

## Ca<sup>2+</sup> Binding to Chromaffin Vesicle Matrix Proteins: Effect of pH, Mg<sup>2+</sup>, and Ionic Strength<sup>†</sup>

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**ABSTRACT:** Recently we found that Ca<sup>2+</sup> within chromaffin vesicles is largely bound [Bulenda, D., & Gratzl, M. (1985) *Biochemistry* 24, 7760-7765]. In order to explore the nature of these bonds, we analyzed the binding of Ca<sup>2+</sup> to the vesicle matrix proteins as well as to ATP, the main nucleotide present in these vesicles. The dissociation constant at pH 7 is 50 μM (number of binding sites, *n* = 180 nmol/mg of protein) for Ca<sup>2+</sup>-protein bonds and 15 μM (*n* = 0.8 μmol/μmol) for Ca<sup>2+</sup>-ATP bonds. When the pH is decreased to more physiological values (pH 6), the number of binding sites remains the same. However, the affinity of Ca<sup>2+</sup> for the proteins decreases much less than its affinity for ATP (dissociation constant of 90 vs. 70 μM). At pH 6 monovalent cations (30-50 mM) as well as Mg<sup>2+</sup> (0.1-0.5 mM), which are also present within chromaffin vesicles, do not affect the number of binding sites for Ca<sup>2+</sup> but cause a decrease in the affinity of Ca<sup>2+</sup> for both proteins and ATP. For Ca<sup>2+</sup> binding to ATP in the presence of 0.5 mM Mg<sup>2+</sup> we found a dissociation constant of 340 μM and after addition of 35 mM K<sup>+</sup> a dissociation constant of 170 μM. Ca<sup>2+</sup> binding to the chromaffin vesicle matrix proteins in the presence of 0.5 mM Mg<sup>2+</sup> is characterized by a *K<sub>d</sub>* of 240 μM and after addition of 15 mM Na<sup>+</sup> by a *K<sub>d</sub>* of 340 μM. The similar affinity of Ca<sup>2+</sup> for protein and ATP, especially at pH 6, in media of increased ionic strength and after addition of Mg<sup>2+</sup>, points to the possibility that the intravesicular medium determines whether Ca<sup>2+</sup> is preferentially bound to ATP or the chromaffin vesicle matrix proteins. Purified chromogranin A, after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, stains with a carbocyanine dye ("Stains-all") and, following blotting onto nitrocellulose, binds to <sup>45</sup>Ca<sup>2+</sup>. A spectrophotometric analysis of dye binding to chromaffin vesicle matrix proteins revealed a strong absorption band at 615 nm for the dye-protein complex. Since the observed spectral changes were unaffected by the presence of Ca<sup>2+</sup> (100 μM free), the sites interacting with the dye and Ca<sup>2+</sup> must be regarded as different.

In resting secretory cells, the cytoplasmic concentration of free Ca<sup>2+</sup> is low. Increase of cytoplasmic Ca<sup>2+</sup> upon stimulation results in the release of secretory products by exocytosis. Secretory vesicles of various types of endocrine cells contain Ca<sup>2+</sup>, and a Ca<sup>2+</sup> transport system dependent on Na<sup>+</sup> has been described in chromaffin (Phillips, 1981; Krieger-Brauer & Gratzl, 1981, 1982, 1983) and neurohypophyseal vesicles (Saermark et al., 1983a,b).

Calculation of the apparent Ca<sup>2+</sup> concentration within the chromaffin vesicles resulted in values between 20 and 40 mM (Borowitz et al., 1965; Phillips et al., 1977; Krieger-Brauer & Gratzl, 1982). Binding of Ca<sup>2+</sup> inside the vesicles would obviously be of great importance for the Ca<sup>2+</sup> transport systems present in the vesicle membrane, because lowering of the Ca<sup>2+</sup> gradient between the cytoplasmic space and the interior of the vesicle can be expected to enhance the efficiency of Ca<sup>2+</sup> uptake.

