Chloroquine augments the binding of insulin to its receptor

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The effect of chloroquine on the interaction of insulin with its receptor has been investigated under both equilibrium and non-equilibrium conditions. Chloroquine was found to augment insulin binding in a pH-dependent manner between pH 6.0 and pH 8.5, with the maximum occurring at approximately pH 7.0. Analysis of the equilibrium binding data in terms of independent binding sites gave equivocal results but suggested an increase in the high-affinity component. Analysis using the negative co-operativity binding model of De Meyts, Bianco and Roth [J. Biol. Chem. (1976) 251, 1877–1888] suggested that the affinity at both high and low occupancy was increased equally. The kinetics of association of insulin with the plasma-membrane receptor indicated that, although the net rate of association increased in the presence of chloroquine, this was due to a reduction in the dissociation rate rather than an increase in the association rate. This was confirmed by direct measurement of the rates of dissociation. Dissociation was found to be distinctly biphasic,

with fast and slow components. Curve fitting suggested that the decrease in dissociation rate in the presence of chloroquine was not due to a decrease in either of the two dissociation rate constants, but rather to an increase in the amount of insulin dissociating by the slow component. It was also found that the increase in dissociation rate in the presence of excess insulin, ascribed to negative co-operativity, could be accounted for by an increase in the amount of insulin dissociating by the faster pathway, rather than by an increase in the dissociation rate constant. Thus chloroquine appears to have the opposite effect to excess insulin, and evidence was found for the induction of positive co-operativity in the insulin–receptor interaction at high chloroquine concentrations. Evidence was also found for the presence of low-affinity chloroquine binding sites with binding parameters similar to the concentration dependence of the chloroquine-induced augmentation of insulin binding.

INTRODUCTION

The anti-malarial drug chloroquine has been shown to have a number of effects on insulin metabolism. Thus in non-insulin-dependent diabetics chloroquine improves glucose tolerance [1], increasing peripheral glucose disposal and decreasing the metabolic clearance rate of insulin [2]. In insulin-dependent diabetics chloroquine has been shown to reduce insulin resistance [3], probably by inhibiting insulin degradation. In normal rats chloroquine causes hepatic retention of insulin [4] with accumulation of undegraded insulin in endosomal vesicles [5]. This accumulation was found to be due to inhibition of endosomal degradation of insulin by chloroquine [6,7]. Inhibition of endosomal degradation is found in detergent-disrupted vesicles as well as in intact vesicles [6], indicating that inhibition is not due solely to elevation of endosomal pH by the acidotrophic action of chloroquine and thus implying a more specific action of chloroquine. Kinetic analysis of the rates of endosomal insulin degradation in control experiments showed that the degradation can be represented as a bi-exponential process, and that the values of the rate constants are very similar to values found for the dissociation of insulin from the insulin receptor [7]. This suggests that dissociation from the receptor is the rate-limiting step in degradation. In the presence of chloroquine, a 2-fold decrease in the value of the slow process was observed, together with a concentration-dependent increase in the proportion of degradation proceeding via the slow process. Chloroquine has also been shown to increase insulin binding in cultured hepatoma cells [8,9]. However, internalization and degradation of insulin in cultured cell systems make a rigorous analysis of surface binding

data somewhat problematical. However, these findings suggest that chloroquine may be affecting the interaction of insulin with its receptor.

The insulin receptor is a heterotetrameric structure composed of two α - and two β -subunits held together by two classes of disulphide bonds [10]. The binding of insulin to its receptor often produces non-linear Scatchard analysis in most cell types considered: adipocytes [11,12], hepatocytes [13], muscle cells [14] and partially purified receptors [15]. This behaviour is often treated as two independent insulin-binding sites with different affinities. Previous studies [16] have suggested that the curvilinearity of the insulin-binding isotherm is a result of negative co-operativity, where the binding affinity of the insulin receptor decreases as a function of the occupancy of the receptor. More recently, it has been suggested that the non-linearity results from the cross-linking of the two α -subunits by a single insulin molecule [17,18]. The present study compares the concentration-dependent interaction of insulin with its receptor in the presence and absence of chloroquine under equilibrium conditions and with respect to the kinetics of association and dissociation. The equilibrium binding data are considered in terms of both independent binding sites and the negative co-operativity model of De Meyts et al. [16].

EXPERIMENTAL

Animals

Male Sprague–Dawley rats, 180–200 g body weight, were used throughout the study.

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Insulin and other chemicals

Monocomponent porcine insulin was prepared from insulin zinc suspension B.P. (80 i.u./ml Monotard, Novo Nordisk Pharmaceuticals, Crawley, U.K.) as outlined by Christensen et al. [19], and was iodinated with lactoperoxidase [20]. The ^{125}I -[A¹⁴-tyrosyl]insulin isomer was separated by HPLC as previously described [21]. All other chemicals were purchased from Sigma Chemical Co., Poole, Dorset, U.K. and were of analytical grade.

Liver plasma membrane preparation

Purified plasma membranes were prepared from rat liver by the method of Dorling and LePage [22]. This preparation routinely gave 20% recovery of plasma membrane as determined by 5'-nucleotidase activity. The final pellet was resuspended in 2 ml of 7 mM Tris/HCl, pH 7.5, buffer and stored at -20°C until use.

