Fusicoccin Binding to Its Plasma Membrane Receptor and the Activation of the Plasma Membrane H\(^+\)-ATPase

I. Characteristics and Intracellular Localization of the Fusicoccin Receptor in Microsomes from Radish Seedlings

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ABSTRACT

The characteristics of fusicoccin binding were investigated in microsomes from 24-h-old radish (Raphanus sativus L.) seedlings. The time course of fusicoccin binding depended on fusicoccin concentration: equilibrium was reached much faster at 10 nanomolar fusicoccin than at 0.3 nanomolar fusicoccin. Scatchard analysis of equilibrium binding as a function of fusicoccin concentration indicated a single class of receptor sites with a K_d of 1.8 nanomolar and a site density of 6.3 picomoles per milligram protein. Similar values (K_d 1.7 nanomolar and site density 7 picomoles per milligram protein) were obtained from the analysis of the dependence of equilibrium binding on membrane concentration at fixed fusicoccin concentrations. Fusicoccin binding comigrated with the plasma membrane H\(^+\)-ATPase in an equilibrium sucrose density gradient; both activities formed a sharp peak (1.18 grams per milliliter) clearly distinct from that of markers of other membranes which all peaked at lower densities. The saturation profiles of fusicoccin binding and of fusicoccin-induced activation of the plasma membrane H\(^+\)-ATPase, measured under identical conditions, were similar, supporting the view that fusicoccin-induced activation of the plasma membrane H\(^+\)-ATPase is mediated by fusicoccin binding to its plasma membrane receptor.

The hypothesis that all the known physiological responses to FC\(^-\) depend on a primary activation of the plasma membrane H\(^+\)-ATPase (20) found recent support in the finding that FC is able to stimulate the hydrolytic and transport activities of the plasma membrane H\(^+\)-ATPase by acting at the membrane level in vesicles isolated from radish seedlings and other plant materials (9, 12, 14, 23, 24).

The stimulating effect of FC on the transport activity of the plasma membrane H\(^+\)-ATPase of radish seedlings has been characterized in some detail (23). The effect of FC is evident only in the pH range of the cytoplasm of higher plant cells (*i.e.* around pH 7.5) and displays structural requirements for the FC molecule similar to those for binding of the toxin to its specific membrane-bound receptors described in other plant materials and induction of the *in vivo* physiological responses (6, 7). Fusicoccin-induced activation of the plasma membrane H\(^+\)-ATPase is saturated by low FC concentrations similar to those required to saturate specific binding of FC to membranes isolated from different plant materials (1, 17, 18, 22).

These results support the view that FC-induced activation of the plasma membrane H\(^+\)-ATPase is a consequence of its binding to the receptor. However, the mechanism through which the binding of FC to the receptor activates the plasma membrane H\(^+\)-ATPase is still unknown. The study of the relations between the FC receptor and the plasma membrane H\(^+\)-ATPase is complicated by the fact that two distinct populations of receptors, with different affinities for FC, have been reported for microsomal membranes isolated from different plant materials (1, 3, 18, 22).

In this paper we show that microsomes isolated from 24-h-old radish seedlings contain a single type of FC receptor, localized at the plasma membrane and saturated by FC at concentrations very similar to those which saturate FC-induced activation of the plasma membrane H\(^+\)-ATPase. Part of these results have been presented elsewhere in a preliminary form (16).

MATERIALS AND METHODS

Plant Material

Radish seeds (*Raphanus sativus* L., cv Tondo Rosso Quarantino, Ingegnoli, Milano, Italy) were incubated in distilled water (5 g of seeds in 60 mL H\(_2\)O in a 750 mL Erlenmeyer flask) at 25°C in the dark in an agitated, thermoregulated water bath. The water was renewed after about 8 h incubation. After 22 h, the seed coats were removed and the seeds were incubated a further 2 h in 60 mL freshly renewed distilled water, blotted dry, weighed (fresh weight roughly doubled during the 24 h incubation), frozen and stored at −80°C.

