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Chloroquine, Quinine and Quinidine Inhibit Calcium Release From Macrophage Intracellular Stores by Blocking Inositol 1,4,5-Trisphosphate Binding to Its Receptor

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Abstract The binding of many ligands to cellular receptors induces a signaling cascade which generates inositol 1,4,5-trisphosphate (IP_3). IP_3 binding to its receptors in various internal compartments causes a rapid Ca^{2+} efflux into the cytosol. We now demonstrate that chloroquine blocks ligand-induced Ca^{2+} mobilization without affecting IP_3 synthesis. The effect is independent of the ligand employed and occurred with five unrelated ligands; namely, α_2-macroglobulin-methylamine, angiotensin II, bradykinin, carbachol, and epidermal growth factor. Chloroquine, quinidine, and quinine, however, block binding of [^{3}H]IP_3 to its receptors by 90%, 88%, and 71%, respectively. These observations suggest a previously undetected mechanism by which these agents may in part function as antimalarials. J. Cell. Biochem. 64:225–232.

Key words: chemotherapy of malaria; inositol trisphosphate receptors; chloroquine; cytosolic calcium; endocytosis

A common mechanism by which ligands activate receptor-mediated signaling cascades is to induce synthesis of inositol 1,4,5-trisphosphate (IP_3) [Berridge, 1993]. IP_3 binding to specific receptors on the membranes of various intracellular compartments then results in a rapid efflux of Ca^{2+} from these stores thus increasing the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$ [Berridge, 1993; Marshall and Taylor, 1993]. The IP_3 receptor consists of four subunits and the complex both binds IP_3 and contains the Ca^{2+} channel which becomes activated on ligand binding [Berridge, 1993; Marshall and Taylor, 1993]. The binding of IP_3 to its receptors is believed to induce a conformational change in the receptor which opens the Ca^{2+} channel [Mignery and Südhof, 1990]. We have recently described a signaling receptor which binds activated forms of the proteinase inhibitor α_2-macroglobulin (α_2M*). Ligation of the α_2M* signaling receptor (α_2M*SR) by α_2M* or a cloned and expressed receptor binding fragment induces rapid synthesis of IP_3 with a subsequent rise in $[Ca^{2+}]_i$ [Misra et al., 1993; Misra et al., 1994a,b]. While employing chloroquine to block endocytosis of α_2M* by macrophages [Howard et al., 1996], we also noted that this reagent abolishes α_2M*-induced increases in $[Ca^{2+}]_i$ by these cells. This unpublished observation has led us to reconsider the mechanism of action of chloroquine on malarial parasites and other cells.

Chloroquine remains a frontline drug in the treatment of malaria, although *Plasmodium* species resistant to chloroquine are now appearing with increasing frequency [Borst and Ouellete, 1995; Krogstad and Schlesinger, 1987]. Chloroquine is a weak base which is able to increase the pH of lysosomes of mammalian cells and the phagocytic vesicles of *Plasmodium* species [Ginsburg and Geary, 1987; Brown, 1993]. The mechanism by which chloroquine

Abbreviations used: IP_3, inositol 1,4,5-trisphosphate; $[Ca^{2+}]_i$, intracellular calcium concentration; α_2M, α_2-macroglobulin; α_2M*, activated forms of α_2M which bind to cellular receptors; α_2MSR, the α_2M* signaling receptor; EGF, epidermal growth factor; Fura-2/AM, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2(-amino-5(-methylphenoxy) ethane-N,N,N(,N(-tetra-acetic acid acetoxymethyl ester.

