# $Ca^{2+}$ Binding to Chromaffin Vesicle Matrix Proteins: Effect of pH, $Mg^{2+}$, and Ionic Strength†

Felicitas U. Reiffen and Manfred Gratzl*
*Abteilung Klinische Morphologie, Universität Ulm, D-7900 Ulm, FRG*
*Received January 16, 1986; Revised Manuscript Received March 21, 1986*

ABSTRACT: Recently we found that $Ca^{2+}$ 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 $Ca^{2+}$ 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 $\mu$M (number of binding sites, $n$ = 180 nmol/mg of protein) for $Ca^{2+}$–protein bonds and 15 $\mu$M ($n$ = 0.8 $\mu$mol/$\mu$mol) for $Ca^{2+}$–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 $Ca^{2+}$ for the proteins decreases much less than its affinity for ATP (dissociation constant of 90 vs. 70 $\mu$M). At pH 6 monovalent cations (30–50 mM) as well as $Mg^{2+}$ (0.1–0.5 mM), which are also present within chromaffin vesicles, do not affect the number of binding sites for $Ca^{2+}$ but cause a decrease in the affinity of $Ca^{2+}$ for both proteins and ATP. For $Ca^{2+}$ binding to ATP in the presence of 0.5 mM $Mg^{2+}$ we found a dissociation constant of 340 $\mu$M and after addition of 35 mM $K^+$ a dissociation constant of 170 $\mu$M. $Ca^{2+}$ binding to the chromaffin vesicle matrix proteins in the presence of 0.5 mM $Mg^{2+}$ is characterized by a $K_d$ of 240 $\mu$M and after addition of 15 mM $Na^+$ by a $K_d$ of 340 $\mu$M. The similar affinity of $Ca^{2+}$ for protein and ATP, especially at pH 6, in media of increased ionic strength and after addition of $Mg^{2+}$, points to the possibility that the intravesicular medium determines whether $Ca^{2+}$ 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 $^{45}Ca^{2+}$. 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 $Ca^{2+}$ (100 $\mu$M free), the sites interacting with the dye and $Ca^{2+}$ must be regarded as different.

In resting secretory cells, the cytoplasmic concentration of free $Ca^{2+}$ is low. Increase of cytoplasmic $Ca^{2+}$ upon stimulation results in the release of secretory products by exocytosis. Secretory vesicles of various types of endocrine cells contain $Ca^{2+}$, and a $Ca^{2+}$ transport system dependent on $Na^+$ 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 $Ca^{2+}$ 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 $Ca^{2+}$ inside the vesicles would obviously be of great importance for the $Ca^{2+}$ transport systems present in the vesicle membrane, because lowering of the $Ca^{2+}$ gradient between the cytoplasmic space and the interior of the vesicle can be expected to enhance the efficiency of $Ca^{2+}$ uptake.

In fact, using secretory vesicles isolated from adrenal medulla, Bulenda and Gratzl (1985) obtained experimental evidence that only a small fraction of total $Ca^{2+}$ (about 0.1%) is in the free state. In order to elucidate the physiological importance of the vesicle matrix proteins, we determined their $Ca^{2+}$ binding properties under conditions comparable to the composition of the chromaffin vesicle content with respect to ionic strength, pH, and the presence of $Mg^{2+}$. In order to facilitate direct comparison, $Ca^{2+}$ binding to the vesicle matrix proteins and to adenosine triphosphate (ATP),[1] another $Ca^{2+}$ 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 $Ca^{2+}$ 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$g_{av}$ in a Beckman L 8-M ultracentrifuge using a 50.2 Ti rotor. In this way mitochondria and lysosomes could be removed as

† This study was supported by the Deutsche Forschungsgemeinschaft (Gr 681/2-2) and by Forschungsschwerpunkt 24 of the State of Baden Württemberg.

[1] 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.

0006-2960/86/0425-4402$01.50/0 

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 (146000$g_{av}$, 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 (146000$g_{av}$ 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 $Ca^{2+}$ Binding.* Prior to the analysis of $Ca^{2+}$ 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 $K^+$, $Na^+$, or $Mg^{2+}$. It should be noted that the original buffer, due to pH adjustment, already contained 15 mM $K^+$ or $Na^+$. 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 $Ca^{2+}$ before titration. After dialysis, $Ca^{2+}$ concentrations between $10^{-6}$ and $10^{-7}$ were determined in the samples. The $Ca^{2+}$ concentrations were quantified by using a $Ca^{2+}$-selective minielectrode operating with a neutral carrier incorporated in a polyvinyl chloride membrane (Simon et al., 1978). The difference in voltage between the $Ca^{2+}$ 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 $10^{-6}$ M $Ca^{2+}$. 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 $Ca^{2+}$ stock solutions. The amount of bound $Ca^{2+}$ was calculated from the difference between free $Ca^{2+}$ in pure buffer and free $Ca^{2+}$ 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 $Ca^{2+}$ bound/mg of protein. $Ca^{2+}$ 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 $\mu$Ci of $^{45}Ca$ (specific activity 13.66 mCi/mg). Then it was washed twice for 5 min in 20 mL of distilled water, dried between filter papers, and placed on a X-ray film which was developed after 3–4 days.

