On the Ca$^{2+}$ Dependence of Non-transferrin-bound Iron Uptake in PC12 Cells*

Non-transferrin-bound iron (NTBI) uptake has been reported to follow two pathways, Ca$^{2+}$-dependent and Ca$^{2+}$-independent (Wright, T. L., Brissot, P., Ma, W. L., and Weisiger, R. A. (1986) J. Biol. Chem. 261, 10909–10914; Sturrock, A., Alexander, J., Lamb, J., Craven, C. M., and Kaplan, J. (1990) J. Biol. Chem. 265, 3139–3145). Studies reporting the two pathways have ignored the weak interactions of Ca$^{2+}$ with the chelator nitrilotriacetate (NTA) and the reducing agent ascorbate. These studies used a constant ratio of total Fe$^{3+}$ to NTA with and without Ca$^{2+}$. We observed Ca$^{2+}$ activation of NTBI uptake in PC12 cells with the characteristics reported for other cells upon using 1 mM ascorbate and a constant ratio of total Fe$^{3+}$ to NTA with or without Ca$^{2+}$. However, Ca$^{2+}$ did not affect NTBI uptake in solutions without NTA. We then determined conditional stability constants for NTA binding to Ca$^{2+}$ and Fe$^{2+}$ by potentiometry under conditions of NTBI uptake experiments (pH, ionic strength, temperature, ascorbate, total Fe$^{2+}$, and total Ca$^{2+}$ concentrations). In solutions based on these constants and taking Ca$^{2+}$ chelation into account, Ca$^{2+}$ did not affect NTBI uptake over a range of free Fe$^{2+}$ concentrations. Thus, the Ca$^{2+}$ activation of NTBI uptake observed using the constant total Fe$^{2+}$ to NTA ratio was because of Ca$^{2+}$-NTA chelation rather than an activation of the NTBI transporter itself. It is suggested that the previously reported Ca$^{2+}$ dependence of NTBI uptake be re-evaluated.

Iron enters cells through multiple pathways (1). The iron entry pathways may be grouped as transferrin-dependent or transferrin-independent (2, 3). The transferrin-dependent pathway involves binding of the iron to transferrin, which then binds to a receptor; the complex is then taken up by endocytosis (4–6). A low affinity non-transferrin-bound iron (NTBI)$^1$ uptake ($K_m$ value, 15 $\mu$m) also occurs in erythroid cells, and a high affinity NTBI uptake has been reported in many cell types (7–13). The high affinity NTBI transporter carries Fe$^{2+}$ with a $K_m$ value of <$1 \mu$m and is temperature-dependent. NTBI uptake has been reported to be inhibited by La$^{3+}$, Cd$^{2+}$, Co$^{2+}$, Mn$^{2+}$, and Cu$^{2+}$ or by chelation with diethylenetriaminepentaacetic acid. Many cell types have also been reported to show an extracellular Ca$^{2+}$-dependent high affinity NTBI uptake that does not distinguish between Fe$^{3+}$ or Fe$^{2+}$ (8, 12–14). The importance of Ca$^{2+}$-dependent high affinity NTBI uptake versus Ca$^{2+}$-independent uptake was presented (8, 12–14). In these experiments, iron was reduced to Fe$^{2+}$ using ascorbate, and the Fe$^{2+}$ was chelated using nitrilotriacetate (NTA). Interactions between Ca$^{2+}$ and NTA were apparently considered too weak to be important, because a constant iron to NTA ratio was used in the presence or absence of Ca$^{2+}$.

The rat adrenergic neural tumor pheochromocytoma cell line, PC12, is used extensively as a neuronal model. Nerve growth factor (NGF)-treated PC12 cells exhibit sympathetic neuron-like properties characterized by neurite outgrowth and electrical excitability. They express cholinergic receptors, Na$^+$ channels, N-type voltage-operated Ca$^{2+}$ channels, and neuronal nitric-oxide synthase (15–17). They also exhibit high levels of expression of sarcolemmal Ca$^{2+}$ pump and low levels of the organellar pump (18). Upon stimulation with acetylcholine, PC12 cells can secrete dopamine, thus making them a model of dopaminergic neurons. Here, we report that in these cells Ca$^{2+}$ produces an apparent activation of NTBI uptake when reported protocols are used. We demonstrate that this activation is an artifact caused by Ca$^{2+}$ binding to NTA and thus indirectly affecting Fe$^{2+}$-NTA chelation rather than an activation of the transporter itself.