Preparation of Microsomes

Frozen seeds were homogenized in 1.5 mL/g fresh weight of ice-cold, freshly prepared extraction medium (0.3 M su-
crose, 0.5% BSA, 3 mM ATP, 5 mM β-mercaptoethanol, 1 mM EDTA, 0.1 mM MgCl₂, 1 mM PMSF, 50 mM Tris adjusted to pH 8 with HCl) by gentle grinding with a mortar and pestle. The homogenate, diluted with extraction medium to a final ratio of 4 mL of medium per gram fresh weight, was filtered through four layers of cheesecloth, and centrifuged for 10 min at 1,000g. The supernatant was centrifuged for 15 min at 13,000g and the resulting supernatant was centrifuged at 30,000 rpm in a Beckman 50.2 Ti rotor for 50 min. The pellet was resuspended in washing medium (0.25 mM sucrose, 0.5% BSA, 3 mM ATP, 5 mM β-mercaptoethanol, 0.1 mM MgCl₂, 5 mM Tris [pH 7]) at 1 mL medium per gram fresh weight and centrifuged at 30,000 rpm (Beckman 50.2 Ti) for 30 min. The resulting pellet (washed microsomes) was resuspended with a syringe and a 26 gauge needle in resuspension medium (0.25 mM sucrose, 0.2% BSA, 0.5 mM β-mercaptoethanol, 1 mM BTP-Hepes [pH 7]) at a concentration of about 4 mg membrane protein per mL (corresponding to 10 g initial fresh weight per mL). The entire procedure was performed at 2 to 3°C. Membrane aliquots (0.5–1 mL) were immediately frozen and stored at −80°C.

Sucrose Gradient

Freshly prepared washed microsomes were resuspended in 0.25 mM sucrose, 1 mM EDTA, 3 mM DTT, 3 mM ATP, 3 mM Mes adjusted to pH 7.2 with Tris, at about 4 mg protein per mL. Two mL of membrane suspension were layered on top of a 36 mL linear sucrose density gradient (20–45% w/w) containing 3 mM ATP, 0.2 mM DTT, 3 mM Mes adjusted to pH 7.2 with Tris. The gradients were centrifuged for 15 h at 27,000 rpm (Beckman SW 28) at 2°C and 2.4 mL fractions were collected with an ISCO 640 density gradient fractionator. Corresponding fractions from three parallel gradients were pooled, diluted 1:1 with 2 mM MgSO₄ and 0.4% BSA, and centrifuged at 40,000 rpm (Beckman 50.2 Ti) for 30 min. The pellets were resuspended in 1 mL of resuspension medium without β-mercaptoethanol and immediately assayed for NADH-dependent Cyt c reductase, H⁺-pumping, and FC binding activities. Aliquots for oligomycin-sensitive ATPase and latent IDPase assays were frozen and stored at −80°C.

Fusicoccin Binding

Aliquots of membrane suspension (usually about 200 μg membrane protein per sample) were incubated in resuspension medium without BSA supplemented with 5 mM MgSO₄, [3H]dihydrofusicoccin (1.3 kBq/pmol, kindly supplied by Prof. G. Randazzo, Cagliari University, Italy) at 0.3 to 0.5 mM (unless otherwise specified) and FC to give the desired concentrations. The assay volume was adjusted to give the specified membrane protein concentrations in the assay. Samples were incubated in glass or polycarbonate tubes at 25°C for the specified times, then centrifuged for 30 min at 50,000 rpm (Beckman 50.2 Ti or 50 Ti), at 2°C unless otherwise noted. The supernatant was removed, the tubes carefully rinsed with distilled water and the pellets resuspended with 1 mL of distilled water. Ten mL of Aqualuma (Lumac) was added, and the samples were counted in a Packard Tricarb liquid scintillation counter. Non-specific binding, measured from the radioactivity associated with the pellets of samples incubated in the presence of 1 to 10 μM unlabeled FC, was subtracted from all the binding values to evaluate specific binding.