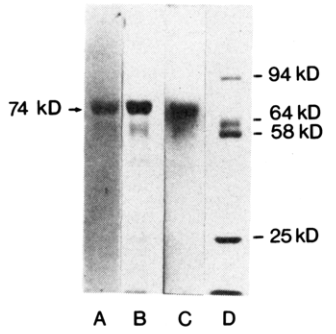


FIGURE 1: $Ca^{2+}$ binding properties of purified chromogranin A. The purified chromogranin A was separated by SDS gel electrophoresis. Nitrocellulose blotting and analysis of its $Ca^{2+}$ binding properties were performed as described under Experimental Procedures. Lane A shows binding of Stains-all,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

## 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 $Ca^{2+}$ (Reiffen & Gratzl, 1986). $^{45}Ca^{2+}$ autoradiography of these proteins separated by SDS–PAGE and blotted onto nitrocellulose indicated that $Ca^{2+}$ binding is mainly limited to a protein with the electrophoretic properties of chromogranin A. $Ca^{2+}$ 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 $^{45}Ca^{2+}$ (lane C) and staining with a carbocyanine dye (lane A), which has been observed to interact with chromogranin A and a variety of $Ca^{2+}$ binding proteins (Campbell et al., 1983; Reiffen & Gratzl, 1986), exhibit bands in corresponding locations.

Since chromogranin A is obviously the only $Ca^{2+}$ binding protein present in the chromaffin vesicle matrix, we further analyzed the $Ca^{2+}$ binding properties using a dialyzed chromaffin vesicle content preparation.

As shown in Figure 2, the number of binding sites for $Ca^{2+}$ provided by the chromaffin vesicle matrix proteins [$n$ = 180 nmol of $Ca^{2+}$/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 $Ca^{2+}$ with a $K_d$ of 50 $\mu$M (Figure 2A). These values should be compared with the data on $Ca^{2+}$ 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 $Ca^{2+}$ binding to ATP in parallel under identical conditions. In this way we found that ATP, at pH 7, binds 0.8 $\mu$mol of $Ca^{2+}$/$\mu$mol with a $K_d$ of 15 $\mu$M (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 $Ca^{2+}$ for ATP (70 $\mu$M) and proteins (90 $\mu$M) are very similar (Figure 2A,B). This is due to the greater influence of the pH change on the affinity of $Ca^{2+}$ binding to ATP (Figure 2B).

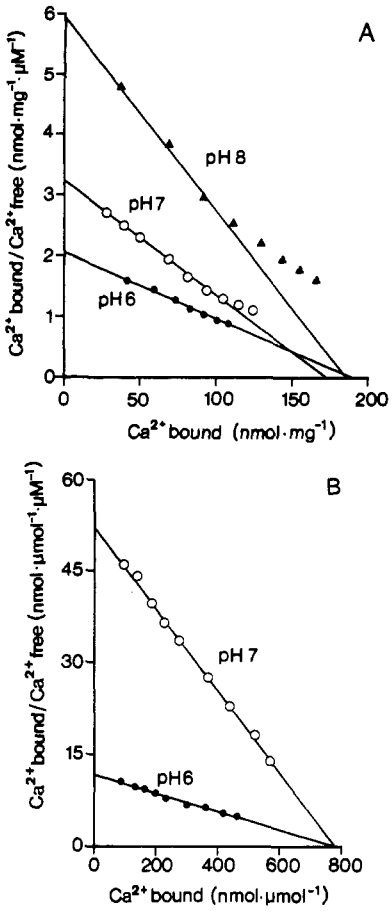


FIGURE 2: Effect of pH on the $Ca^{2+}$ binding to chromaffin vesicle matrix proteins and ATP. The chromaffin vesicle matrix proteins bind about 180 nmol of $Ca^{2+}$/mg of protein (A). The dissociation constant, calculated by linear regression analysis, decreases with increasing pH. At pH 6 we found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

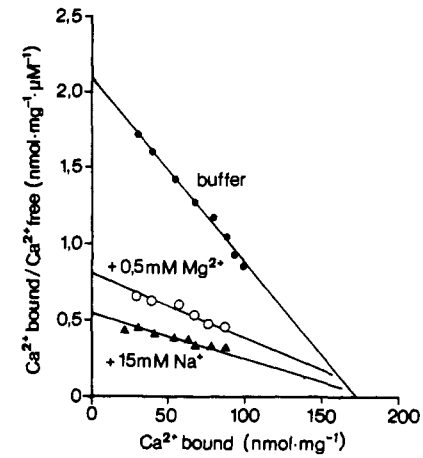


FIGURE 3: Effect of $Mg^{2+}$ and ionic strength on the $Ca^{2+}$ binding to chromaffin vesicle proteins. At pH 6 which is closer to the pH found in intact chromaffin vesicles, addition of 15 mM or 35 mM $Na^{+}$ reduces the affinity for $Ca^{2+}$ to the proteins. For example, additional 15 mM $Na^{+}$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

