Antilipolytic effects of chlorophenoxyisobutyrate and warfarin in adipocytes

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Abstract This study was designed to examine whether displacement of free fatty acids (FFA) from albumin (BSA) can explain the antilipolytic effect of chlorophenoxyisobutyrate (CPIB). Warfarin, which binds to albumin with equal affinity, was used to test the generality of this mechanism. The procedure was to measure the concentration of free drug needed to inhibit hormone-stimulated lipolysis in isolated rat epididymal fat cells, and the effect on this process of albumin, which binds both FFA ligand and drug. The free drug concentration was initially obtained by ultrafiltration studies with albumin and ^{14}C-labeled CPIB or ^{14}C-labeled warfarin in the absence of cells. When epinephrine-activated lipolysis was measured, inclusion of 0.3 mM albumin decreased the free CPIB concentration required for 50% inhibition from 1.8 mM (−BSA) to 0.08 mM. Warfarin also inhibited lipolysis more effectively in the presence of albumin, with 50% inhibition at 0.06 mM (+BSA) vs. 0.7 mM (−BSA). Both drugs showed a similar high-affinity binding constant to albumin of $n = 1$, $k = 2-4 \times 10^5$ M^{-1}, and both competitively displaced [^{14}C]stearate, provided that a hydrophobic trap was present. The results are consistent with the possibility that the antilipolytic effect of CPIB and warfarin is mediated by way of a competitive displacement of FFA from albumin, or an analogous cellular binding site, with subsequent feedback inhibition of lipolysis.

Supplementary key words free fatty acids · epinephrine · lipolysis

Several reports have demonstrated that the hypolipidemic drug chlorophenoxyisobutyrate (CPIB) reduces basal and hormone-stimulated lipolytic activity of isolated fat pads and adipocytes (1–4). It has been proposed that one mechanism by which hypolipidemic drugs work is through a reduction in cyclic AMP concentration (3–5). Evidence that procaine (4) and tolbutamide (6) stimulate cyclic AMP formation but inhibit lipolysis cannot be reconciled easily with this concept. Thorp (7, 8) originally proposed that the hypolipidemic effect of CPIB resulted from a displacement of thyroxine and FFA from albumin. Although this theory has been disclaimed (4), at pharmacological levels, between 90 and 96% is bound to albumin (7, 9) and is therefore available for interaction with circulating FFA. It is known that CPIB increases FFA uptake from the FFA–albumin complex into ascites cells (10) and enhances hepatic oxidation and esterification of [^{14}C]palmitate from the palmitate–albumin complex (11, 12). We have shown that CPIB displaces long chain fatty acids from albumin in an apparently competitive manner, provided that a hydrophobic phase is present to trap unbound FFA (9). Since the concentration of intracellular and extracellular FFA is thought to serve as a negative feedback modifier of triglyceride breakdown in adipose tissue (13–15), the displacement of FFA by CPIB, and the subsequent increase in FFA concentration, could explain the antilipoytic effects. Toward this end, we have examined the effect of albumin on the concentration of free CPIB required to achieve 50% inhibition of hormone-stimulated lipolysis. Warfarin, an anticoagulant that binds to albumin with the same affinity as CPIB, has been included to test the generality of this effect. The results show that in the presence of albumin, inhibition of hormone-stimulated lipolysis by both CPIB and warfarin is achieved at lower free drug levels.

METHODS

Epididymal fat cells from 140–200 g rats were isolated by the method of Rodbell (16), using 2 mg of crude collagenase (Worthington Chemicals, Freehold, NJ) per fat pad. Cells were washed four times in 0.128 M NaCl, 1.4 mM $MgSO_4$, 5.2 mM KCl, and 10 mM phosphate buffer, pH 7.45, containing 2% BSA (fraction V, Sigma, St. Louis, MO). This fraction contained about 0.3 mol FFA/mol albumin. Total lipid was measured by extracting cells three times with ether and drying overnight at 140°C. Immediately before

Abbreviations: CPIB, chlorophenoxyisobutyrate; BSA, bovine serum albumin; FFA, free fatty acids.