EXPERIMENTAL PROCEDURES

PC12 Cell Cultures—PC12 cells in passages 12 to 30 were cultured in bovine Achilles tendon collagen-coated plates in RPMI 1640 medium supplemented with penicillin and streptomycin (5000 units/5000 $\mu$g), 10% fetal calf serum, and 5% calf serum as described previously (18). At each passage, the cells were split 1 to 4. The cells were treated with 40 ng/ml 2.5 $\times\ 10^6$ NGF, which was purified as described previously (19). The medium with NGF was replenished 2, 5, and 7 days after the initial plating. The cells were harvested in Ca$^{2+}$-free physiological saline solution (PSS) after another 2 days. PSS contained 10 mM HEPES, 140 mM NaCl, 5 mM KCl, and 5 mM MgCl$_2$ at pH 7.4 (pH at 37 °C).

Non-transferrin-bound Iron Uptake Experiments—NTBI uptake was carried out using the PC12 cells in suspension with shaking. Initially, we used a constant iron to NTA ratio (12, 13, 20). Typically, the uptake was for 2 min in a 300-ml reaction mixture at 37 °C in an ascorbate (1 mM)-PSS solution containing 0.43 mM total iron including $^{55}$FeCl$_3$ (1–2 $\mu$Ci/ml, specific activity 13 $\mu$Ci/g), 28 $\mu$m NTA, and PC12 cells containing 0.4–0.8 $\mu$g of protein. 200 $\mu$l of the sample was filtered under suction through a 0.45-$\mu$m nitrocellulose filter, which was prewashed in 1% bovine serum albumin and 100 $\mu$l KCl. The filter was washed 3 times with 5 ml of a chilled solution containing 30 mM imidazole-HCl, pH 6.8, 0.5 mM EGTA, and 250 mM sucrose. The amount of radioactivity remaining on the filter was determined by scintillation counting. Blank values obtained without any cells were subtracted before any further computations. A number of specific variations made in this protocol will be as described under “Results.”

Determination of Conditional Stability Constants—The stability con-

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2The abbreviations used are: NTBI, non-transferrin bound iron; $K_{\text{cond}}$, conditional association constant; NGF, nerve growth factor; NTA, nitrilotriacetate; PSS, physiological saline solution.
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Iron Uptake at Constant Iron to NTA Ratio—We followed the literature and examined NTBI uptake initially using fixed concentrations of iron and NTA in ascorbate-PSS with and without 2 mM Ca$^{2+}$ (12, 13, 20). The experiment clearly shows a higher rate of NTBI uptake in the presence of 2 mM CaCl$_2$ than in its absence (Fig. 1A). Subsequently, the uptake was determined for only 2 min. In 16 such experiments, 2 mM CaCl$_2$ increased the NTBI uptake by 151 ± 23%. Activation of the NTBI uptake was [Ca$^{2+}$]-dependent (Fig. 1B). The [Ca$^{2+}$] required to produce half-maximum activation was 0.5–1 mM. Consistent with the processes being associated with an activation energy, the Ca$^{2+}$-activated and Ca$^{2+}$-independent components of the NTBI uptake were greater at 37 than at 4 °C (Fig. 1C). We also examined whether Ca$^{2+}$ activation occurred for NTBI uptake using Fe$^{3+}$ (FeCl$_3$) plus NTA in ascorbate-free PSS. In 5 experiments, there was an activation of 251 ± 43% by Ca$^{2+}$. These properties are consistent with those reported in the literature for the two components of NTBI uptake, one being Ca$^{2+}$-dependent and the other Ca$^{2+}$-independent.

NTBI Uptake without NTA—Using literature values for the stability constants for various interactions of Fe$^{3+}$, Mg$^{2+}$, Ca$^{2+}$, and H$^+$ with NTA and ascorbate (23) and correcting these constants for the temperature and ionic strength, 0.43 μM total iron and 28 μM NTA gave different values for free [Fe$^{3+}$] with 0 and 2 mM CaCl$_2$ (4.8 and 9.5 μM, respectively). Therefore, we conducted an NTBI uptake experiment in ascorbate-PSS without NTA (Fig. 2). It is noted that this experiment is limited in that high concentrations of Fe$^{3+}$ cannot be sustained at this pH without chelation. In the range of Fe$^{3+}$ concentrations used, 2 mM Ca$^{2+}$ did not activate the uptake. Because Ca$^{2+}$ activated NTBI uptake when NTA was used (Fig. 1) but not without it (Fig. 2), Ca$^{2+}$-NTA interactions were explored further.