Equilibrium binding studies of insulin with the plasma membrane

The method of Sivaprasadarao and Findlay [23] was used. In brief, plasma membrane (70 μl , 1.5 mg of protein per ml) was added to a range of native insulin concentrations (8.7×10^{-7} – 1.7×10^{-11} M) and approx. 500 Bq of tracer ^{125}I -[A¹⁴-tyrosyl]insulin, in the presence or absence of 1 mM chloroquine in 50 mM Tris buffer, pH 7.4, containing 0.5% BSA (Fraction V RIA grade), in a final volume of 200 μl . After incubation at 25°C for 1 h, insulin binding was assessed by sedimentation and washing of bound ligand through a 500 μl mixture of ice-cold phthalate oils (dibutyl:diethyl, 1:1; density = 1.012 g/cm^3) at 12000 g in an Eppendorf centrifuge. The aqueous phase and oils were then aspirated and the precipitate was assayed for radioactivity.

Determination of parameters for equilibrium binding

The data obtained in equilibrium binding studies were initially characterized using the computer program LIGAND [24] to determine the most appropriate model assuming independent binding sites. Once this was established, LIGAND was then used to compare data sets obtained in the presence and absence of chloroquine to determine if there were significant differences between the data sets. If differences existed, the binding parameters were examined in a systematic manner, to determine which were most likely to be responsible for the observed differences. It was also considered possible that chloroquine might be affecting binding through alteration of the negative cooperativity that may be associated with the insulin–receptor interaction. This aspect of insulin binding is often examined using the average affinity plot of De Meyts [25]. However, this requires an accurate estimate of R_0 , the total receptor concentration, and does not take into account the contribution to total binding of non-specific binding. The standard Scatchard equation was therefore modified in accordance with De Meyts' proposal ($K_d \propto$ receptor occupancy) and with the addition of a term representing non-specific binding. The resulting equation was solved using Mathematica (Wolfram Research Ltd.) in terms of receptor-bound insulin, to give a quadratic expression to which the equilibrium binding data were fitted by non-linear regression analysis. The equations are presented in Appendix 1.

Rate of association of insulin with plasma-membrane receptors

Plasma membrane (1.3 mg of protein) was added to a final volume of 2.5 ml of Tris/HCl buffer (50 mM, pH 7.4) containing 6.25 kBq ^{125}I -[A¹⁴-tyrosyl]insulin and a range of native insulin

concentrations (final total insulin concentration in the range 0.04–9 nM). In addition, each incubation was performed in the presence or absence of 1 mM chloroquine. Samples (0.1 ml) were taken in triplicate immediately and at timed intervals to 60 min. Each sample was placed on to 0.5 ml of ice-cold phthalate oil mixture and centrifuged for 2 min in an Eppendorf microfuge. The supernatant was aspirated and the tip of the tube was removed for assay of radioactivity.

Rate of dissociation of insulin from plasma-membrane receptors

Dissociation rates were measured as proposed by De Lean and Rodbard [26], using a 100-fold dilution. Plasma membrane (2 mg of protein) was added to Tris/HCl buffer (50 mM, pH 7.4) containing 0.5% BSA and 10.5 kBq of ^{125}I -[A¹⁴-tyrosyl]insulin (0.13 pmol) in the presence or absence of 1 mM chloroquine, to give a final volume of 55 μl , and incubated at 25°C for 1 h. At the end of this period 40 μl was removed and diluted to 4.0 ml with Tris buffer (50 mM, pH 7.4) containing 0.5% BSA in the presence and absence of 1 mM chloroquine and/or in the presence and absence of 0.2 mM native insulin, and incubated at 25°C . Samples ($3 \times 100\text{ }\mu\text{l}$) were removed at timed intervals over a period of 1 h and centrifuged in a Beckman airfuge at 122000 g for 1 min. The supernatant was removed and the pellet was counted for radioactivity.

Equilibrium binding studies of chloroquine with the plasma membrane

A 1:1 mixture of dibutyl phthalate:diethyl phthalate (0.5 ml; final density 1.012 g/cm^3) was overlaid with 0.17 ml of buffer [20 mM NaH_2PO_4 (pH 7.4)/150 mM NaCl/0.5% BSA], containing 2.5 kBq [^{14}C]chloroquine (96.2 MBq/mmol, Amersham International), unlabelled chloroquine diphosphate, to give a final concentration range of 0–6.5 mM, and plasma membrane (0.2 mg of protein). The tubes were incubated at 37°C for 1 h, then chilled on ice for 5 min and centrifuged at 12000 g for 2 min. The supernatant was then aspirated and the bottoms of the tubes containing the pelleted membrane were removed and placed in 3 ml of Monofluor (National Diagnostics). The radioactivity in the pellet was assessed by liquid-scintillation counting.

Assays

5'-Nucleotidase was assayed by the method of Avruch and Wallach [27] as modified by Seymour and Peters [28]. Protein was measured as described by Lowry et al. [29], with the modifications of Schacterle and Pollack [30].

RESULTS

Equilibrium binding of ^{125}I -[A¹⁴-tyrosyl]insulin to purified plasma-membrane insulin receptors in the presence or absence of chloroquine

The possibility that chloroquine was altering the binding of insulin to plasma-membrane receptors was initially examined using a single fixed quantity of radiolabelled insulin. Using the standard assay conditions described in the Experimental section, ^{125}I -insulin (2.4 kBq; 0.03 pmol) was incubated with plasma membrane for 1 h at 25°C in the presence or absence of 0.2 mM native insulin to distinguish specific and non-specific binding. The incubations were performed in the presence and absence of 1 mM chloroquine and over a pH range of 5.5–8.5. At the end of the incubation period membrane-bound and free radioactivity were separated by sedimentation through phthalate oils. Figure

Figure 1 Effect of pH on insulin binding to the plasma membrane in the absence and presence of chloroquine

The graph shows the percentage of radiolabelled insulin bound to plasma membrane after 1 h incubation in the absence (open symbols) and presence (closed symbols) of 1 mM chloroquine. Binding was carried out for 1 h at 25 °C with label alone (●/○) and in the presence of 2.5 mM native insulin (■/□) at the indicated pH. The results represent the mean of two separate experiments.