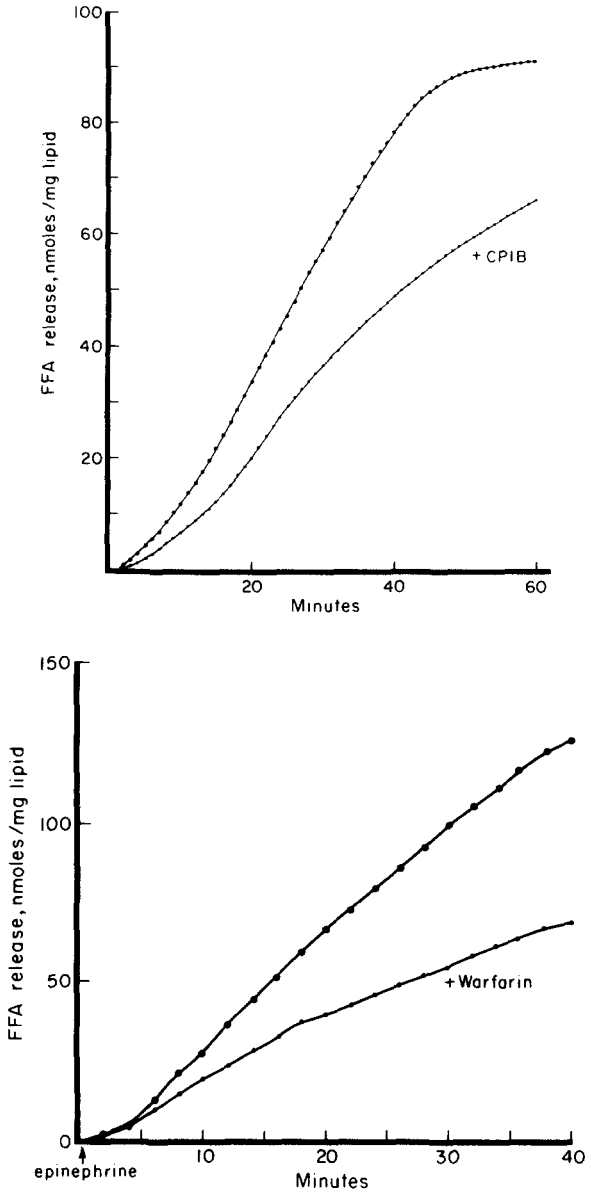

Fig. 1. Effect of CPIB (*A*) and warfarin (*B*) on catecholamine-stimulated FFA release by isolated adipocytes. Fat cells (*A*, 53 mg lipid; *B*, 30 mg lipid) were preincubated at 37°C in buffer containing 2% BSA and drug for 10 min before adding catecholamine. Final concentrations were *A*, 1 μg/ml norepinephrine and 0.5 mM CPIB; *B*, 0.5 μg/ml epinephrine and 0.75 mM warfarin. FFA release was measured as a decrease in pH of the medium, assuming a stoichiometric relationship between H^+ and FFA appearance during activated lipolysis (19). Each curve is derived from a single experiment.

use, cells were washed once to remove endogenous material such as adenosine, which is known to lower cyclic AMP concentration and lipolysis (17). Cells (between 15–25 mg of lipid) were incubated at 37°C for 20 min, with shaking, in buffer containing 2% BSA. Samples were immediately cooled to 0°C, and the cell-free infranatant was removed after a brief centrifugation. An aliquot was extracted with isopropyl alcohol–heptane–1 N H_2SO_4 40:10:1 (v/v) and FFA were measured by the ^{63}Ni method of Ho (18).

In experiments where albumin was omitted, the cells were washed once without albumin, then incubated under the appropriate experimental conditions at 37°C. After 20 min, cells were diluted with 5 ml of ice-cold buffer containing 0.1% BSA and filtered onto 0.8-μm cellulose discs (Millipore). Lipids were extracted from the filters with Dole's medium and analyzed for FFA as described above. As Cushman, Heindel, and Jeanrenaud (13) have found, and we have confirmed here, inclusion of 0.1% BSA had no effect on cell-associated fatty acid levels.