Proton Pumping

The initial rate of H⁺-pumping was measured by monitoring the quenching of fluorescence of quinacrine, with a Jasco FG-770 spectrophotometer. The incubation mixture contained 2 μM quinacrine, 0.5 mM EGTA, 5 mM MgSO₄, 0.1 M KBr, and 0.05 M KNO₃ in 17.5 mM Mes-Tris buffer (pH 6.6) for assays of H⁺-ATPase in gradient fractions, or 2 μM quinacrine, 0.2 mM EGTA, 5 mM MgSO₄, 0.15 M KBr, and 50 mM valinomycin in 40 mM BTP-Hepes buffer at pH 7.5 for FC-stimulated H⁺-ATPase assays. In both cases the reaction was initiated by addition of 3 mM Na-ATP from a 0.3 mM stock solution adjusted at the desired pH with NaOH.

Fusicoccin-induced activation of the H⁺-ATPase was assayed on membranes pretreated with or without FC at the specified concentrations for 15 min at 25°C under the same experimental conditions of FC binding assays, at 0.8 to 1 mg membrane protein per mL. At the end of this period the membranes were diluted into H⁺-pumping assay medium without added FC to 0.12 to 0.15 mg protein per mL and incubated at 25°C for 30 min to allow for transmembrane pH equilibration, before initiating the reaction by addition of ATP.

Other Assays

NADH-dependent Cyt c reductase was assayed as described (25), IDPase as described by Chanson et al. (10) except that assays were run at 25°C, and oligomycin-sensitive ATPase as the activity inhibited by 5 μg/mL oligomycin at pH 8.5 (15). Protein was determined as previously described (13).

Statistics

All the experiments were performed at least three times with three or more replicates. Since the activities were somewhat variable in different membrane preparations, results are from representative experiments. Variability of replicates within each experiment did not exceed ±4%.

RESULTS

Characteristics of FC Binding to Microsomal Membranes

Several requisites must be fulfilled before using Scatchard analysis of binding values measured by competitive displacement of radiolabeled ligand by unlabeled native ligand, to determine the affinity of a receptor for a ligand and the density of binding sites. In particular: (a) nonspecific binding must be correctly evaluated, (b) the ligand-receptor interaction must be at equilibrium at all the ligand concentrations tested, (c) the ligand concentration must be in excess of the receptor concentration, (d) the receptor must have the same affinity for the radioiodinated and for the unlabeled ligand (11, 21, 27). An initial set of experiments was thus performed to check the aforementioned in our experimental system.
Figure 1 shows that when microsomes from 24-h-old radish seedlings (12 μg protein per mL) are incubated in the presence of a fixed (0.34 nm) concentration of [3H]FC and increasing concentrations of unlabeled FC, the amount of radioactivity associated with the membranes decreases with the increase of unlabeled FC concentration, until a steady level corresponding to about 0.3% of total applied is reached, which is unaffected by further increasing the concentration of unlabeled FC. This low level of radioactivity associated with the membranes at higher FC concentrations (≥μM), which is independent of the length of incubation, represents unsaturable nonspecific binding of FC to the membranes. In all the experiments reported in the following, specific binding of FC was evaluated as the difference between radioactivity associated with the pellet at the relevant FC concentrations and that associated with the pellets of samples incubated in parallel in the presence of 1 to 10 μM unlabeled FC.

Figure 2 shows that the time course of specific binding of FC to the membranes depends on FC concentration in the manner expected for a bimolecular reaction between FC and its receptor: equilibrium is reached within 60 min when total FC concentration is 10 nm, but only after about 180 min when FC concentration is 0.34 nm. At the FC and membrane concentrations utilized in this experiment, binding of FC to the membrane accounts for less than 4% of the supplied FC, indicating that the ligand is in large excess with respect to receptor concentration even at the lowest FC concentration tested. Table I shows that FC binding is not influenced by a 20-fold increase in the specific activity of FC, indicating that the affinity of the receptor for [3H]dihydrofusicoccin is not significantly different from that for native fusicoccin.