Figure 2 Effect of chloroquine on the insulin-binding isotherm

The graph shows a binding displacement curve for radiolabelled insulin binding to the plasma membrane at pH 7.4 in the absence (○) or presence (●) of 1 mM chloroquine at a range of native insulin concentrations. The points represent the mean $\pm$ S.D. of five separate experiments. $[B]/[T]$ represents the fraction of total labelled insulin that is bound. The limits indicate where points in the presence of chloroquine are significantly different at the 5% level from those in its absence using a paired *t*-test.

1 shows the pH profile for specific and non-specific binding expressed as a percentage of the total quantity of radiolabelled insulin in the incubation.

Specific insulin binding shows a maximum at approx. pH 7.5. In the presence of 1 mM chloroquine, binding again shows a maximum at pH 7.5, but the total ligand bound at this pH increased from 26 to 34%. The enhancement of binding is not observed below pH 6.0 and above pH 8.5. The non-specific binding component does not show a defined pH optimum over the range studied although it increases sharply below pH 6.5. There also appears to be an increase in non-specific binding between pH 6.0 and 8.0 in the presence of chloroquine, although this difference is not statistically significant.

Figure 2 shows the displacement of tracer ^{125}I -[A¹⁴-tyrosyl]-

Table 1 Effect of chloroquine on the parameters for equilibrium binding of insulin

The binding parameters ($\pm$ S.E.M., $n = 5$) obtained from LIGAND for insulin binding to plasma membrane are given. The model assumes two independent binding sites. K_1 and K_2 are the affinities of the two receptor sites, of concentration R_1 and R_2 respectively. N represents the non-specific component of binding.

Parameter	Control	Chloroquine
K_1 (nM ⁻¹)	1.285 ± 0.398	0.491 ± 0.039
R_1 (nM)	0.222 ± 0.093	1.750 ± 0.157
K_2 (nM ⁻¹)	0.114 ± 0.016	0.010 ± 0.011
R_2 (nM)	2.439 ± 0.122	3.438 ± 0.227
$N \times 10^2$	3.934 ± 0.078	3.759 ± 0.260
$K_1 \times R_1$	0.285 ± 0.037	0.858 ± 0.017
$K_2 \times R_2$	0.277 ± 0.039	0.036 ± 0.014
Total binding	0.562	0.894

insulin binding by native insulin over the concentration range 8.7×10^{-7} – 1.7×10^{-11} M and in the presence or absence of 1 mM chloroquine. The enhancement in binding found in the presence of chloroquine is significant ($P < 0.05$) from the lowest insulin concentration used up to 8.7×10^{-9} M insulin. Initial data analysis using LIGAND indicated that for all data sets, both in the presence and absence of chloroquine, a model with binding to two sites gave a significantly better fit than binding to a single site ($P = 0.0001$). The values obtained for the binding parameters where no constraints are placed on the possible relationships for the parameters in the presence or absence of chloroquine are given in Table 1. The relationship $K \times R$ (where K is the affinity of the receptor site of concentration R) gives a measure of the binding capacity; 1 mM chloroquine increased the total binding by nearly 60%. This appears to be due to an increase in binding by high-affinity receptors with an accompanying decrease in the binding by low-affinity receptors. Although for the high-affinity population there appears to be a decrease in affinity accompanied by an increase in receptor concentration, an accurate assessment of the source of the increased binding is difficult since K_1 and R_1 are highly correlated ($r = 0.98$). Similar problems occur when considering the low-affinity site.

The data were subjected to a systematic examination where all ten data sets (five control, five chloroquine) were fitted simultaneously to a two-site binding model. As suggested in [24], the fit was performed as the various binding parameters were forced to be considered equal in the control and chloroquine data sets. This was performed for each binding parameter both singly and in combination. Table 2 shows the results of two alternative scenarios where in the presence of chloroquine either (1) the concentration of receptors does not change or (2) the affinities of the receptors do not change. Both of these cases give fits to the data that are not significantly worse than when all parameters are free. They indicate that if the receptor concentration is assumed to remain constant, then the increased binding is a result of a 50–80% increase in the affinity of both high- and low-affinity receptors. If the affinities are assumed to remain unchanged, then the increased binding is a result of a 2-fold increase in the concentration of high-affinity receptors with no corresponding decrease in the concentration of low-affinity receptors. The former case would seem to be more plausible since it does not invoke the generation, *de novo*, of new binding sites, although the uncovering of cryptic binding sites by chloroquine would be a possibility.

An alternative explanation of the curvature of the insulin-

Table 2 Effect of chloroquine on the parameters for equilibrium binding of insulin: effect of model constraints

The binding parameters ($\pm$ S.E.M., $n = 5$) obtained from LIGAND for insulin binding to plasma membrane are given. The model assumes two independent binding sites and shows the values obtained when (1) the concentration of receptors is assumed to be unchanged in the presence of chloroquine and (2) when the affinities of the binding sites are assumed to be unchanged in the presence of chloroquine. K_1 and K_2 are the affinities of the two receptor sites, of concentration R_1 and R_2 respectively. N represents the non-specific component of binding. *Parameters where the effect of chloroquine is statistically significant at the 5% level.