Proton release as a measure of the rate of FFA efflux from adipocytes was carried out as described (19), using a digital pH meter connected to a Datel DPP-7 printer (Datel Systems, Inc., Canton, MA). Under the experimental conditions, H^+ and FFA release were stoichiometric.

Equilibrium partitioning between long chain FFA and drug was carried out as described previously (9, 20). Generally, 1.0 ml of 0.2 mM BSA in pH 7.45 salts buffer (0.1 M NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, and 25 mM phosphate) was equilibrated with 1.0 ml of hexane containing [^{14}C]stearate for 24 hr. at 37°C, with shaking. The drugs were added to, and remained in, the aqueous phase. Scintillation counting of 0.2-ml aliquots of both phases was carried out in a 2:1 toluene–Triton X-100 solution containing 0.4% 2,5-diphenyloxazole. Free aqueous concentrations of stearate were calculated from partition ratios and the concentration present in the hexane phase at equilibrium (9). Free aqueous concentrations of [^{14}C]-warfarin and ^{14}C-labeled CPIB in the presence of long chain FFA were determined by ultrafiltration experiments under identical conditions (i.e., both phases present) (21).

Synthesis of [2-^{14}C]chlorophenoxyisobutyrate from *p*-chlorophenol and [2-^{14}C]acetone was carried out as described (20, 21). Purity was greater than 95% as determined by thin-layer chromatography in methylcyclohexane– acetone–acetic acid 70:30:1, (v/v). Radioactive [^{14}C]warfarin was purchased from Amersham Corp., Arlington Heights, IL, and was used without further purification.

ATP was measured by the luciferase method (23). FFA was determined by the ^{63}Ni method of Ho (18).

Warfarin was provided by Endo Labs, Garden City, NY, and sodium chlorophenoxyisobutyrate by Ayerst Labs, New York, NY.

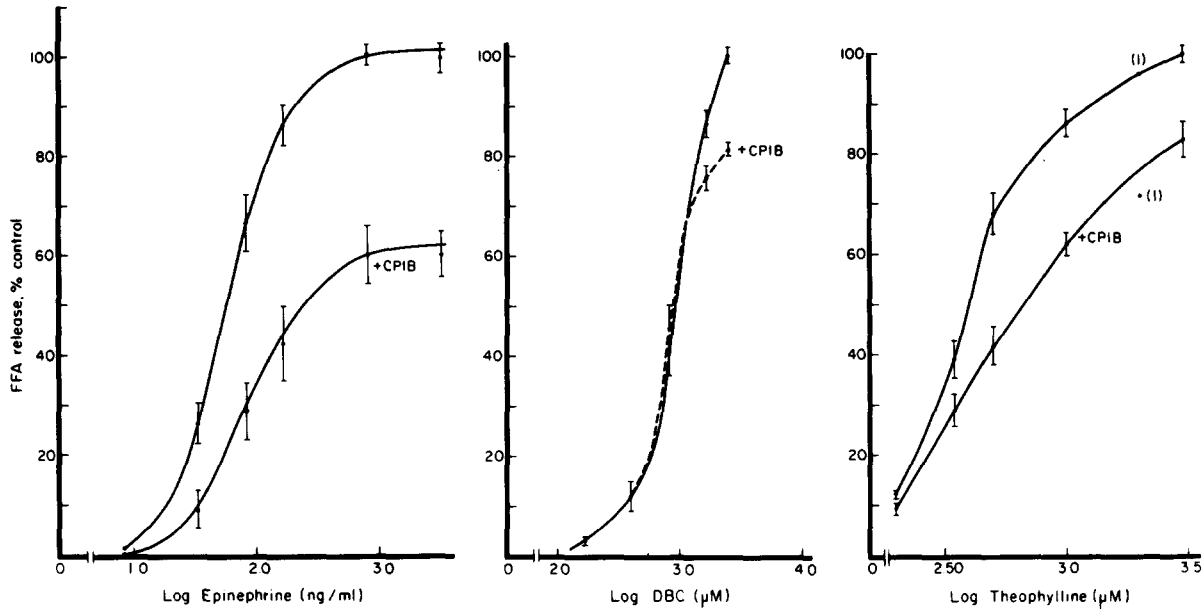

Fig. 2. Dose-response curves of the antilipolytic effect of CPIB on (*A*) epinephrine; (*B*) dibutyryl cyclic AMP; and (*C*) theophylline-stimulated lipolysis. Incubation time for activated lipolysis was 20 min.; CPIB was present at 0.67 mM. Bars represent SEM of 5–6 data points except where noted. DBC, dibutyryl cyclic AMP.