In fact, using secretory vesicles isolated from adrenal medulla, Bulenda and Gratzl (1985) obtained experimental evidence that only a small fraction of total Ca<sup>2+</sup> (about 0.1%) is in the free state. In order to elucidate the physiological importance of the vesicle matrix proteins, we determined their Ca<sup>2+</sup> binding properties under conditions comparable to the composition of the chromaffin vesicle content with respect to ionic strength, pH, and the presence of Mg<sup>2+</sup>. In order to facilitate direct comparison, Ca<sup>2+</sup> binding to the vesicle matrix proteins and to adenosine triphosphate (ATP),<sup>1</sup> another Ca<sup>2+</sup>

binding compound present within the chromaffin vesicle, was analyzed under identical conditions by means of a specific electrode. It was found that the vesicle matrix protein chromogranin A provides significant amounts of the Ca<sup>2+</sup> binding sites within chromaffin vesicles.

### EXPERIMENTAL PROCEDURES

#### Materials

EGTA, Mes, Mops, and Hepes were from Serva, Heidelberg. ATP was vanadate free and from Sigma, München. Chelex-100 (200-400 mesh, sodium form) was obtained from Bio-Rad. "Stains-all" was from Aldrich. All other reagents were of analytical grade.

#### Methods

**Isolation of Chromaffin Vesicles.** A fraction of crude chromaffin secretory vesicles was obtained from bovine adrenal medullae homogenized in a medium containing 340 mM sucrose and 20 mM MOPS/KOH, pH 7.3, as described earlier (Gratzl et al., 1981). This sample was put on a sucrose step gradient consisting of 2.4/2.0/1.8/1.7 M sucrose in 20 mM MOPS/KOH, pH 7.0, and centrifuged for 1 h at 146000*g<sub>av</sub>* in a Beckman L 8-M ultracentrifuge using a 50.2 Ti rotor. In this way mitochondria and lysosomes could be removed as

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<sup>1</sup> Abbreviations: EGTA, ethylene glycol bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; ATP, adenosine triphosphate; Stains-all, 3,3'-diethyl-9-methyl-4,5,4',5'-dibenzothiacarbocyanine bromide; Tris, tris(hydroxymethyl)aminomethane; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

described for a similar gradient centrifuged in a swing out rotor (Gratzl, 1984). After centrifugation, the secretory vesicles were concentrated around the 1.8/2.0 M sucrose interface. In order to lower the sucrose concentration, the collected secretory vesicles were dialyzed for 60 min in homogenization buffer and condensed by centrifugation (146000g<sub>av</sub>, 30 min). Afterward they were lysed in 20 mM MOPS/KOH, pH 7.0 (1 volume of vesicle fraction diluted with 40 volumes of buffer). The secretory vesicle membranes were spun down by centrifugation (146000g<sub>av</sub> for 30 min), and the supernatant was lyophilized. The material obtained was dissolved in a small volume of bidistilled water and dialyzed twice for 24 h in 20 mM MOPS/KOH, pH 7.0, in order to remove catecholamines, nucleotides, and other low molecular weight components.

**Measurement of Ca<sup>2+</sup> Binding.** Prior to the analysis of Ca<sup>2+</sup> binding properties, isolated matrix proteins were dialyzed overnight in a large volume of 20 mM MOPS/KOH or NaOH at pH 7.0 either alone or also containing additional K<sup>+</sup>, Na<sup>+</sup>, or Mg<sup>2+</sup>. It should be noted that the original buffer, due to pH adjustment, already contained 15 mM K<sup>+</sup> or Na<sup>+</sup>. Mes and Hepes were used as buffer substances at pH 6.0 and 8.0, respectively. All buffers used for dialysis also contained 0.5 g of Chelex-100/200 mL, in order to remove residual Ca<sup>2+</sup> before titration. After dialysis, Ca<sup>2+</sup> concentrations between 10<sup>-6</sup> and 10<sup>-7</sup> were determined in the samples. The Ca<sup>2+</sup> concentrations were quantified by using a Ca<sup>2+</sup>-selective minielectrode operating with a neutral carrier incorporated in a polyvinyl chloride membrane (Simon et al., 1978). The difference in voltage between the Ca<sup>2+</sup> electrode and the reference electrode was recorded with a Knick pH meter connected to a chart recorder. The calibration curve of this electrode was linear down to 10<sup>-6</sup> M Ca<sup>2+</sup>. The dialyzed chromaffin vesicle matrix proteins were titrated in a volume of 1 mL (0.3–0.4 mg of protein/mL) with 5 or 10 mM Ca<sup>2+</sup> stock solutions. The amount of bound Ca<sup>2+</sup> was calculated from the difference between free Ca<sup>2+</sup> in pure buffer and free Ca<sup>2+</sup> in buffer containing the vesicle matrix proteins. Scatchard plots were constructed with the aid of linear regression analysis using values up to 100 nmol of Ca<sup>2+</sup> bound/mg of protein. Ca<sup>2+</sup> binding to ATP (0.5 mM) was measured in freshly prepared solutions from a commercial preparation. One drawback of this technique is that one titration takes about 20–30 min. The instability of nucleotides during this period might slightly modify their binding data.