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kills malarial organisms is believed to involve at least in part this alkalinization of the phagocytic vesicles in the parasites [Ginsburg and Geary, 1987; Brown, 1993]. The change in pH inhibits acid proteinases employed by the parasites to digest host hemoglobin which is essential for supplying the organism with amino acids. Other mechanisms by which chloroquine may exert its actions include its ability to intercalate into DNA and its ability to bind to ferriprotoporphyrin IX in the phagocytic vesicles of the parasite [Ginsburg and Geary, 1987; Brown, 1993]. These effects appear to promote red cell and plasmodial lysis. Both alkalinization of phagocytic vesicles and binding to porphyrins occurs at micromolar concentrations of chloroquine; however, DNA binding occurs only at a drug concentration of about 1–2 mM. Chloroquine has been shown to affect a number of metabolic events in cells and their organelles [Kubo and Hostetler, 1985; Hurst et al., 1986; Ginsburg and Geary, 1987; Jancinová et al., 1994; Nosál, et al., 1991, 1995]. Among these effects, the drug inhibits lysosomal phospholipase A_1 promoting lysosomal storage of both acidic and neutral phospholipids [Pappu and Hostetler, 1983; Kubo and Hostetler, 1995] and it inhibits platelet phospholipase A_2 in thrombin-stimulated platelets [Nosál et al., 1995] thereby affecting the arachidonic acid pathway. Chloroquine affects phospholipid turnover in stimulated mast cells inhibiting release of histamine and calcium uptake [Pecivová et al., 1994]. The compound has also been reported to inhibit cAMP-dependent protein kinase [Wang et al., 1991].

The current investigation probes the observation that chloroquine blocks the rise in macrophage $[Ca^{2+}]_i$ triggered by α_2M* and demonstrates that the phenomenon is independent of the ligand employed to induce a rise in $[Ca^{2+}]_i$. Our studies suggest that the compound does not block IP_3 synthesis but its effect is directly dependent on the ability of chloroquine to block IP_3 binding to its receptor.

MATERIALS AND METHODS

Reagents

Sources of reagents have been described in our previous publications [Misra et al., 1993; Misra et al., 1994a,b; Howard et al., 1996]. α_2M was activated by use of methylamine and characterized with regard to purity, receptor-recognition and signaling properties as described [Misra et al., 1993; Misra et al., 1994a,b; Howard et al., 1996].

Digital Imaging Microscopy of Fura-2/AM Preloaded Macrophages

Murine peritoneal macrophages were incubated overnight on glass coverslips in RPMI 1640 medium containing 5% fetal bovine serum with glutamine, 1 mM, penicillin, 12.5 units/ml, and streptomycin, 6.5 µg/ml, at 37°C in a humidified CO_2 (5%) incubator as reported previously [Misra et al., 1993; Misra et al., 1994a; Misra et al., 1994b]. Fura-2/AM (1–1.5 µM) was then added and the culture dishes incubated for 30 min. The monolayers were washed 3 times in Hanks' balanced salt solution containing Hepes, 10 mM, and $NaHCO_3$, 3.5 mM (pH 7.4) and then placed on the stage of the digital imaging microscope for determination of $[Ca^{2+}]_i$ [Misra et al., 1993; Misra et al., 1994a; Misra et al., 1994b]. Basal $[Ca^{2+}]_i$ levels in the Fura-2/AM preloaded cells were measured for 3 min prior to addition of the stimulant. Thapsigargin (100 nM) was employed to further probe the mechanism by which chloroquine blocks ligand-induced increases in $[Ca^{2+}]_i$ in macrophages. Thapsigargin is a sesquiterpene lactone derived from plants which specifically inhibits Ca^{2+} transport and blocks reuptake of cytosolic Ca^{2+} into both IP_3-sensitive and insensitive storage pools [Thastrup et al., 1990; Bian et al., 1991].

IP_3 Synthesis

IP_3 synthesis was measured as previously described employing [2-^{3}H] myo-inositol (specific activity, 10–20 Ci/mmol) as previously described [Misra et al., 1993; Misra et al., 1994a,b; Howard et al., 1996]. For each determination 4×10^6 cells were employed.

IP_3 Binding to Microsomal Preparations

The assay was performed as described by Mignery et al. [1989, 1990] except that macrophages were substituted for brain tissue to obtain the microsomal preparations. Macrophages ($10–12 \times 10^6$ cells) were incubated in a humidified CO_2 (5%) incubator for 16 to 18 h at 37°C in RPMI 1640 medium containing the additions listed above. Monolayers were washed three times with cold Hepes, 10 mM, and