RESULTS

In **Fig. 1,** the effect of drugs on hormone-stimulated lipolysis is determined by recording changes in H^+ concentration, which we have shown to be a convenient and stoichiometric indicator of FFA release (23, 24). An immediate inhibition of catecholamine-stimulated FFA release by 0.5 mM CPIB (Fig. 1*A*) and 0.75 mM warfarin (Fig. 1*B*) can be observed.

The dependence of lipolysis on epinephrine, dibutyryl cyclic AMP, and theophylline concentration, and the effects of CPIB at a drug/BSA molar ratio of 1.4, is shown in **Fig. 2.** The effect of CPIB on epinephrine-stimulated lipolysis (Fig. 2*A*) is partially competitive, with the inhibition falling from 65% to 40% at 0.02 and 2.0 μg epinephrine/ml, respectively. No effect of the drug is observed until a dibutyryl cyclic AMP level of 1.7–2.5 mM is reached, a finding that confirms the data of D'Costa and Angel (4). The possibility that dibutyryl cAMP was bound to albumin, particularly at low concentrations, was eliminated by ultrafiltration experiments with [^{14}C]monobutyryl cyclic AMP, which showed that insignificant binding occurred. Theophylline (Fig. 2*C*) yielded a relatively constant 25–30% inhibition by CPIB. Thus, with respect to the mode of stimulation of lipolysis, the dose-response curves do not reveal any consistent pattern of antilipolytic effect of CPIB.

The possible role of albumin in mediating the antilipolytic effects of CPIB and warfarin was examined by varying the albumin concentration. Albumin acts as an acceptor of FFA released during activated lipolysis (25). Without albumin, triglyceride breakdown continues, but FFA accumulates intracellularly (13). In order to compare results, the free CPIB concentration was obtained from ultrafiltration studies carried out with ^{14}C-labeled CPIB and 2–5% BSA (**Fig. 3**). When albumin was absent, it was assumed that all CPIB was free. Based on these results, the free CPIB

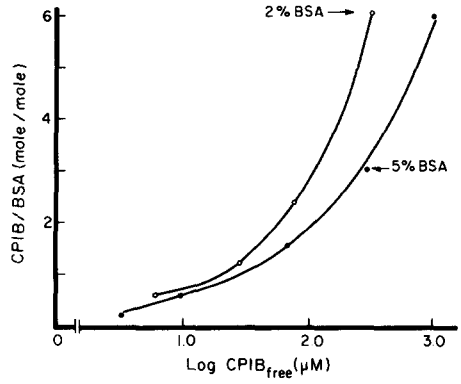

Fig. 3. Binding of ^{14}C-labeled CPIB to albumin. Mixtures of ^{14}C-labeled CPIB and 2% or 5% BSA at pH 7.4 were ultrafiltered through Amicon CF25 conical membranes. Free drug concentration was determined by sampling the ultrafiltrate. Ordinate, total drug/albumin molar ratio initially present in the incubation mixture. Data are means of duplicate samples.