**Other Procedures.** Protein was determined according to Lowry et al. (1951). Chromogranin A was purified according to Kiang et al. (1982).

**Electrophoresis and Nitrocellulose Blotting.** Gel electrophoresis was performed according to Laemmli (1970) with 10% acrylamide in the separation gel and 4% acrylamide in the stacking gel.

After electrophoresis the separated proteins were blotted onto nitrocellulose. The transfer buffer contained 192 mM glycine/25 mM Tris/20% methanol (final pH 8.3). The blotting apparatus was supplied with 60 V for 1.5 h.

**Staining Procedures and Autoradiography.** Protein staining was performed with 0.1% amido black/45% methanol/10% acetic acid. Staining with the cationic carbocyanine dye Stains-all was performed in the original gel according to Campbell et al. (1983).

**Autoradiography.** The nitrocellulose sheet was washed 3 times for 20 min in 20 mM MOPS/KOH, pH 7.0. Afterward it was incubated and continually shaken for 10 min in 15 mL of the same buffer containing 15 μCi of <sup>45</sup>Ca (specific activity 13.66 mCi/mg). Then it was washed twice for 5 min in 20

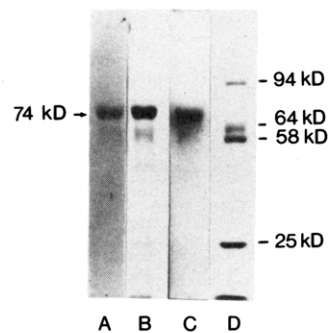


FIGURE 1: Ca<sup>2+</sup> binding properties of purified chromogranin A. The purified chromogranin A was separated by SDS gel electrophoresis. Nitrocellulose blotting and analysis of its Ca<sup>2+</sup> binding properties were performed as described under Experimental Procedures. Lane A shows binding of Stains-all, lane B, the protein stain with amido black, lane C, <sup>45</sup>Ca binding, and lane D, the molecular weight marker proteins.

mL of distilled water, dried between filter papers, and placed on a X-ray film which was developed after 3–4 days.

## RESULTS

Chromaffin vesicles isolated from adrenal medulla contain in addition to catecholamines a variety of small molecular weight substances, (glyco)proteins, and proteoglycans [cf. Winkler & Carmichael (1982)]. Although the overall composition of the vesicle interior is well-defined, the molecular organization of these contents within the vesicle core is largely unknown. Recently we learned that chromaffin vesicle matrix proteins avidly bind to Ca<sup>2+</sup> (Reiffen & Gratzl, 1986). <sup>45</sup>Ca<sup>2+</sup> autoradiography of these proteins separated by SDS-PAGE and blotted onto nitrocellulose indicated that Ca<sup>2+</sup> binding is mainly limited to a protein with the electrophoretic properties of chromogranin A. Ca<sup>2+</sup> binding to purified chromogranin A is shown in Figure 1. This protein, separated as described (see Methods), displays one major band in the protein stain (lane B). Bound <sup>45</sup>Ca<sup>2+</sup> (lane C) and staining with a carbocyanine dye (lane A), which has been observed to interact with chromogranin A and a variety of Ca<sup>2+</sup> binding proteins (Campbell et al., 1983; Reiffen & Gratzl, 1986), exhibit bands in corresponding locations.

Since chromogranin A is obviously the only Ca<sup>2+</sup> binding protein present in the chromaffin vesicle matrix, we further analyzed the Ca<sup>2+</sup> binding properties using a dialyzed chromaffin vesicle content preparation.