$NaHCO_3$, 3.5 mM (pH 7.2). To the monolayers was then added a buffer containing Tris-HCl, 50 mM, EDTA, 1 mM, phenylmethylsulfonyl fluoride, 1 mM, and leupeptin, 10 µM (pH 8.3). The cells were scraped off the tissue culture wells into tubes and maintained at 4°C. The suspensions were then passed through a sterile 27 gauge needle 40 to 45 times on ice and the lysate centrifuged at 289,000*g* for 30 min at 4°C. The pellet was suspended in another volume of the Tris-HCl buffer described above and the protein content measured [Bradford, 1976]. The binding of [^{3}H]IP3 (New England Nuclear, specific activity 21 Ci/mmol) to membranes (75–100 µg protein in a volume of 100 µl) was studied in 1.5 ml Eppendorf tubes at 4°C under the desired conditions. For competition studies, [^{3}H]IP_3 (5 pM) was incubated with unlabeled IP_3, chloroquine, quinine, or quinidine at the concentrations indicated in the text and figure legends. After incubation, the tubes were centrifuged in an Eppendorf microcentrifuge for 4 min at 4°C and the pellet washed four times with centrifugation in the Tris-HCl buffer described above. The pellet was then dissolved in a volume of 1N NaOH and radioactivity determined by liquid scintillation counting. The concentration of [3_H]IP_3 was chosen based on the measured K_d for binding to the IP_3 receptor preparation. The rationale for the concentrations of antimalarials and other compounds tested in these studies is based on the known pharmacology of antimalarials [see for example, White, 1988]. Tissue concentrations of chloroquine at the time of drug administration range from 100 to 500 µM.

RESULTS

Chloroquine and Ligand-Induced Increases in Macrophage $[Ca^{2+}]_i$

Binding of α_2M-methylamine to murine peritoneal macrophages causes a biphasic increase in $[Ca^{2+}]_i$ [Misra et al., 1993, 1994a,b]. The initial increase results from IP_3-induced release of Ca^{2+} from intracellular Ca^{2+} stores while the later increase is due to entry of Ca^{2+} from the medium by a capacitative mechanism [Misra et al., 1993]. Incubation of intact murine peritoneal macrophages with chloroquine (75 µM) for 5 min at 37°C prior to stimulation of the cells with α_2M -methylamine (100 nM) completely suppressed the initial IP_3-induced increase in $[Ca^{2+}]_i$ (Fig. 1a, Table I). However, chloroquine had little or no effect on the capacitative entry of Ca^{2+} from the medium (Fig. 1a). Since capacitative Ca^{2+} entry is believed to be activated by the depletion of Ca^{2+} from internal stores [Berridge, 1993; Marshall and Taylor, 1993; Putney and Bird, 1993], this latter observation was unexpected.

