate cyclase, exist on an intracellular pool of membranes (light vesicle pool, Waldo *et al.* 1983). They may form a reservoir of receptors cycling to and from the cell surface (Mahan *et al.* 1985; Waldo *et al.* 1983). In addition, β-adrenoreceptors may be present at least transiently on the cell surface in a low-affinity, uncoupled state, probably befor

being sequestered into intracellular light vesicles (Strasser *et al.* 1984). Exposure of intact cells to β-adrenergic agonists promotes uncoupling of the β-adrenoreceptor from adenylate cyclase and sequestration in intracellular sites (Marsh *et al.* 1985; Van der Larsse *et al.* 1984). Therefore, it appears that our protocol may allow us to model the localization and mobilization of erythrocyte β-adrenoreceptors as has been accomplished in other receptor systems.

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