As shown in Figure 2, the number of binding sites for Ca<sup>2+</sup> provided by the chromaffin vesicle matrix proteins [ $n = 180$  nmol of Ca<sup>2+</sup>/mg of protein (Figure 2A)] does not change with pH. However, there affinity is sensitive to the pH of the medium. At pH 7 the proteins bind Ca<sup>2+</sup> with a  $K_d$  of 50 μM (Figure 2A). These values should be compared with the data on Ca<sup>2+</sup> binding to ATP, another chelating substance present within chromaffin vesicles. Earlier investigation of the divalent cation/ATP equilibria, possibly due to the different techniques used, yielded quite different values [cf. Yount et al. (1971) and Mohan & Rechnitz (1972)]. In order to facilitate direct comparison with the proteins, we determined Ca<sup>2+</sup> binding to ATP in parallel under identical conditions. In this way we found that ATP, at pH 7, binds 0.8 μmol of Ca<sup>2+</sup>/μmol with a  $K_d$  of 15 μM (Figure 2B), i.e., with a higher affinity than that of the proteins investigated. Interestingly enough, in contrast to the situation observed at pH 7, the affinities at pH 6 of Ca<sup>2+</sup> for ATP (70 μM) and proteins (90 μM) are very similar (Figure 2A,B). This is due to the greater influence of the pH change on the affinity of Ca<sup>2+</sup> binding to ATP (Figure 2B).

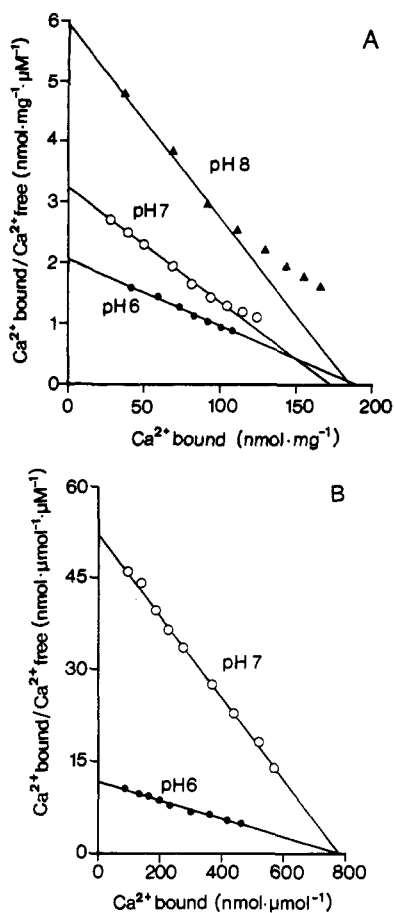


FIGURE 2: Effect of pH on the  $\text{Ca}^{2+}$  binding to chromaffin vesicle matrix proteins and ATP. The chromaffin vesicle matrix proteins bind about 180 nmol of  $\text{Ca}^{2+}$ /mg of protein (A). The dissociation constant, calculated by linear regression analysis, decreases with increasing pH. At pH 6 we found 90  $\mu\text{M}$ , at pH, 7, 50  $\mu\text{M}$ , and at pH 8, 30  $\mu\text{M}$ . ATP (B) binds 800 nmol of  $\text{Ca}^{2+}$ / $\mu\text{mol}$ , and the dissociation constant calculated from the experimental values obtained at pH 6 (70  $\mu\text{M}$ ) decreases by a factor of 5 to 15  $\mu\text{M}$  at pH 7. Thus, the change in the pH from 7 to 6 results in a decrease in the affinity for  $\text{Ca}^{2+}$  for both components, but the decrease in the pH affects  $\text{Ca}^{2+}$  binding to ATP much more than  $\text{Ca}^{2+}$  binding to the chromaffin vesicle matrix proteins.

Since pH 6 is closer to the pH present within the intact chromaffin vesicle (Johnson & Scarpa, 1976; Pollard et al., 1976; Bashford et al., 1976), most of the following experiments were carried out at this pH. The affinity of  $\text{Ca}^{2+}$  binding to chromaffin vesicle matrix proteins is decreased by monovalent ions and by  $\text{Mg}^{2+}$  at pH 6 (Figure 3). Addition of 15 mM  $\text{Na}^+$  results in a considerable decrease of the binding affinity ( $K_d = 340 \mu\text{M}$ ). In the presence of 0.5 mM  $\text{Mg}^{2+}$ , a  $K_d$  of 240  $\mu\text{M}$  was found. There was no difference whether  $\text{K}^+$  or  $\text{Na}^+$  was used in these experiments (data not shown).