Parameter	Constant [receptor]		Constant affinities	
	Control	Chloroquine	Control	Chloroquine
K_1 (nM ⁻¹)	1.206 $\pm$ 0.277	1.813 $\pm$ 0.470*	1.170 $\pm$ 0.246	1.170 $\pm$ 0.246
R_1 (nM)	0.244 $\pm$ 0.075	0.244 $\pm$ 0.075	0.250 $\pm$ 0.072	0.543 $\pm$ 0.141*
K_2 (nM ⁻¹)	0.109 $\pm$ 0.012	0.197 $\pm$ 0.026*	0.111 $\pm$ 0.012	0.111 $\pm$ 0.012
R_2 (nM)	2.450 $\pm$ 0.070	2.450 $\pm$ 0.070	2.416 $\pm$ 0.072	2.647 $\pm$ 0.238
$N \times 10^2$	3.918 $\pm$ 0.078	4.198 $\pm$ 0.197	3.933 $\pm$ 0.079	3.981 $\pm$ 0.159
$K_1 \times R_1$	0.294 $\pm$ 0.026	0.442 $\pm$ 0.079*	0.293 $\pm$ 0.026	0.635 $\pm$ 0.051*
$K_2 \times R_2$	0.267 $\pm$ 0.029	0.483 $\pm$ 0.063*	0.267 $\pm$ 0.029	0.293 $\pm$ 0.047
Total binding	0.561	0.925*	0.497	0.928*

binding isotherm is that of negative co-operativity, as proposed by De Meyts et al. [31,32]. Hill plots of the data shown in Figure 2 produce Hill coefficients of less than 1.0 for both the control and the chloroquine curves (control = 0.769 ± 0.057 , chloroquine = 0.712 ± 0.015 , given as means $\pm$ S.E.M.). Although heterogeneity of binding sites can give rise to Hill coefficients of less than unity, this may also be interpreted as being indicative of negative co-operativity. Under this latter interpretation, both control and chloroquine data show moderate negative co-operativity. However, there is no significant difference ($P = 0.34$) in the Hill coefficients, indicating that if negative co-operativity is the cause of the negative values for the Hill coefficients, then chloroquine is not affecting the degree of negative co-operativity. The data were also analysed, as described in the Experimental section, by non-linear regression analysis to the modified equations for negative co-operativity, as shown in the Appendix. Table 3 shows the values obtained for the parameters describing binding with negative co-operativity, and the resultant fit to the data is shown in Figure 3 in the form of an average affinity plot [33]. The values obtained for the receptor concentration in the presence of chloroquine are not significantly different from those obtained for the control. Both K_e (affinity when receptor occupancy = 0) and K_t (affinity when receptor occupancy = 1) are

Table 3 Effect of chloroquine on insulin binding—negative cooperativity model

Mean values ($\pm$ S.E.M., $n = 5$) for the binding parameters assuming the negatively cooperative model of De Meyts as described in the Appendix are given. R_0 = receptor concentration, K_e and K_t are the affinity constant for the receptor population when occupancy = 0 (no receptors occupied) and 1 (all receptors occupied) respectively, and N is the non-specific component of binding which is directly proportional to the concentration of free ligand. *Parameters are significantly different from the control at the 5% level in a paired *t*-test.

	Control	Chloroquine
R_0 (nM)	3.009 $\pm$ 0.323	3.343 $\pm$ 0.231
K_e (nM ⁻¹)	0.185 $\pm$ 0.019	0.275 $\pm$ 0.019*
K_t (nM ⁻¹)	0.060 $\pm$ 0.018	0.092 $\pm$ 0.018
N	0.039 $\pm$ 0.001	0.039 $\pm$ 0.001

Figure 3 Average affinity plot of insulin binding data

The data shown in Figure 2 were fitted to the modified equation for negative co-operativity (Appendix; eqn. A3) to generate the parameters describing negatively co-operative binding shown in Table 3. The values obtained for the total receptor concentration [R_0] and the non-specific binding were then used to calculate the average affinity and occupancy, where average affinity = ([specifically bound insulin]/[free insulin])/([R_0] - [specifically bound insulin]) and occupancy = [specifically bound insulin]/[R_0]. The continuous lines represent the fitted curves: (○), control data; and (●), data obtained in the presence of 1 mM chloroquine.

elevated in the presence of chloroquine, although only that of K_e is statistically significant ($P = 0.005$). K_t is less well determined in the fitting procedure due to the large contribution to total binding of non-specifically bound insulin at high receptor occupancy. However, α , the interaction factor, which is the ratio K_t/K_e , is unchanged in the presence of chloroquine ($0.32 \rightarrow 0.33$), indicating that chloroquine is not affecting aspects of the interaction that produce the negative co-operativity. Thus, irrespective of the analytical model used, the effect of chloroquine appears to shift the insulin-receptor complex to an overall higher affinity state.

Association kinetics

The effects of chloroquine on the interaction of insulin with its receptor were further investigated by examining the rates of association and dissociation of insulin in the presence and absence of 1 mM chloroquine. Figure 4 shows the effect of chloroquine on the apparent rate of association of insulin with its receptor at various concentrations of native insulin. It can be clearly seen that in the presence of chloroquine there is an increase in the amount of membrane-bound insulin at equilibrium. There is also an increase in the rate at which that equilibrium value is obtained.