To evaluate the dissociation constant (K\textsubscript{d}) and the site density of FC receptor(s) in our membrane fraction, we measured FC binding as a function of FC concentration in membrane samples (10 μg protein per mL) incubated for 3 h in the presence of FC concentrations ranging from 0.3 to 30 nm. The results (Fig. 3A) show that FC binding shows simple saturation behavior. When plotted according to Scatchard, the data fit a single straight line (Fig. 3B), which allows calculation of a K\textsubscript{d} value of 1.8 nm and a site density of 6.3 pmol receptor per mg protein.

A major pitfall in Scatchard analysis of binding data is the lack of an independent variable, as both binding and the bound/free ratio are experimentally determined parameters (11, 21, 27). An independent evaluation of K\textsubscript{d} and of the site density of FC receptor(s) can be obtained by measuring FC binding at equilibrium as a function of membrane concentration at various fixed concentrations of FC. As detailed in Eq. 4 of the Appendix, the relation between membrane concentration (mg protein/mL) and the fraction of bound FC (bound FC/total FC) can be derived from the mass law equation for the reaction between FC and its receptor:

\[
\frac{\text{mg prot}}{\text{mL}} = b \times \frac{\text{FC}_T}{\text{SD}} + \frac{b}{(1 - b)} \times \frac{K_\text{d}}{\text{SD}}
\]

where b represents the ratio: bound [FC]/total [FC] at equilibrium, FC\textsubscript{T} is the total FC concentration, and so the site density (i.e. the specific activity of the receptor). The relation between fractional binding (b) and protein concentration depends on the value of FC\textsubscript{T} relative to K\textsubscript{d} when the term \(b \times \text{FC}_T/\text{SD}\) is high with respect to \(b/(1-b) \times K_\text{d}/\text{SD}\), Eq. 4 simplifies to:

\[
\frac{\text{mg prot}}{\text{mL}} = b \times \frac{\text{FC}_T}{\text{SD}}
\]

**Table I. Independence of FC Binding on the Concentration of [3H] Dihydrofusicoccin**

<table>
<thead>
<tr>
<th>Concentration (nm)</th>
<th>Total FC concentration (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]FC</td>
<td>3</td>
</tr>
<tr>
<td>1.5</td>
<td>3.8</td>
</tr>
<tr>
<td>3.0</td>
<td>3.5</td>
</tr>
<tr>
<td>10.0</td>
<td>ND</td>
</tr>
<tr>
<td>30.0</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Not determined.
which means that under these conditions bound FC is linearly related to the receptor concentration. The linear relation between \( b \) and protein concentration holds for higher protein concentrations and \( b \) values the higher FC\( T \) is with respect to \( K_d \). Conversely, when FC\( T \) is small with respect to \( K_d \) (FC\( T / K_d < 0.1 \)), the term \( b \times FC_T \) becomes negligible. Eq. 4 simplifies to:

\[
\frac{\text{mg prot}}{\text{mL}} = \frac{b}{(1-b)} \times \frac{K_d}{SD}
\]

and the relation between \( b \) and protein concentration becomes hyperbolic.

Figure 4 shows the relation between \( b \) and membrane protein concentration, measured in the presence of two FC concentrations. When FC is supplied at 0.86 nM, the relation between \( b \) and protein concentration is roughly exponential, while when FC concentration is raised to 10 nM the relation between \( b \) and protein concentration is linear at least up to a protein concentration of about 0.5 mg protein/mL, corresponding to a \( b \) value of about 0.3. These results are fully consistent with a \( K_d \) value intermediate between the two FC concentrations assayed, as indicated by Scatchard analysis (Fig. 3B).

Eq. 4 can be solved for any \( i \) value of \( b \) leading to Eq. 5 of the Appendix:

\[
\left( \frac{\text{mg prot}}{\text{mL}} \right)_{i/b} = \frac{b_i}{SD} \times FC_T + \frac{b_i}{(1-b_i)} \times \frac{K_d}{SD}
\]

which shows that for a single class of noncooperative binding sites (\( i.e. \) \( SD \) and \( K_d \) constant) the membrane concentration corresponding to a given \( i \) value of \( b \) is linearly related to FC\( T \), with a slope depending on the site density (slope = \( b_i/SD \)) and a \( y \) intercept depending on both the site density and the \( K_d \) (\( y \) intercept = \( b_i/(1-b_i) \times K_d/SD \)).