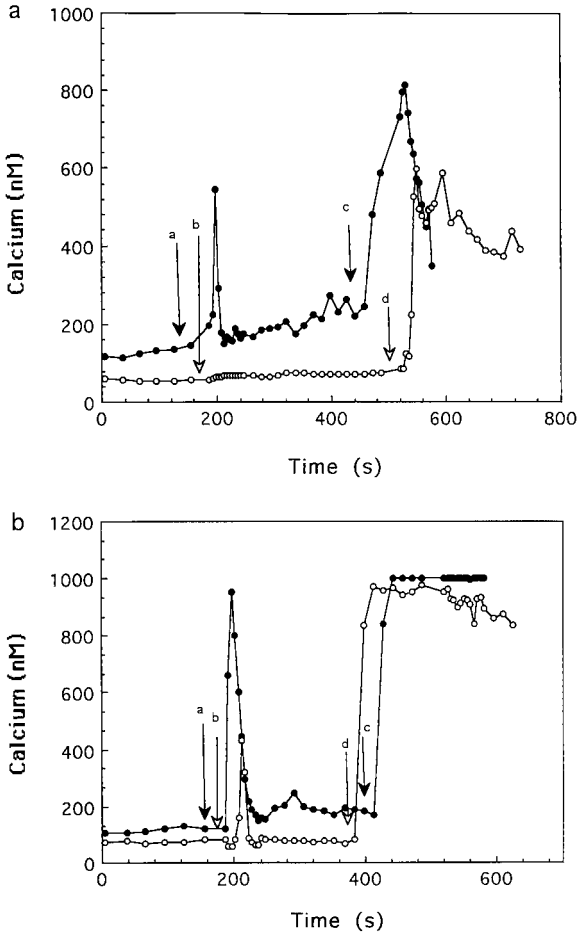

Fig. 1. $[Ca^{2+}]_i$ in intact macrophages stimulated with α_2M-methylamine or thapsigargin in the presence and absence of chloroquine. **a**: Compares the effect of α_2M-methylamine (100 nM) added at arrow "a" alone (●) or added at arrow "b" to cells pretreated with chloroquine (75 µM) for 5 min prior to the addition of ligand (○). Extracellular calcium (1 mM), was added at arrow "c" in chloroquine-untreated cells and at arrow "d" in chloroquine-treated cells. **b**: Thapsigargin (100 mM) was added at arrow "a" in the absence (●) or at arrow "b" in the presence (○) of chloroquine (75 µM). Extracellular calcium (10 nM) was added at arrow "c" in chloroquine-untreated cells and at arrow "d" in chloroquine-treated cells. The Ca^{2+} concentrations chosen for these studies were based on previous protocols for use of α_2M-methylamine [Misra et al., 1993] and thapsigargin [Thastrup et al., 1990]. For both panels a and b between 200 and 250 cells were studied in four separate experiments. The single cells are representative of typical responses. Table I shows the averaged data for all cells studied.

TABLE I. Changes in $[Ca^{2+}]_i$ in Macrophages Treated with Ligands in the Presence and Absence of Chloroquine

Additions	n	Cell number studied	Cells responding (%)	$[Ca^{2+}]_i$ nM	
				Basal	Peak
Chloroquine (75 μM)	2	50–60	—	74.41 ± 4.28	72.99 ± 3.91
α_2M-methylamine (100 nM)	4	200–250	90–95	146.14 ± 9.28	358.15 ± 20.75
α_2M-methylamine + Chloroquine (75 μM)	4	250–300	—	56.61 ± 0.98	59.91 ± 1.42
Angiotensin II (100 nM)	2	30–35	90–95	59.88 ± 6.36	136.68 ± 8.34
Angiotensin II + Chloroquine (75 μM)	2	45–50	—	59.70 ± 2.72	58.93 ± 3.53
Bradykinin (50 nM)	2	45–50	90–95	74.14 ± 5.46	185.09 ± 9.83
Bradykinin + Chloroquine (75 μM)	2	30–35	—	62.70 ± 3.38	60.32 ± 3.43
Carbachol (100 μM)	2	30–35	90–95	62.70 ± 3.38	353.03 ± 40.36
Carbachol + Chloroquine (75 μM)	2	25–30	—	68.70 ± 2.25	67.29 ± 3.12
EGF (100 ng/ml)	3	50–55	80–85	108.16 ± 3.93	263.55 ± 25.00
EGF + Chloroquine (75 μM)	2	25–30	—	56.92 ± 1.38	57.19 ± 2.15

In order to further define these effects, experiments were then performed with thapsigargin. This compound has been used extensively to study the relationship of the filling status of intracellular Ca^{2+} stores and capacitative Ca^{2+} entry. Thapsigargin causes release of Ca^{2+} from intracellular Ca^{2+} stores independently of IP_3 [Thastrup et al., 1990; Bian et al., 1991; Mignery et al., 1989]. Chloroquine pretreatment of macrophages prior to addition of thapsigargin resulted in a significant decrease in the thapsigargin-dependent increase in $[Ca^{2+}]_i$ but little effect on capacitative entry (Fig. 1b). Chloroquine itself did not cause an increase in $[Ca^{2+}]_i$ but rather a decrease in basal levels of $[Ca^{2+}]_i$ (Table I). These studies suggest that one effect of chloroquine may be to increase leakiness of cellular membranes to Ca^{2+}. This leakiness could result in internal Ca^{2+} stores emptying and thus triggering Ca^{2+} entry from the extracellular medium. This effect was not further explored in the present study.

The effect of chloroquine on IP_3-induced increases in $[Ca^{2+}]_i$, however, was studied in greater detail by probing its effect on the increase in macrophage $[Ca^{2+}]_i$ induced by other ligands. Four unrelated ligands angiotensin II, bradykinin, carbachol (carbamylcholine), and epidermal growth factor (EGF) were chosen for study. In each case, chloroquine inhibited the IP_3-dependent rise in $[Ca^{2+}]_i$ induced by these ligands (Table I). These observations suggest that the effect of chloroquine on IP_3-dependent Ca^{2+} stores is independent of the nature of the agonist employed to stimulate the cells. Chloroquine did not significantly affect α_2M-methylamine-induced IP_3 synthesis by stimulated macrophages (Fig. 2b). These results suggest that chloroquine exerts its effect subsequent to IP_3 synthesis. Given the role of chloroquine as an antimalarial, we also studied the effects of quinine and quinidine on α_2M-methylamine-induced increases in IP_3 synthesis and $[Ca^{2+}]_i$. Both agents also suppressed ligand-induced increases in $[Ca^{2+}]_i$ as effectively as chloroquine (data not shown) without affecting IP_3 synthesis (Fig. 2b).