It is not possible, from a simple inspection of the data, to estimate whether the increase in the rate of association is due to an increase in the rate constant for association or to a decrease in the rate constant for dissociation of the insulin-receptor complex. However, an indication may be obtained by an analysis of the data as described by Gammeltoft [34] according to eqn. (1).

$$[RI] = \frac{[R_T] \cdot [L_T](1 - e^{-k_2 + k_1 \cdot [L_T]})}{[L_T] + \frac{k_1}{k_2}} + N \cdot [L_T] \quad (1)$$

where [RI] = insulin-receptor complex concentration, [R_T] = total receptor concentration, [L_T] = ligand concentration, k_1 and k_2 are the rate constants for association and dissociation of the insulin receptor complex respectively and N is a dimensionless

Figure 4 Rate of association of insulin with plasma-membrane receptors

The association of insulin with plasma-membrane receptors was followed as described in the Experimental section, with sampling at the indicated time-points for (a) control and (b) in the presence of chloroquine. The total concentration of insulin in each association mixture is indicated (in nM) at the end of each curve. The points represent the means of four separate experiments and the solid lines are the curves obtained by simultaneously fitting the data obtained at the six insulin concentrations to eqn. (1) by multivariate non-linear regression analysis.

Table 4 Effect of chloroquine on the rates of association of insulin with plasma membrane

Means ($\pm$ S.E.M., $n = 4$) for the kinetic parameters obtained by fitting the data shown in Figure 4, to eqn. (1) are given. * The decrease in the dissociation rate constant (k_2) in the presence of chloroquine is significant in a paired *t*-test at the 5% level.

	Control	Chloroquine
k_1 (nM^{-1})	0.0232 ± 0.0089	0.0239 ± 0.0067
k_2 (min^{-1})	0.0841 ± 0.0279	$0.0680 \pm 0.0244^*$
R_T (nM)	0.8627 ± 0.7016	1.2173 ± 1.0498

constant representing the proportion of ligand bound in a non-specific manner.

The data shown in Figure 4 were fitted to eqn. (1) using non-linear regression analysis and the results are presented in Table 4. The values obtained for the kinetic parameters indicated that there is no significant change in either the receptor concentration or the magnitude of the association rate constant in the presence of chloroquine. However, chloroquine appears to induce a significant decrease (19.1%, $P = 0.041$) in the rate constant for dissociation of the insulin-receptor complex. It should be noted that this analysis does not make any allowance for insulin binding at more than one site or the operation of negative co-operativity at one or more sites. The increasing complexity of the resultant equations, embodying just some of these facets of insulin's interaction with its receptor [26], suggests that an explicit analytical solution can probably not be obtained.

Dissociation kinetics

The above results suggest that chloroquine is exerting its effect through the dissociation of the insulin-receptor complex. Experiments were therefore carried out to confirm this suggestion and to quantify any changes. Figure 5 shows the time-courses representing the dissociation of receptor-bound insulin in the presence and absence of chloroquine and excess native insulin. It can be seen that, as found in previous studies [16,35], the dissociation curves are not asymptotic to zero. This was confirmed when attempting mathematical analysis of the time

Figure 5 Rate of dissociation of insulin from plasma-membrane receptors

Radiolabelled insulin was allowed to associate with plasma membrane for 1 h as described in the Experimental section. The mixture was diluted 100-fold with either dissociation buffer alone (○) or buffer containing 1 mM chloroquine (△), 2×10^{-7} M insulin (□) or 1 mM chloroquine + 2×10^{-7} M insulin (▽). Samples were taken at the indicated time-points and membrane-bound insulin was determined by centrifugation. The points represent the means $\pm$ S.E.M. of at least four experiments in each case.

courses, when fitting to a single exponential expression produced systematic deviation of the points about the fitted line. Although the points could be matched, with a significant improvement in fit, to a double exponential expression, as suggested previously [16,36], the kinetic constants were extremely poorly determined. It was found that an equally good fit and improved confidence limits on the kinetic parameters were obtained using the simpler model of a single exponential plus residual bound insulin not undergoing significant dissociation (eqn. 2)

$$[\text{RI}] = A_0 \cdot e^{-kt} + \text{Res} \quad (2)$$

where [RI] is the concentration of receptor-bound insulin, A_0 is the amount of bound insulin undergoing degradation, k is the first-order rate constant for dissociation and 'Res' is the residual bound insulin not undergoing significant dissociation within the time-frame of the experiment.

Table 5 Kinetic parameters obtained for the dissociation of insulin from its receptor

The individual data sets comprising the mean data shown in Figure 5 were fitted by non-linear regression to an expression comprising a single exponential term with a rate constant k , and amount A_0 of bound insulin undergoing dissociation. The expression also contained a quantity Res, which is the residual not undergoing significant dissociation during the time-frame of the experiment. A = control; B = 1 mM chloroquine; C = 0.2 mM insulin; D = 1 mM chloroquine + 0.2 mM insulin. The values of k and A_0 represent the means $\pm$ S.D. of five experiments. Res = $100\% - A_0$. The initial velocity of dissociation, v_i , is the product of k and A_0 . * Significant differences from the control at the 5% level using an unpaired t -test.