$\text{Mg}^{2+}$  decreases also the affinity of  $\text{Ca}^{2+}$  to ATP (Figure 4). In the presence of 0.5 mM  $\text{Mg}^{2+}$  a  $K_d$  of 340  $\mu\text{M}$  was determined.  $\text{K}^+$  at 15 mM reduces the binding constant in a similar magnitude as does 0.1 mM  $\text{Mg}^{2+}$  (not shown).

Studies using total chromaffin vesicle matrix proteins (Reiffen & Gratzl, 1986) as well as experiments with purified chromogranin A (Figure 1) demonstrated that  $\text{Ca}^{2+}$  binding and carbocyanine binding are shared by the same proteins. Thus, it seemed to be interesting to know whether both substances bind to the same sites or not. When studying the interaction of the carbocyanine dye with the chromaffin vesicle matrix proteins, we added increasing amounts of the proteins to 20  $\mu\text{M}$  Stains-all. As can be seen in Figure 5, only a few micrograms of protein per milliliter drastically modify the dye

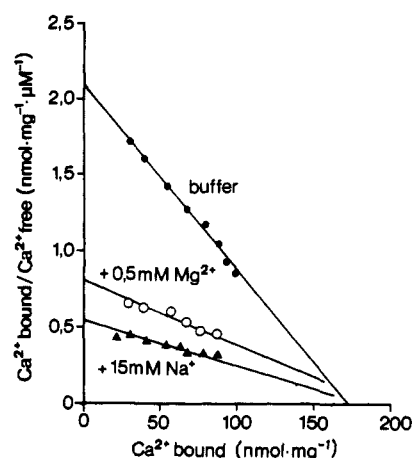


FIGURE 3: Effect of  $\text{Mg}^{2+}$  and ionic strength on the  $\text{Ca}^{2+}$  binding to chromaffin vesicle proteins. At pH 6 which is closer to the pH found in intact chromaffin vesicles, addition of 15 mM or 35 mM  $\text{Na}^+$  reduces the affinity for  $\text{Ca}^{2+}$  to the proteins. For example, additional 15 mM  $\text{Na}^+$  results in an increase in the  $K_d$  from 90  $\mu\text{M}$  to 340  $\mu\text{M}$ .  $\text{Mg}^{2+}$  at 0.5 mM decreases the affinity to 240  $\mu\text{M}$ .

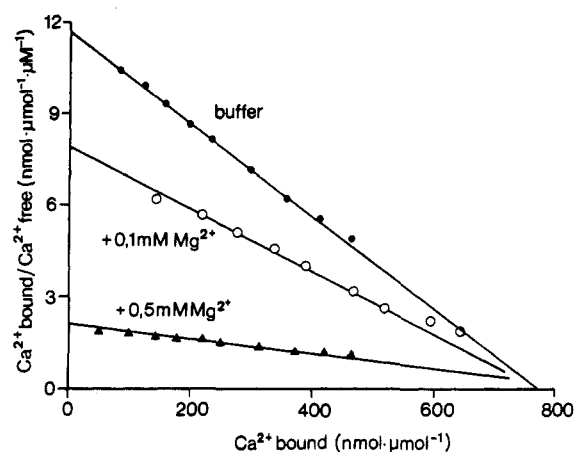


FIGURE 4: Effect of  $\text{Mg}^{2+}$  on  $\text{Ca}^{2+}$  binding to ATP. The titration of the potassium salt of ATP was carried out in 20 mM MES/KOH at pH 6.0. The presence of  $\text{Mg}^{2+}$  (0.1 and 0.5 mM) results in a marked decrease in the affinity of ATP for  $\text{Ca}^{2+}$ . This divalent cation does not affect the number of binding sites for  $\text{Ca}^{2+}$ . The dissociation constant changes from 70 to 340  $\mu\text{M}$  in the presence of 0.5 mM  $\text{Mg}^{2+}$ .

spectrum. As the concentration of protein increases, the Stains-all peak (510 nm) decreases and the protein dye complex (615 nm) increases in parallel. In further experiments we adjusted the amount of free  $\text{Ca}^{2+}$  with the specific electrode to 100  $\mu\text{M}$  (i.e., twice the value of the  $K_d$  at pH 7 for  $\text{Ca}^{2+}$  binding to the proteins). Since we did not observe quantitative or qualitative changes in the spectra, we concluded that  $\text{Ca}^{2+}$  and Stains-all bind to different sites of the matrix proteins.