NTBI Uptake Using NTA Concentrations Based on Literature Values—Based on pH, ionic strength, and temperature corrected literature values of the stability constants for Fe$^{3+}$, Mg$^{2+}$, and Ca$^{2+}$ binding to NTA and ascorbate, 0.43 μM total Fe$^{3+}$ in ascorbate-PSS with 0 mM Ca$^{2+}$ and 28 μM NTA gave an identical free Fe$^{3+}$ concentration (4.8 μM) as using 2 mM Ca$^{2+}$ and 74 μM NTA. Using these concentrations of NTA gave a lower value for the NTBI uptake with 2 than with 0 mM Ca$^{2+}$ (Table I). However, the computations for the experiment presented in Table I were based on 16 stability constants. Each of these had been determined at different metal ion concentrations, temperatures, and ionic strengths as reported in the literature and corrected to our experimental conditions. A dis-
crepancy in any one of them could give erroneous values. Therefore, we set out to determine stability constants under conditions of the NTBI uptake experiment.

**Determination of Conditional Stability Constants**—We determined the various conditional stability constants based on competition between Cd\(^{2+}\) and other cations to bind NTAs under conditions of the NTBI experiment (in the presence of ascorbate at the pH, ionic strength, temperature, and range of total [Fe\(^{2+}\)] and [Ca\(^{2+}\)]). We computed the conditional stability constant for Cd\(^{2+}\)-NTA binding using 0.94 NTA/Cd\(^{2+}\). The Cd\(^{2+}\)-NTA binding constants were determined first on each day of the experiment. These data fit best with the model in which NTA binds Ca\(^{2+}\) and Cd\(^{2+}\). These experiments fit a model in which NTA binds Ca\(^{2+}\) with a stoichiometry of 0.907 NTA/Ca\(^{2+}\) and \(K_{\text{cond}}\) 2.2 \times 10^4 M\(^{-1}\). We then used 2 and 5 \(\mu\)M FeSO\(_4\) in the absence of added Ca\(^{2+}\) to obtain a stoichiometry of 1.04 NTA/Fe\(^{2+}\) and \(K_{\text{cond}}\) \(= 1.9 \times 10^6\) M\(^{-1}\) (Fig. 3A). Based on the binding constants for NTA to Ca\(^{2+}\) and Fe\(^{2+}\), we computed the \(K_{\text{cond}}\) value for NTA binding to Fe\(^{2+}\) in the presence of 2 mM Ca\(^{2+}\) as 2.29 \times 10^6 M\(^{-1}\). We then carried out the binding experiment using 2 and 5 \(\mu\)M FeSO\(_4\) in the presence of 2 mM Ca\(^{2+}\). This experiment gave \(K_{\text{cond}}\) \(= 2.34 \times 10^6\) M\(^{-1}\), which was consistent with the computed value (Fig. 3C).

**NTBI Uptake Using [NTA] Based on Conditional Stability Constants**—Fig. 4A compares NTBI uptake in 0 and 2 mM Ca\(^{2+}\) in ascorbate-PS buffer using a fixed total iron concentration and NTA concentrations based on the conditional stability constants to clamp free Fe\(^{2+}\) concentration at 0.1 \(\mu\)M. Ca\(^{2+}\) did not produce an activation. In three experiments, activation of iron uptake in 2 min by 2 mM Ca\(^{2+}\) was 9 ± 8\%. This experiment indicated that Ca\(^{2+}\) chelation to NTA was the explanation for the 151 ± 23% activation we had observed in the initial protocol using a constant amount of NTA with and without Ca\(^{2+}\). To determine whether the lack of activation in Fig. 4A was because of the free [Fe\(^{2+}\)] chosen for the experiment, we conducted an experiment at various concentrations of free Fe\(^{2+}\), which we prepared using a fixed concentration of NTA and a series of concentrations of total Fe\(^{2+}\) based on the conditional stability constants and correcting for NTA binding to Ca\(^{2+}\). There was no effect of Ca\(^{2+}\) on NTBI uptake (Fig. 4B).