	A	B	C	D
k (min^{-1})	0.050 ± 0.022	0.045 ± 0.015	0.047 ± 0.020	0.045 ± 0.010
A_0 (%)	60.23 ± 6.01	$40.77 \pm 7.81^*$	$79.22 \pm 10.32^*$	$71.27 \pm 6.06^*$
Res (%)	39.77	61.23	20.78	28.73
v_i ($\% \cdot \text{min}^{-1}$)	3.00	1.87	3.72	3.21

The kinetic parameters obtained are shown in Table 5 (see also Figure 5). In the control dissociation approx. 60% of the membrane-bound insulin appears to be available for dissociation with a $t_{1/2}$ of nearly 14 min. The presence of chloroquine or insulin, either alone or in combination, produces dissociation curves that are different from the control. The presence of chloroquine alone clearly reduces dissociation, whereas the presence of excess native insulin increases the rate of dissociation. Insulin in the presence of chloroquine again increases the rate of dissociation. These changes are reflected in the calculated initial rates for dissociation (v_i) as shown in Table 5. Table 5 also shows that, in all of the test groups, the rate constant for dissociation was not significantly different from the control. The changes in dissociation rate appear to result from alterations in the amount of insulin (A_0) available for dissociation with a $t_{1/2}$ of 14 min. In the presence of chloroquine, A_0 is significantly decreased by 32% ($P = 0.002$), and in the presence of insulin, A_0 is significantly increased by 31.5% ($P = 0.007$). There is no significant difference in the values of A_0 obtained in the presence of insulin alone and in combination with chloroquine.

Concentration dependence of chloroquine effect

A single concentration of radiolabelled insulin was used to examine the dependence of the enhancement of binding on the concentration of chloroquine. Figure 6 shows the specific and non-specific binding, determined as for Figure 1 at pH 7.4, over a range of chloroquine concentrations. The enhancement increases in a curvilinear manner, reaching a maximum at 8 mM chloroquine, with 50% stimulation being achieved at approx. 2 mM chloroquine. Since this experiment indicates a distinct concentration dependence of the chloroquine augmentation of binding, the effect of a higher concentration of chloroquine on the insulin-binding isotherm was examined. Lower concentrations of plasma membrane were used than for Figure 2 in order to decrease the percentage of labelled insulin initially bound in the absence of chloroquine and native insulin. The results are shown in Figure 7 as displacement curves, comparing the effect of 1 and 5 mM chloroquine. It can be seen that at these lower membrane concentrations the effects of chloroquine are more marked, and that, especially in the case of 5 mM chloroquine, there is a distinct increase in the amount of label bound with increasing native insulin concentration, reaching a maximum at approx. 2×10^{-10} M insulin, before the percentage of bound label begins to decrease in the expected manner. This

Figure 6 Concentration dependence of the effect of chloroquine on insulin binding

Radiolabelled insulin (2.7 kBq; 0.034 pmol) was incubated with 0.08 mg of plasma membrane for 1 h at 25 °C in the presence of the indicated concentrations of chloroquine (●) in a final volume of 0.2 ml. Duplicate incubations were performed in the presence of 2×10^{-7} M insulin (■) to determine non-specific binding. Membrane-bound radiolabel was estimated by centrifugation of the samples through phthalate oils as described for Figure 1. The points represent the means $\pm$ S.E.M. of three experiments.

behaviour is consistent with the induction of positive cooperativity into the insulin-receptor interaction.

Chloroquine binding to the plasma membrane

The binding of chloroquine itself to the plasma membrane was also investigated, as described in the Experimental section, and a

Figure 7 Effect of high concentrations of chloroquine on the insulin-binding isotherm

The graph shows the variation in the ratio [bound tracer]:[total tracer] as the total concentration of insulin is increased. Radiolabelled insulin (1.2 kBq; 0.015 pmol) was incubated with plasma membrane (0.04 mg of protein) for 1 h at 25 °C in the presence of increasing concentrations of insulin and in the absence (○) or presence of 1 mM chloroquine (□) and 5 mM chloroquine (▽). The final volume of the assay was 0.2 ml. The amount of membrane-bound insulin was determined as described for Figure 2. The points represent the means of two separate experiments in each case.

Figure 8 Chloroquine binding to plasma membrane

(a) pH dependency of binding. [^{14}C]Chloroquine (2.5 kBq) was incubated with plasma membrane (0.2 mg of protein) at 37 °C for 1 h in a buffer cocktail (50 mM each of acetate, Mes, Tris and glycine) adjusted to the appropriate pH with NaOH. The membrane-bound chloroquine was then determined by centrifugation as stated in the Experimental section. (b) Scatchard plot for chloroquine binding. The Figure shows a representative Scatchard plot of data obtained from the variation in [^{14}C]chloroquine binding to plasma membrane as the concentration of unlabelled chloroquine was varied. The assay was performed at pH 7.5 in Tris buffer as described in the Experimental section.

typical Scatchard plot is shown in Figure 8. Analysis of the data using LIGAND suggested a single binding site of relatively low affinity. The mean parameters ($n = 4$) characterizing the binding were $K_d = 1.7 \pm 0.56$ mM ($\pm$ S.E.M.), $R = 2.27 (\pm 0.72) \times 10^{16}$ sites/mg of protein.