Chloroquine and IP_3 Binding to Its Receptor

One possible mechanism by which chloroquine, quinine, and quinidine might exert their action is at the level of the IP_3 receptor. We, therefore, tested the effect of chloroquine on the binding of $[^3H]IP_3$ to IP_3 receptors in preparations of microsomal vesicles from macrophages. The maximal binding of $[^3H]IP_3$ to this microsomal preparation was achieved within 5 min and thereafter plateaued (Fig. 3a). Unlabeled IP_3 competed for the binding of $[^3H]IP_3$ to the preparation (Fig. 3). The binding of $[^3H]IP_3$ was concentration-dependent (Fig. 3b) and Scatchard analysis demonstrated a single class of binding sites with a K_d of 4.3 ± 0.6 pM (Fig. 3c). Chloroquine inhibited the binding of $[^3H]IP_3$ to IP_3 receptors in the microsomal preparation in a dose-dependent manner (Fig. 3d) and maximal inhibition under the experimental conditions was achieved at ~100 μM chloroquine (Fig. 3d). The maximal inhibition of $[^3H]IP_3$ by chloroquine showed some variation between microsomal preparations. For this reason, all of the experiments shown in Figure 3 were performed

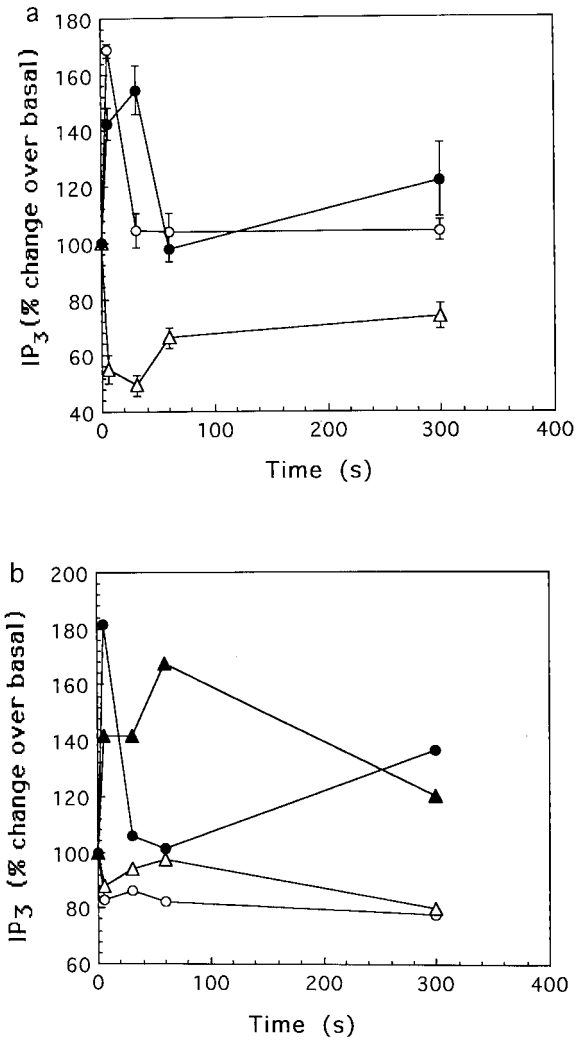

Fig. 2. IP_3 synthesis by intact macrophages treated with α_2M-methylamine in the presence and absence of chloroquine. **a**: The formation of IP_3 in macrophages stimulated with α_2M-methylamine (100 nM) in the absence (●) or presence (○) of chloroquine (75 µM). The sharp rise of IP_3 in the absence of chloroquine is consistent with previous studies (Misra et al., 1993, 1994a,b). Chloroquine added alone (△) caused a decrease in IP_3 synthesis. The values reported are the mean (SEM from three separate experiments. **b**: Either quinine (●) or quinidine (▲) was substituted for chloroquine in duplicate experiments where macrophages were treated with α_2M-methylamine (100 nM). Like chloroquine, quinine (○) or quinidine (△) added without ligand somewhat suppressed IP_3 synthesis. For each experiment chloroquine quinine and quinidine were added 5 min before the assay as in Figure 1.

on the same day. In this particular study chloroquine, 100 µM, blocked 70% of the binding of the $[^3H]IP_3$. The range of inhibition observed in multiple experiments with different microsomal preparations was 70% to 90%.