#### DISCUSSION

The characterization of the  $\text{Ca}^{2+}$  binding properties of the chromaffin vesicle matrix proteins as well as those for ATP, the latter constituting the main small molecular weight substance capable of binding to  $\text{Ca}^{2+}$ , was carried out in order to clarify the possible molecular organization of the interior of the chromaffin vesicle. This in turn may help to understand the function of the  $\text{Ca}^{2+}$  transport systems present in the chromaffin vesicle membrane.

Chromaffin vesicles contain about 2.5  $\mu\text{mol}$  of catecholamines/mg of protein [cf. Winkler & Carmichael (1982)]. Since the molar ratio of ATP to catecholamines is roughly 1:4, about 600 nmol of ATP/mg of protein is present within the vesicles which can bind about 500 nmol of  $\text{Ca}^{2+}$  (Figure 2B).

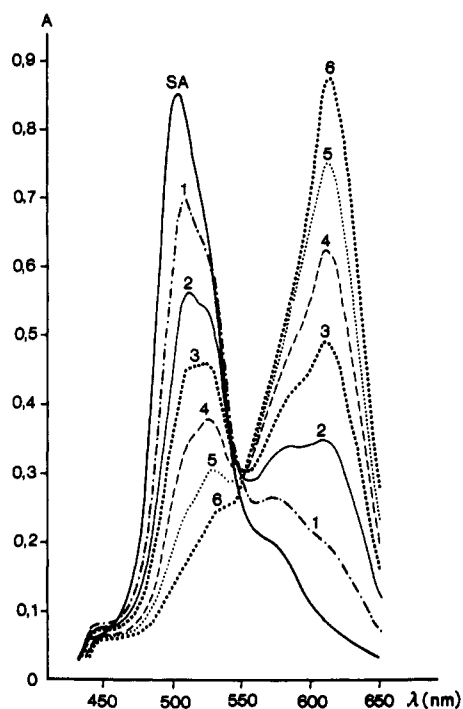


FIGURE 5: Effect of chromaffin vesicle matrix proteins on the spectrum of Stains-all. Stains-all at 20  $\mu$ M in a medium containing 2 mM MOPS/KOH (pH 7) and 0.1 mM EGTA exhibits a maximum at 510 nm (full line). Successive addition of chromaffin vesicle matrix proteins results in a reduction of the absorbance at 510 and a concomitant appearance of a new peak at 615 nm. The numbers 1–6 indicate the amount of protein present in the cuvette ( $\mu$ g/mL). Stains-all was dissolved in ethanol (1 mM) and injected directly into the cuvette.

The chromaffin vesicle matrix proteins, comprising about 80% of the total chromaffin vesicle protein, maximally bind about 180 nmol of Ca<sup>2+</sup>/mg of protein (Figure 3). Therefore, ATP and chromaffin vesicle matrix proteins have the total capacity to bind approximately 680 nmol of Ca<sup>2+</sup>/mg of protein, i.e., 10 times more than is actually present in the vesicles (Borowitz et al., 1965; Phillips et al., 1977; Krieger-Brauer & Gratzl, 1982).

At pH 6, the affinity of the two components for Ca<sup>2+</sup> is very similar (see Figure 2); they compete for the Ca<sup>2+</sup> ion. The affinities may be even more alike in intact vesicles, where a lower pH (around 5.5) has been determined (Johnson & Scarpa, 1976; Pollard et al., 1976; Bashford et al., 1976). The acid pH within chromaffin vesicles is due to a proton-transporting ATPase. It may very well be that the activity of this enzyme plays a direct role in the control of intravesicular Ca<sup>2+</sup> binding.

The maximal Ca<sup>2+</sup> binding to the proteins or ATP was neither changed by variation of the pH (Figure 2) nor changed by addition of monovalent cations or Mg<sup>2+</sup> (Figures 3 and 4). However, increasing the ionic strength or adding Mg<sup>2+</sup> results in a further decrease in the affinity to Ca<sup>2+</sup>. Ca<sup>2+</sup> binding to ATP after addition of 15 mM K<sup>+</sup> exhibits almost the identical value as found with 0.1 mM Mg<sup>2+</sup> present (170  $\mu$ M) whereas Ca<sup>2+</sup> binding to the chromaffin vesicle content proteins exhibit a  $K_d$  of 340  $\mu$ M in the presence of 15 mM Na<sup>+</sup>, which is to be compared to that found with 0.5 mM Mg<sup>2+</sup> present (240  $\mu$ M). Certainly, the dilute solutions of the chromaffin vesicle matrix proteins investigated here cannot directly be compared with the densely packed chromaffin vesicle content. Still it can be concluded that the affinity of Ca<sup>2+</sup> binding to the individual components, namely, the matrix proteins and ATP, is modified by other ions present within the chromaffin vesicle; i.e., they could also have a regulatory function as does the pH

of the intravesicular compartment.