DISCUSSION

Examination of the binding of insulin to the plasma membrane at a single low concentration of insulin showed that the presence of chloroquine increased the specific binding of insulin and that the increase in binding was pH dependent. The increase in binding was maximal at pH 7.5 and disappeared below pH 6.0. This suggests that if chloroquine is exerting its inhibitory effect on endosomal insulin degradation via a binding phenomenon, then either the pH within the insulin endosome is normally near neutrality or 'acidotrophic' neutralization of the insulin endosome pH must be a necessary pre-requisite to augmentation of binding. A number of studies have shown that various endosome populations [37], including those internalizing insulin [38,39], rapidly become acidic following internalization and before lysosomal fusion. However, insulin degradation in the liver does not proceed via the lysosomal compartment but occurs within the endosome [5,40,41] by a neutral metalloprotease activity [6], and recent studies [39] have indicated that the pH of

the insulin-containing endosome in liver may in fact be near to neutrality.

The displacement curve (Figure 2) confirms that the increased binding in the presence of chloroquine is primarily in the specific-binding component, and analysis of the data with LIGAND indicated that, in both the absence and presence of chloroquine, a two-site model gave a much better fit to the data than a single-site model. Using this model it is clear that 1 mM chloroquine produced a 60% increase in total binding and that the major part of the increase is in terms of high-affinity binding.

As indicated earlier, an alternative explanation of the non-linearity in insulin binding is that the insulin-receptor interaction is negatively co-operative [32]. However, no evidence was found either in the values of the Hill coefficients or in the average affinity analysis to indicate that chloroquine is affecting the parameters describing the negative co-operativity. Thus if negative co-operativity is indeed a feature of insulin-receptor interaction, then it is unlikely that chloroquine is enhancing binding through the mechanism by which negative co-operativity operates.

Further characterization of the effects of chloroquine on the insulin-receptor interaction were carried out by examination of the rates of association and dissociation of the insulin-receptor complex. The net rates of association (Figure 4) were clearly increased in the presence of chloroquine at each of the insulin concentrations used. However, analysis of the curves as suggested by Gammeltoft [34] indicated that the rate constant for association was unchanged and that the increase in the net association rate was in fact due to a decrease in the rate constant for dissociation (Table 4).

Explicit measurement of the rates of dissociation in the absence and presence of chloroquine under conditions where association would be minimal confirmed the suggestion that chloroquine caused a decrease in the rate of dissociation (v_i , Figure 5 and Table 5). The values obtained for the rate constant for dissociation (k_1 , Table 5) show good agreement with those obtained in the association experiment. However, the presence of chloroquine was found to make no significant difference to the value of this rate constant. Although this may at first seem to contradict the conclusion from the analysis of net rates of association, it should be remembered that the analysis put forward by Gammeltoft is based on a simple bi-molecular reaction, with no allowance for multiple binding sites or negative co-operativity. The decrease in dissociation rate appears to result from a decrease in the amount of insulin available for dissociation by the 'fast' component of a biphasic process. This is very similar to the situation observed for inhibition of endosomal degradation of insulin by chloroquine [7]. In this study, insulin internalized into hepatic endosomes was found to undergo degradation in a biphasic manner, and chloroquine inhibited degradation by increasing the proportion undergoing degradation by the slower process. Thus it is clear that at neutral pH chloroquine induces increased binding of insulin to its receptor, through a decreased rate of dissociation. The effects of native insulin in increasing the dissociation rate of the insulin-receptor complex are often taken as evidence for negatively co-operative binding. The effects of chloroquine are in the opposite direction to, and are reversed by, excess native insulin. This suggests that chloroquine may be inducing a positively co-operative phase to insulin binding. This is confirmed by examination of the binding displacement curve at higher chloroquine concentrations (Figure 7), which shows increased binding of tracer with increasing native insulin concentrations up to 0.2 nM insulin. At higher insulin concentrations, the displacement curve behaves as expected, and analysis of this part of the curve (results not shown) indicates

two-site or negatively co-operative binding. Thus chloroquine appears to induce positively co-operative interactions within the insulin receptor, resulting in increased binding at low insulin concentrations. Positive co-operativity of the insulin–receptor interaction has been shown before but only at either very high insulin concentrations [35,42] or with insulin analogues [35]. As mentioned previously, increases in insulin receptor affinity have also been demonstrated in the presence of various antibodies to the insulin receptor [35,43]. Two of the antibodies, MA20 and MA51 [35], show a small (10%) degree of binding enhancement at low insulin concentrations, indicating positive co-operativity, although, paradoxically, MA51 increases the rate of insulin–receptor complex dissociation rather than decreasing it.

Previous studies have also provided evidence for the transformation of the initial complex to give a tightly bound form, the K_{super} state [34,43], which, as mentioned earlier, may be a reflection of insulin forming a cross-link between receptor α -subunits [17,18]. The kinetic analysis of the dissociation rates indicates that it is not the dissociation rate constant that is changing in the presence of chloroquine but the proportion of insulin in a tightly bound form. It is therefore possible that chloroquine is increasing the proportion of insulin–receptor complex in the cross-linked or K_{super} state. However, it is not clear how this would give rise to the appearance of positively co-operative behaviour as discussed above.