**DISCUSSION**

The results presented here demonstrate that the Ca\(^{2+}\) activation of NTBI uptake observed using the established literature protocol is an artifact caused by Ca\(^{2+}\)-NTA interaction. The "Discussion" will focus on the validity of the methods used, the need for determining conditional stability constants, possible implications of these observations to the field of NTBI uptake in general, and the biological relevance of these findings.

NTBI transport has been reported in many cell types, and in some cases, shown to be activated by Ca\(^{2+}\) with an EC\(_{50}\) value of 0.5–1 mM (8, 12, 13). In these studies, iron was maintained as Fe\(^{2+}\) using ascorbate and chelated using NTA. In several studies, a constant iron to NTA ratio was used with and without Ca\(^{2+}\), and in others it is not clear how the required concentrations of NTAs were determined (8, 12–14). Using the established constant ratio method, PC12 cells showed NTBI uptake activation by Ca\(^{2+}\) with the characteristics previously reported. However, this protocol is based on the binding of Ca\(^{2+}\) to NTA to be too weak to affect the Fe\(^{2+}\)-NTA buffering system. This issue has not been adequately addressed in the literature concerning NTBI uptake. Our computations using the previously reported absolute stability constants showed that 2 mM Ca\(^{2+}\) bound 65% of the NTA in our initial experiments, which led to a doubling of the free Fe\(^{2+}\) concentration. However, the matter is even more complex because Fe\(^{2+}\)-NTA binding stoichiometry is not 1:1 (24). Furthermore, it is not known how this stoichiometry depends on metal ion concentration, pH, temperature, and ionic strength. Sixteen such constants were used in these...
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computations. Consequently, the validity of the classical methods for correcting standard constants to experimental conditions is questionable. We resolved this problem by determining conditional stability constants. These values were determined under the conditions of the NTBI uptake experiments. The results obtained using the conditional stability constants were in agreement with the experiments without using NTA in that NTBI uptake is not activated by Ca$^{2+}$. The results using the established protocols, however, are flawed. Based on the conditional stability constants using 0.43 μM total Fe$^{3+}$ and 28 μM NTA in the experiments in Fig. 1, the free Fe$^{2+}$ concentration would be higher in the presence of 2 mM Ca$^{2+}$ (52 nM) than at 0 mM (28 nM). Computations using the conditional stability constants (Table I) show that Ca$^{2+}$ decreased the NTBI uptake because the NTA concentration used resulted in lower free Fe$^{2+}$ in the presence of Ca$^{2+}$ (20 nM) than in its absence (28 nM). Thus, all four experiments are consistent with the conclusion that Ca$^{2+}$ binds NTA, indirectly affecting the free Fe$^{2+}$ concentration, but it has no direct effect on the NTBI transporter. Several studies have used citrate instead of NTA to chelate Fe$^{2+}$ (1), but the chelation problem highlighted in this study may also apply to citrate. These results suggest a need to re-evaluate the Ca$^{2+}$-activated pathway for NTBI uptake.

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FIG. 4. Effect of calcium on NTBI uptake based on conditional stability constants. A, time course. NGF-treated PC12 cells were incubated at 37 °C in the ascorbate-PSS solution containing 2 μM total iron, 0 or 2 mM CaCl$_2$, and NTA added to clamp the free Fe$^{2+}$ concentration at 0.1 μM. NTA concentration = 28 μM at 0 mM CaCl$_2$ and 56.5 μM at 2 mM CaCl$_2$. Ca$^{2+}$-dependent uptake is the difference between uptake at 2 and 0 mM CaCl$_2$. All of the values are the means ± S.E. of 6 replicates. These values were significantly different for 4, 6, and 10 min but not 2 and 8 min. B, free [Fe$^{3+}$] dependence of NTBI uptake at 0 or 2 mM CaCl$_2$. [NTA] = 14.3 μM and total [Fe$^{2+}$] varied from 1 to 25 μM. Free [Fe$^{3+}$] was computed by correcting for NTA binding to Ca$^{2+}$ using the conditional stability constants. Repeating the experiments from A and B gave similar results; there was no Ca$^{2+}$ activation of the NTBI uptake.