Like chloroquine, quinine (100 µM), and quinidine (100 µM) also inhibited $[^3H]IP_3$ binding to the IP_3 microsomal preparation. When the ability of chloroquine, quinidine, and quinine to inhibit $[^3H]IP_3$ binding to IP_3 receptors was examined with the same preparation of macrophage microsomes at the same time, these compounds caused a 90%, 88%, and 71% inhibition, respectively of the binding of $[^3H]IP_3$ to its receptor. We also studied the effects of a number of other compounds containing one or more rings with aromatic character on $[^3H]IP_3$ binding to the receptors in the microsomal preparation. Pyrimidine, purine, α-naphthol, and 1,4-benzoquinone, all 100 µM, decreased $[^3H]IP_3$ binding to the microsomal preparation by 11, 7, 30, and 20%, respectively suggesting that there is specificity to this competitive effect.

DISCUSSION

The data presented here raise implications pertinent to the therapy of malaria and the mechanism by which chloroquine affects endocytosis of receptor-ligand complexes by mammalian and other cells. The present study suggests that chloroquine and other antimalarials may have an additional mode of action other than effects on intracellular pH, binding to ferriprotoporphyrin II or DNA intercalation; namely, abrogation of IP_3-dependent signal transduction events mediated by $[Ca^{2+}]_i$. The potential effects of disrupting IP_3 binding to its receptors in *Plasmodium* species is at present speculative. These organisms, however, require IP_3 synthesis to regulate a number of cellular processes not unlike other organisms. Ogwan'g et al. [1993] have shown, for example, that IP_3 synthesis is essential to malarial gamete formation. They found that both chloroquine and quinine at concentrations of 500 and 250 µM, respectively completely blocked gamete formation [Ogwan'g et al., 1993]. These investigators did not determine the EC_{50} for these antimalarials, but clearly the concentrations employed are not significantly greater than those where we observed the blockade of IP_3 binding to its receptors. Moreover, *Plasmodium* species highly regulate their intracellular concentrations of both Na^+ and Ca^{2+} [Ginsburg, 1990; Tanabe, 1990]. The intracellular concentrations of these cations increases at the expense of K^+ and it seems likely that the elevated Na^+ and Ca^{2+} levels are important for the function of malarial