By means of histochemical and biochemical techniques, Ca<sup>2+</sup> has been found in the secretory vesicles of the adrenal medulla (Ravazzola, 1976), the pancreatic islet (Herman et al., 1973), and the adenohypophysis (Stoeckel et al., 1975). It is worth noting that chromogranin A exists not only in the chromaffin cell but also in other endocrine cells: It has been found in pancreatic islet cells, C-cells of the thyroid gland, chief cells of the parathyroid gland, and the adenohypophysis (O'Connor et al., 1983; O'Connor & Frigon, 1984; Cohn et al., 1982; Lloyd & Wilson, 1983). Within the pancreatic islet it seems to coexist with insulin, glucagon, and somatostatin, not only within the same cell but even within the same vesicle (Ehrhart et al., 1986).

ATP is present in chromaffin vesicles in high amounts [cf. Winkler & Carmichael (1982)]. However, this nucleotide is much less abundant (at least 2 orders of magnitude) in insulin-containing (Leitner et al., 1975) or in neurohypophysal vesicles (Poisner & Douglas, 1968; Gratzl et al., 1980). Thus, the matrix proteins may be even more important for storage and binding of Ca<sup>2+</sup> in these vesicles than in chromaffin vesicles.

Ca<sup>2+</sup> binding within other subcellular structures participating in the control of cytoplasmic Ca<sup>2+</sup> has been noted in mitochondria (Hansford & Castro, 1982; Joseph et al., 1983; Reinhardt et al., 1984), sarcoplasmic reticulum (Chiu & Haynes, 1977), secretory vesicles containing chromogranins (Bulenda & Gratzl, 1985), and other organelles the matrix proteins of which have not yet been described to contain chromogranins (Grinstein et al., 1983).

The Ca<sup>2+</sup> binding properties of chromaffin vesicle matrix proteins can be compared with those of calsequestrin, a well-known Ca<sup>2+</sup> binding protein present in the lumen of the sarcoplasmic reticulum. Calsequestrin binds nearly 1000 nmol of Ca<sup>2+</sup>/mg of protein, and Mac Lennan and Wong (1971) measured a dissociation constant for the Ca<sup>2+</sup>–calsequestrin complex of about 40  $\mu$ M at pH 7.5, which is very close to the value of 50  $\mu$ M at pH 7 determined in this study for the chromaffin vesicle matrix protein. Increasing the ionic strength shifts the dissociation constant for Ca<sup>2+</sup>–calsequestrin binding to higher values (about 1 mM) but does not change the number of binding sites (Ostwald & Mac Lennan, 1974; Mac Lennan, 1974; Ikemoto et al., 1972). The same is true for the chromaffin vesicle matrix proteins (this study). Mg<sup>2+</sup> decreases Ca<sup>2+</sup> binding affinity of both types of proteins (Mac Lennan & Wong, 1971; Ikemoto et al., 1973).

The same influence of the monovalent ion K<sup>+</sup> in increasing the dissociation constant has also been reported for the S-100b protein (Mani et al., 1983).

The specific interaction of the major matrix protein of chromaffin vesicles chromogranin A (Reiffen & Gratzl, 1986; this study) with a positively charged carbocyanine dye is an interesting finding that parallels observations reported for other Ca<sup>2+</sup> binding proteins (Campbell et al., 1983). Since the binding of the dye is not affected by low concentrations of Ca<sup>2+</sup> (see Results), the binding sites for both substances must be regarded as different. This is in contrast to the properties of calmodulin, where the dye binding is sensitive to the presence of Ca<sup>2+</sup> (Caday & Steiner, 1985). The low amount of protein required for the dramatic change in the dye spectrum opens a new possibility for a simple and efficient assay for chromogranins.

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**Registry No.** ATP, 56-65-5; Stains-all, 7423-31-6; Ca, 7440-70-2; Mg, 7439-95-4.

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