For chloroquine to be affecting insulin–receptor binding, it is either interacting directly with the receptor or modifying the receptor environment to achieve the necessary conformational change. The effect of chloroquine in augmenting the binding of insulin was shown to be concentration dependent, with a maximum effect at 8 mM chloroquine, 50% of maximum occurring at approx. 2 mM chloroquine. This suggests that chloroquine may be binding to specific sites on either the receptor or the plasma membrane. Systematic investigation of the binding characteristics, using radiolabelled chloroquine, showed that chloroquine binding was pH dependent, with maximum binding at pH 6.5–7.0. The studies also indicated a single class of low-affinity binding sites with a K_d of 1.7 ± 0.6 mM. The similarity between the K_d and the estimate for 50% of maximum effect on insulin binding suggests that binding to these low-affinity sites is responsible for the effect of chloroquine on the insulin–receptor interaction. However, calculation of the number of binding sites per mg of protein (2.27×10^{16}) and comparison with the number of insulin receptors (1.4×10^{12} per mg of protein) indicates that the ratio of chloroquine-binding sites to insulin-binding sites is $> 10000:1$. Thus it is unlikely that the interaction is specifically with the insulin receptor itself.

The kinetics of insulin dissociation seen in this study are very similar to those observed for the endosomal degradation of insulin [7]. Both are biphasic exponential events, described by almost identical kinetic parameters. The behaviour of both degradation and dissociation with respect to the presence of chloroquine are also very similar. In the case of endosomal degradation, increasing chloroquine concentration causes a progressive shift in the flux of degradation from the faster to the slower of the two exponential processes. Thus it would seem likely that some aspect of insulin dissociation is the rate-limiting step in endosomal insulin degradation and that chloroquine is acting by slowing dissociation. Previous studies [44,45] have shown that chloroquine can accumulate in lysosomal vesicles to give chloroquine concentrations in the millimolar range, thus achieving the concentrations found to be effective in this *in vitro* study. As discussed previously, the overall inhibition may also depend on the ability of chloroquine to accumulate in acidic organelles via proton trapping, raising the pH by neutralization

of the acidic contents. This elevated pH will then enable chloroquine to increase the affinity of the insulin–receptor interaction. This increase in affinity, with the associated decrease in receptor-mediated insulin degradation, could affect insulin signalling through the following mechanism. The presence of insulin on the receptor initiates autophosphorylation [46] and activation of the tyrosine kinase [47] and at the same time triggers endocytosis of the insulin–receptor complex [48]. In the absence of chloroquine, dissociation/degradation of insulin occurs [5,40] and the phosphotyrosine phosphatase can dephosphorylate the receptor [49], attenuating the signal and triggering return of endosome with free receptor to the cell surface [50]. In the presence of chloroquine, dissociation/degradation of receptor-bound insulin is slowed, increasing the half-life of the activated insulin–receptor complex. Thus, although the phosphotyrosine phosphatase(s) can dephosphorylate the receptor, the continuing presence of insulin on the receptor will induce rephosphorylation and thus potentiate the signal.

The present studies do not prove that the effects of chloroquine in patients with non-insulin-dependent diabetes are mediated through the insulin receptor. However, they clearly show that chloroquine has marked effects on the interaction of insulin with its receptor, which could easily give rise to alterations in the insulin/glucose homeostatic mechanisms and thus affect glucose balance in diabetics. The studies provide a basis for investigating the mechanisms by which chloroquine is exerting these effects, which may result in alternative treatment regimes for diabetes.

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APPENDIX

Expressions for negative co-operativity after DeMeyts [33]

Single-site binding is normally represented and analysed using the Scatchard equation (A1).

$$[B]/[F] = ([R_0] - [B]) \cdot K_a \quad (\text{A1})$$

where: [B] = bound ligand concentration, [F] = free ligand concentration, R_0 = total receptor concentration and K_a = the affinity constant.

According to DeMeyts, K_a is directly proportional to occupancy and therefore the relationship of K_a (inverse of K_d) to occupancy is given by:

$$K_a = \frac{K_e}{1 + \frac{[B]}{R_0} \cdot \left(\frac{K_e}{K_t} - 1 \right)}$$

where K_e = affinity when receptor occupancy = 0 and K_t = affinity when receptor occupancy = 1.

Substituting for K_a in the Scatchard equation we obtain

$$\frac{[B]}{[F]} = (R_0 - [B]) \times \frac{(K_e \cdot R_0 \cdot K_t)}{(R_0 \cdot K_t + [B] \cdot K_e - [B] \cdot K_t)} \quad (\text{A2})$$

However, the total bound [B] term contains a non-specifically bound element. The amount of non-specifically bound ligand is directly proportional to [F]; thus the amount of non-specifically bound ligand = $N \cdot [F]$, where $0 \leq N \leq 1$. Thus

$$\frac{[B]}{[F]} = N + \left[\{R_0 - ([B] - (N \cdot [F]))\} \times \frac{(K_e \cdot R_0 \cdot K_t)}{\{R_0 \cdot K_t + ([B] - (N \cdot [F])) \cdot K_e - ([B] - (N \cdot [F])) \cdot K_t\}} \right] \quad (\text{A3})$$

where N is the non-specific binding constant represented as a decimal fraction. Eqn. (A3) can be solved in terms of [B] to give eqn. (A4).

$$[B] = \frac{(2 \cdot [F] \cdot N) - ([F] \cdot K_t \cdot R) - \frac{K_t \cdot R}{K_e - K_t} - \frac{[F] \cdot K_t^2 \cdot R}{K_e - K_t} + \frac{\sqrt{K_t} \cdot \sqrt{(4 \cdot [F] \cdot K_e^2 + K_t - 2 \cdot [F] \cdot K_e \cdot K_t + [F]^2 \cdot K_e^2 \cdot K_t)} \cdot R}{K_e - K_t}}{2} \quad (\text{A4})$$