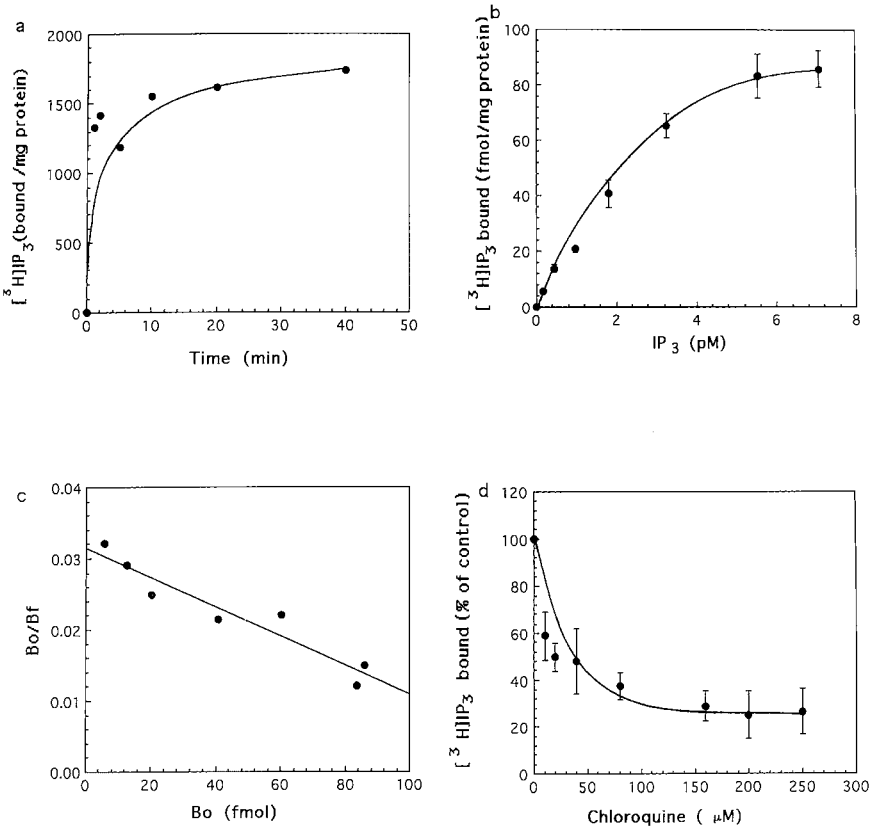

Fig. 3. The binding of $[^3H]IP_3$ to IP_3 receptors in macrophage microsomal preparations. **a**: A time course study performed to determine equilibrium binding conditions for $[^3H]IP_3$. **b**: The specific binding of $[^3H]IP_3$ as a function of $[^3H]IP_3$ was determined by subtracting the non-specific binding determined in the presence of a 100-fold excess of unlabeled IP_3. The microsomal preparation was incubated with $[^3H]IP_3$ for 10 min at 4°C. The nonspecific binding was then subtracted from the total binding. **c**: The Scatchard analysis of the data presented in b. The data showed an R^2 of 0.917. **d**: The effect of chloroquine on $[^3H]IP_3$ binding. The time course was performed in duplicate while the other studies were performed in triplicate and reported as the mean ± SEM.

parasites. Supporting this notion is the observation that depletion of extracellular Ca^{2+} blocks the development of *P. falciparium* in phagocytized human red blood cells [Wasserman et al., 1982].

Our studies also demonstrated that chloroquine had a much greater effect on the initial IP_3-induced increase in cellular $[Ca^{2+}]_i$ than on the capacitative entry of Ca^{2+} from the medium. This was unexpected since it is currently believed that depletion of internal Ca^{2+} stores triggers capacitative Ca^{2+} entry from the external environment of the cell [Berridge, 1993; Marshall and Taylor, 1993; Putney and Bird, 1993]. In part our data suggest that chloroquine may increase leakiness of cellular membranes to Ca^{2+} thus explaining our observation. However, some studies [Nasmith and Grinstein, 1987; Demaurex et al., 1992; Shuttleworth and Thompson, 1996] have called into question the current view with regard to the relationship between capacitative Ca^{2+} entry and mobilization of internal Ca^{2+} stores. Shuttleworth and Thompson (1996) have recently reported that the initial exposure of cells to an agonist is capable of activating Ca^{2+} entry from the medium prior to release of Ca^{2+} from internal stores. It is conceivable, therefore, that our data also reflect the fact that capacitative Ca^{2+} entry, at least in part, is independent of mobilization of internal stores. This observation will require further investigation with respect to these issues.

The current observations with chloroquine may also have implications for our understanding of the effects of chloroquine on inhibiting endocytosis of ligand-receptor complexes. This is generally believed to result from the weak base effect of this agent in lysosomes where it raises the pH and interferes with ligand disso-

ciation from the receptor and subsequent recycling of the receptor [Stahl et al., 1980; Tietze et al., 1980; Dautry-Varsat et al., 1993; Hunyady et al., 1991]. The elevation of pH also interferes with the ability of lysosomal acid proteinases to degrade protein ligands. More recently, Hunyady et al. [1991] have suggested that internalization of angiotensin II-receptor complexes by adrenal glomerular cells is dependent on polyphosphoinositide hydrolysis to produce IP_3 and the subsequent rise in $[Ca^{2+}]_i$. The ability of chloroquine to block IP_3 binding to its receptor suggests a novel mechanism whereby this agent may disrupt ligand-receptor uptake and signal transduction. Whether chloroquine and other antimalarials disrupt IP_3 binding to *Plasmodium* IP_3 receptors will require study; however, these receptors are likely to be highly conserved and such an effect is predicted.

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