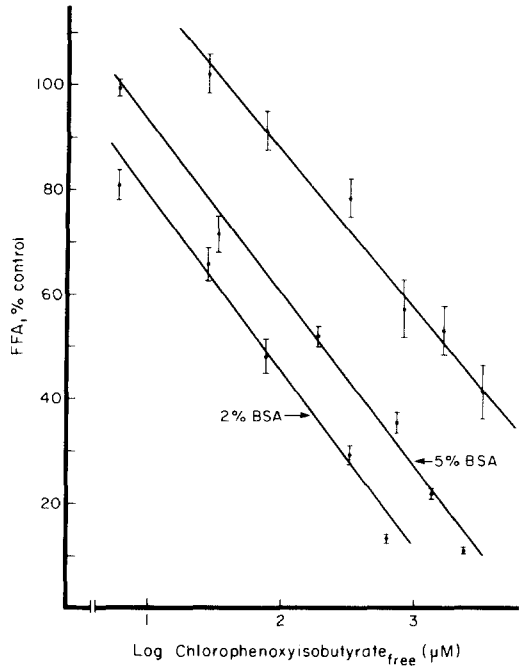

Fig. 4. Effect of albumin on the free CPIB concentration required to inhibit epinephrine-stimulated lipolysis. Adipocytes were preincubated with 0, 2%, or 5% BSA plus drug for 10 min at 37°C. Lipolysis was initiated with 0.05 μg/ml epinephrine and, after 20 min, cell-associated fatty acid (−BSA) or FFA released into the medium (+BSA) was measured as described in Methods. Final FFA concentrations in the presence of epinephrine, without drug, were 4.4, 31.1, and 63.9 nmol/mg lipid for 0, 2%, and 5% BSA, respectively. Control values at 20 min (without epinephrine or drug) of 2.0, 6.0, and 6.4 nmol/mg lipid for 0, 2%, and 5% BSA, respectively, were subtracted from all experimental values. Addition of CPIB had no effect on FFA values in the absence of hormones. The free CPIB concentration was calculated from Fig. 3. Bars represent standard error of 5–6 determinations.

concentration at a particular CPIB/BSA ratio was obtained and was used to assess the effect of albumin on the inhibition of lipolysis (**Fig. 4**). At 2% BSA, a 50% inhibition of lipolysis by CPIB was reached at

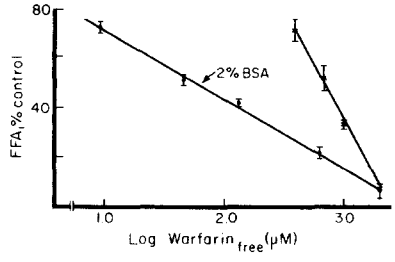

Fig. 5. Effect of albumin on the free warfarin concentration required to inhibit epinephrine-stimulated lipolysis. Final FFA concentrations in the presence of epinephrine, without drug, were 4.1 and 22.2 nmol/mg lipid for 0 and 2% BSA, respectively. Control values of 2.6 and 2.9 nmol/mg lipid for 0 and 2% BSA were subtracted from all experimental values. Bars represent SEM of six samples. Other details are exactly as described in Fig. 4.

0.08 mM free drug. Raising albumin to 5% shifted the inhibition curve slightly, resulting in a free drug concentration of 0.19 mM required for 50% inhibition. In the presence of albumin, the effect of CPIB is dependent upon the rate of epinephrine-stimulated lipolysis (Fig. 2*A*). This rate is approximately twofold higher in 5% than in 2% BSA. Thus, the higher drug concentration required to yield 50% inhibition of lipolysis may be ascribed to the partially competitive nature of the reaction (Fig. 2*A*). In the absence of BSA, CPIB effectively blocks lipolysis, but in this case approximately 10–20 times as much free drug (1.8 mM) is required to achieve the same degree of inhibition. Although not shown, ^{14}C-labeled CPIB was not incorporated into cells to a noticeable degree; however, the total cytoplasmic volume occupied by the cell suspension, estimated at approximately 1.5 μl, is too small to allow accurate determination of uptake.

The effect of BSA on the ability of warfarin to inhibit epinephrine-stimulated lipolysis is examined in **Fig. 5.** Free warfarin was calculated from ultrafiltration studies carried out in 2% BSA, similar to that described in Fig. 3. In the presence of albumin, warfarin gave a 50% inhibition of FFA release at 0.06 mM free drug. Furthermore, the slope of the inhibition, and concentration range of free drug, is identical to CPIB plus BSA (Fig. 4). In the absence of albumin, a 50% inhibition of cell-associated fatty acid accumulation occurs at 0.7 mM free warfarin, but over a narrower concentration range, so that, at the highest level of warfarin, the free drug concentration required to block lipolysis is independent of albumin. [^{14}C]-Warfarin was not incorporated to a measurable extent into the cytoplasmic space.