If we assume that such a linear relation holds for our experimental system, the approximate values of \( SD \) and \( K_d \) can be calculated from the membrane concentrations (extrapolated from Fig. 4) corresponding to any given \( i \) value of \( b \) at the two FC\( T \) tested. This has been done for seven different values of \( b \) ranging from 0.1 to 0.7 and the results are shown in Table II. The various values of \( SD \) and of \( K_d \) so obtained are remarkably similar, thus indicating that our data can be satisfactorily described as representing binding to a single class of receptor, with constant \( K_d \). The mean site density (7 pmol per mg protein) and \( K_d \) (1.7 nM) values are very similar to those independently obtained from the Scatchard plot of binding measured at constant receptor concentration, with varying FC concentrations. Thus, no evidence for the presence of more than one class of FC receptors in this microsomal fraction comes from the measurements of affinity of the receptor for FC. The discrepancy between these data and those reported in the literature for microsomes from other plant materials (1, 3, 18, 22) will be discussed in the “Discussion” section.

**Membrane Localization of the FC Receptor**

The equilibrium distribution of FC binding in a sucrose density gradient was compared with that of marker enzymes of different cell membranes.

Figure 5 shows that the different cell membranes form quite distinct peaks. The distribution of marker enzymes for the plasma membrane (plasma membrane H+-ATPase) and for the ER (NADH-dependent Cyt c reductase) is qualitatively similar to that previously reported (25), but the peaks for both activities are at higher densities. This difference depends on the fact that the previously reported gradient, which was run for only 3 h, had not reached equilibrium (data not shown). A striking feature of this gradient is the low density of the peak of the mitochondrial marker (oligomycin-sensitive ATPase [pH 8.5]), which reflects the development of the inner membrane of mitochondria in the seedlings at this early stage of germination (28).

Fusicoccin binding forms a distinct peak in the denser zone of the gradient (1.18 g/mL), virtually coincident with that of
Table II. Evaluation of the Site Density and Dissociation Constant of FC Binding Sites from the Analysis of Binding as a Function of Membrane Concentration

The membrane concentrations corresponding to the selected \( i \) values of \( b \) at 0.86 and 10 nm FC were extrapolated from Figure 4. The values of \( b/so \) and \( b/(1-b) \times K_d/so \) were computed assuming a linear relation between the membrane concentrations and FC, at each \( i \) value of \( b \) (see Eq. 5).

<table>
<thead>
<tr>
<th>( b )</th>
<th>FC</th>
<th>( b/so )</th>
<th>so</th>
<th>( b/1-b \times K_d/so )</th>
<th>( K_d/so )</th>
<th>( K_d )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.04</td>
<td>0.17</td>
<td>0.0142</td>
<td>7.03</td>
<td>0.028</td>
<td>0.252</td>
</tr>
<tr>
<td>0.2</td>
<td>0.08</td>
<td>0.34</td>
<td>0.0284</td>
<td>7.03</td>
<td>0.056</td>
<td>0.224</td>
</tr>
<tr>
<td>0.3</td>
<td>0.15</td>
<td>0.53</td>
<td>0.0416</td>
<td>7.22</td>
<td>0.114</td>
<td>0.266</td>
</tr>
<tr>
<td>0.4</td>
<td>0.21</td>
<td>0.73</td>
<td>0.0569</td>
<td>7.03</td>
<td>0.161</td>
<td>0.242</td>
</tr>
<tr>
<td>0.5</td>
<td>0.31</td>
<td>0.98</td>
<td>0.0733</td>
<td>6.82</td>
<td>0.247</td>
<td>0.247</td>
</tr>
<tr>
<td>0.6</td>
<td>0.43</td>
<td>1.22</td>
<td>0.0864</td>
<td>6.94</td>
<td>0.356</td>
<td>0.237</td>
</tr>
<tr>
<td>0.7</td>
<td>0.62</td>
<td>1.51</td>
<td>0.0974</td>
<td>7.19</td>
<td>0.536</td>
<td>0.230</td>
</tr>
<tr>
<td>Mean ( \pm ) SE</td>
<td>7.04 ( \pm ) 0.05</td>
<td>0.243 ( \pm ) 0.04</td>
<td>1.70 ( \pm ) 0.04</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

30 min to allow for pH equilibration between the medium and the intravesicular volume, prior to initiate H\(^+-\)pumping by addition of ATP. Centrifugation of the membranes for the measurement of FC binding was thus performed at 25°C after dilution in H\(^+-\)-pumping medium. The results reported in Figure 6 show that under these conditions FC binding and FC-induced activation of the plasma membrane H\(^+-\)ATPase are both saturated at about 40 nM FC. The relatively low activation of the plasma membrane H\(^+-\)ATPase observed at the lowest FC concentrations tested is discussed below.

**DISCUSSION**

In the present work the \( K_d \) and the concentration of the FC receptor in microsomes isolated from 24-h-old radish seedlings were determined both by Scatchard analysis of equilib-

### Figure 5
Separation of membranes on a linear sucrose density gradient. Data are expressed as percentage of the peak values. Values of the peak fractions were: 13.8 nmol/min per fraction for NADH-Cyt c reductase (△); 4.2 μmol/h per fraction for latent IDPase (△); 4.4 μmol/h per fraction for oligomycin-sensitive ATPase (○), 260% quenching/min per fraction for H\(^+-\)-pumping driven by the plasma membrane H\(^+-\)ATPase (○) and 4.6 pmol per fraction for specific binding of FC (○) measured over 2 h of incubation in the presence of 1 nM FC. All assays were performed as described in "Materials and Methods."

### Figure 6
Comparison between the dependence of FC binding (○) and of stimulation of H\(^+-\)pumping driven by the plasma membrane H\(^+-\)ATPase (○) on the concentration of FC. Membranes (0.8 mg membrane protein per mL) were treated with FC at the indicated concentrations for 15 min, then diluted into H\(^+-\)-pumping assay medium (to 0.12 mg membrane protein per mL) and either centrifuged for 30 min at 25°C (FC binding measurements) or incubated at 25°C for 30 min before initiating H\(^+-\)pumping by the addition of ATP. Data are expressed as percentage of maximum values, which were 3.9 pmol/mg protein for FC binding and 105% stimulation for the activation of H\(^+-\)-pumping.

### Relationships between FC Binding and Activation of the Plasma Membrane H\(^+-\)ATPase

The concentration dependence of FC binding of the FC-induced activation of the plasma membrane H\(^+-\)ATPase were compared under identical conditions. Since membrane vesicles become leaky to protons upon prolonged incubation at 25°C, binding was not measured at equilibrium but rather after 15 min of incubation at 25°C. For the measurements of H\(^+-\)pumping this treatment was followed by dilution of the membranes in H\(^+-\)-pumping medium at pH 7.5 (details in "Materials and Methods") and further incubation at 25°C for
rium binding as a function of FC concentration and by analysis of equilibrium binding as a function of membrane concentration at fixed FC concentrations. The two independent experimental approaches lead to very similar estimates of $K_d$ (1.8 and 1.7 nM) and of the density of binding sites (6.3 and 7.0 pmol per mg membrane protein), strongly supporting the conclusion that a single class of FC receptors is present in the microsomal membranes.