The ability of CPIB and warfarin to displace [^{14}C]-stearate from BSA in a hexane–aqueous phase partitioning system is shown in **Fig. 6.** As we have demonstrated previously (9, 21), CPIB competitively displaces [^{14}C]stearate, yielding in this case a K_I for CPIB of 0.9×10^3 M^{-1}. We show here that 5 mM warfarin can also displace [^{14}C]stearate, with a K_I warfarin of 0.56×10^3 M^{-1}. The ability of both drugs to displace stearate to the same extent compares well with a similar high-affinity association constant of warfarin ($n = 0.4$, $k = 4.3 \times 10^5$ M^{-1})[1] and CPIB ($n = 1$, $k = 2 \times 10^5$ M^{-1}) (21) and is consistent with an equivalent effectiveness in blocking activated lipolysis.

The coumarin ring in warfarin suggested that the antilipolytic effect might be explained by an uncoupling of oxidative phosphorylation. However, as **Table 1** shows, ATP levels were unaffected by 0.83 mM warfarin despite a 33% inhibition of lipolysis.

[1] Meisner, H. Unpublished data.

Raising the cell concentration threefold *did* reduce ATP concentration and the rate of lipolysis, but the antilipolytic effect of warfarin was unchanged.

DISCUSSION

The evidence reported here that the concentration of free CPIB and warfarin required to inhibit epinephrine-stimulated lipolysis by 50% is considerably less in the presence than in the absence of albumin is consistent with the possibility that the antilipolytic effect of the drugs is related to a competitive displacement of FFA. This is supported by the fact that CPIB and warfarin competitively displace stearate from albumin in a two-phase partitioning system, despite the fact that the primary association constant of both drugs to albumin is $2-4 \times 10^5$ M^{-1} (21, 26, 27), compared to 10^7 M^{-1} for long chain FFA (20, 28). The ability of warfarin and CPIB to displace the more tightly bound FFA is based on the presence of a hydrophobic phase to trap displaced FFA. In the presence of a given concentration of drug, significantly higher unbound FFA levels are attained at any given FFA/albumin molar ratio (9). The magnitude of the displacement will depend on the partitioning of FFA in the organic phase, which in adipocytes can be the cell membrane or analogous hydrophobic area. Since the partition ratio is unknown, the extent of increase in unbound FFA cannot be quantitated. The fact that the antilipolytic effect is not blocked completely in the absence of albumin can be taken to mean that CPIB and warfarin compete much more effectively with albumin for hydrophobic FFA binding sites than with analogous cellular structures. In this manner, the rate of release of FFA during activated lipolysis is retarded, due to a buildup of unbound intracellular FFA or product thereof.

One consequence of the proposed mechanism is that any agent that binds to albumin with a relatively high affinity ($K_a > 5 \times 10^4$ M^{-1}) should be antilipolytic, provided that a sufficient free plasma concentration is achieved. It has been found that agents as chemically distinct as chloroquine (29, 30, nystatin (31), digitoxin (32), CPIB (1, 4), and sodium salicylate (33) inhibit hormone-stimulated lipolysis in vitro. A feature of all the drugs is a tight binding to albumin (34), with affinity constants of about 10^5 for the primary site. Dawkins, McArthur, and Smith (35) have shown that salicylate displaces FFA from albumin, provided that an organic phase is present to trap the released FFA. The present report shows that the anticoagulant drug warfarin, which has a high affinity association constant of 4.3×10^5 M^{-1}, also effectively

TABLE 1. Effect of warfarin on cellular ATP content during lipolysis

Lipid mg	0.75 mM Warfarin			
	ATP pmol/mg lipid		FFA nmol/mg lipid/hr	
	−	+	−	+
23.3	251	249	97.9	64.6
70	118	127	27.7	18.6

Adipocyte suspensions were incubated with 2% BSA in a total volume of 0.6 ml. After 10 min at 37°C, either perchloric acid (final concentration, 3%) was added for ATP determination or lipolysis was activated with 50 ng of epinephrine, and the mixture was incubated for an additional 10 min before separating cells and medium.