This result is in contrast with that reported for microsomes from other plant materials, where two distinct classes of receptor sites, endowed with different affinities for FC, have been reported (1, 3, 18, 22). This discrepancy might arise from the young developmental stage of radish seedlings at which our microsomes were extracted. However, this possibility seems unlikely, if both classes of receptor sites are involved in FC biological action, given the full capability of radish seedlings at this or earlier stages of germination to respond to in vivo FC treatments (5, 19). It seems possible that the two classes of binding sites reported in other plant materials represent two different states of the FC receptor, possibly derived during the extraction procedure due to the activity of hydrolytic enzymes (2). In fact, the apparent $K_d$ values for the two classes of binding sites reported for membranes isolated from the same plant material, are fairly variable, ranging from 0.3 to 1.2 nM for the high affinity site in maize coleoptiles, and the relative incidence of the higher affinity site is also variable (ranging from 2 to 70% of total binding sites) (1, 3, 22).

The FC receptor comigrates with the plasma membrane H+-ATPase in a sucrose density gradient, thus confirming the previous observation (8, 17, 18, 26) that it is localized at the plasma membrane.

Comparison of the dependence of FC binding and FC-induced activation of the plasma membrane H+-ATPase on FC concentration under identical conditions shows that the two phenomena show similar behavior, being saturated at about 40 nM FC (Fig. 6). The finding that at low FC concentrations FC-induced activation of the plasma membrane H+-ATPase is relatively lower than FC binding, might suggest that a threshold fraction of receptors must be occupied before a biological response can be observed (11). However, an alternative explanation might arise from the fact that the binding data reflect the binding of FC to plasma membrane vesicles independently of their leakiness and orientation, while H+-pumping measurements reflect only the activity of sealed inside-out plasma membrane vesicles. Since FC binding sites are exposed to the outer side of the plasma membrane (4, 18), FC permeation through the plasma membrane might be the rate-limiting step of FC binding to the receptor in inside-out plasma membrane vesicles. This would be of relevance at the lowest FC concentrations tested, for which binding is far from equilibrium, while it would not influence the data obtained at higher FC concentrations, for which equilibrium is reached within the length of this experiment (Fig. 2). So, the results reported in Figure 6 are consistent with the view that FC-induced activation of the plasma membrane H+-ATPase is the consequence of FC binding to its plasma membrane receptor. This conclusion is strengthened by the recent finding that FC is able to stimulate MgATP-dependent H+-pumping in proteoliposomes reconstituted with partially purified plasma membrane H+-ATPase and FC receptor (P. Aducci, personal communication).

**APPENDIX**

According to the simplest model describing the ligand (L)-receptor (R) interaction, the reaction between $L$ and $R$ is a simple bimolecular reaction: $L + R \leftrightarrow LR$. At equilibrium:

$$ \frac{L \times R}{LR} = K_d $$

where $L$, $R$, and $LR$ represent the equilibrium concentrations, respectively, of free ligand, free receptor, and ligand-receptor complex; $K_d$ is the dissociation constant.

Since $L = LR - LR$ (where $T$ stands for total concentration), Eq. 1 can be rewritten to give:

$$ R = \frac{b}{(1 - b)} \times K_d $$

where $b = LR/L_T$. Since: $R = R_T - LR = R_T - b \times L_T$, it comes:

$$ R_T = b \times L_T + \frac{b}{(1 - b)} \times K_d $$

This relation can be divided by the site density, i.e., the specific activity of the receptor, $S_D$, leading to the relation:

$$ \frac{R_T}{S_D} = \text{mg prot} \frac{mL}{mL} = b \frac{L_T}{S_D} + \frac{b}{(1 - b)} \times K_d \frac{S_D}{S_D} $$

For any $b$ value of $b$ we can write:

$$ \left( \frac{\text{mg prot}}{S_D} \right) = \text{mg prot} \frac{S_D}{S_D} \frac{L_T}{S_D} + \frac{b}{(1 - b)} \times K_d \frac{S_D}{S_D} $$

which shows that the relation between the membrane concentration corresponding to a given $j$ value of $b$ and $L_T$ is linear. The values of $S_D$ and of $K_d$ can be evaluated, respectively, from the slope ($b/(1 - b) \times K_d/S_D$) and the $y$ intercept ($b/(1 - b) \times K_d/S_D$) of the straight line relating the membrane concentration to $L_T$.

**LITERATURE CITED**

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