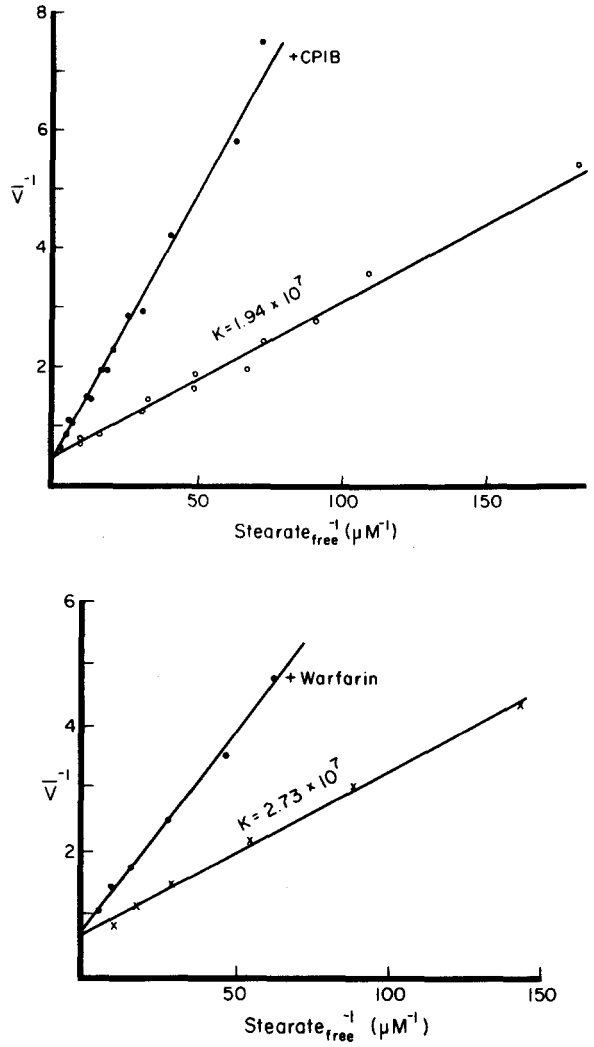

Fig. 6. Competitive displacement of [^{14}C]stearate from albumin by CPIB (*A*) and warfarin (*B*) in a two-phase hexane–salts partitioning system. When indicated, samples contained initially 2 mM CPIB or 5 mM warfarin. $\bar{V}$, mol [^{14}C]stearate bound/mol albumin. Free [^{14}C]stearate was determined from partition ratios, as described in Methods.

blocks epinephrine-stimulated FFA release from isolated adipocytes. In the presence of albumin, the concentration required for 50% inhibition of lipolysis and the association constant for the primary binding site are identical to those for CPIB. In vivo, the therapeutic level of warfarin is 2.5 μg/ml (36), which is too low for an observable antilipolytic effect.

The proposed increase in unbound FFA as the mechanism of the antilipolytic effect and the known hypotriglyceridemia produced by CPIB in vivo appear to be incompatible, since it is known that the rate of hepatic triglyceride formation is proportional to plasma FFA concentration (37, 38). However, plasma triglycerides have been found to fall sharply during a 24–48 hr fast in rats (39) and in exercising dogs (40), while plasma FFA levels rise. It has been shown that CPIB activates heparin-releasable lipoprotein lipase in animals (41) and man (42). The mechanism of this hypotriglyceridemic effect may be via a reduction in circulating FFA, which are known to inhibit lipoprotein lipase (43). Thus, unlike the perfused liver system, no simple relationship between plasma triglycerides and unbound FFA may exist in vivo.

Not all hypolipidemic drugs act in the proposed manner by competing with FFA for albumin sites. Nicotinic acid is representative of a drug that reduces FFA by a different mechanism. The concentration required in vivo to decrease lipolysis is about 0.1 mM (44), which is quite low compared to the K_a of 10^4 M^{-1} for the high-affinity binding site (34). This drug, as well as adenosine and PGE_1 (45), probably reduces serum FFA by inhibiting the synthesis of cyclic AMP, with secondary and lesser reduction in FFA concentration (46).

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