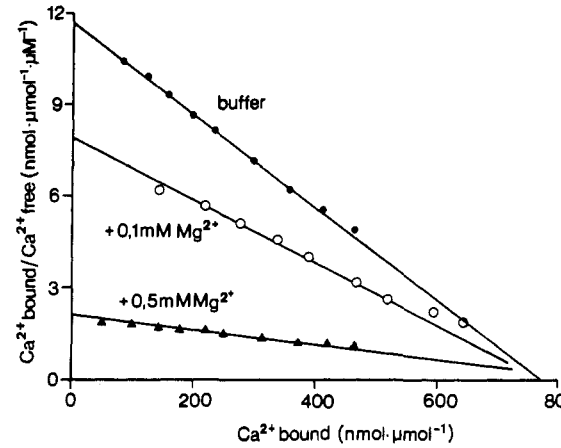


FIGURE 4: Effect of $Mg^{2+}$ on $Ca^{2+}$ binding to ATP. The titration of the potassium salt of ATP was carried out in 20 mM MES/KOH at pH 6.0. The presence of $Mg^{2+}$ (0.1 and 0.5 mM) results in a marked decrease in the affinity of ATP for $Ca^{2+}$. This divalent cation does not affect the number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

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 $Ca^{2+}$ binding to chromaffin vesicle matrix proteins is decreased by monovalent ions and by $Mg^{2+}$ at pH 6 (Figure 3). Addition of 15 mM $Na^{+}$ results in a considerable decrease of the binding affinity ($K_d$ = 340 μM). In the presence of 0.5 mM $Mg^{2+}$, a $K_d$ of 240 μM was found. There was no difference whether $K^{+}$ or $Na^{+}$ was used in these experiments (data not shown).

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

Studies using total chromaffin vesicle matrix proteins (Reiffen & Gratzl, 1986) as well as experiments with purified chromogranin A (Figure 1) demonstrated that $Ca^{2+}$ 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 μM Stains-all. As can be seen in Figure 5, only a few micrograms of protein per milliliter drastically modify the dye 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 $Ca^{2+}$ with the specific electrode to 100 μM (i.e., twice the value of the $K_d$ at pH 7 for $Ca^{2+}$ binding to the proteins). Since we did not observe quantitative or qualitative changes in the spectra, we concluded that $Ca^{2+}$ and Stains-all bind to different sites of the matrix proteins.

## Discussion

The characterization of the $Ca^{2+}$ 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 $Ca^{2+}$, 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 $Ca^{2+}$ transport systems present in the chromaffin vesicle membrane.

Chromaffin vesicles contain about 2.5 μmol 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 $Ca^{2+}$ (Figure 2B).

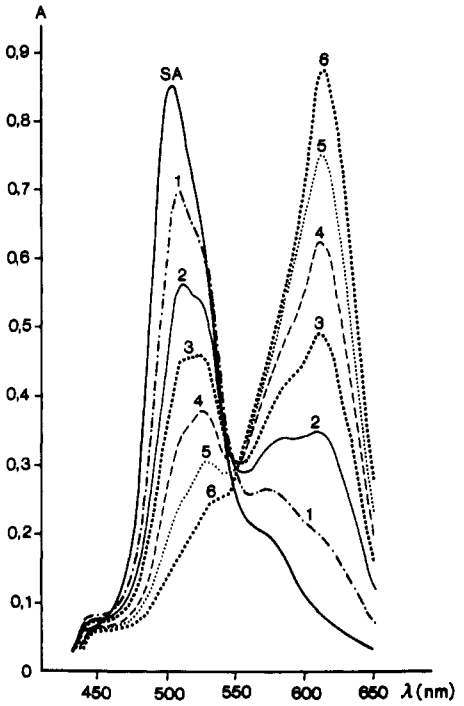


FIGURE 5: Effect of chromaffin vesicle matrix proteins on the spectrum of Stains-all. Stains-all at 20 $\mu$M in a medium containing 2 mM MOPS/KOH (pH 7) and 0.1 mM EGTA exhibits a maximum at 510 nm (full line). Successive addition of chromaffin vesicle proteins results in a reduction of the absorbance 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 $Ca^{2+}$/mg of protein (Figure 3). Therefore, ATP and chromaffin vesicle matrix proteins have the total capacity to bind approximately 680 nmol of $Ca^{2+}$/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 $Ca^{2+}$ is very similar (see Figure 2); they compete for the $Ca^{2+}$ 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 $Ca^{2+}$ binding.

The maximal $Ca^{2+}$ binding to the proteins or ATP was neither changed by variation of the pH (Figure 2) nor changed by addition of monovalent cations or $Mg^{2+}$ (Figures 3 and 4). However, increasing the ionic strength or adding $Mg^{2+}$ results in a further decrease in the affinity to $Ca^{2+}$. $Ca^{2+}$ binding to ATP after addition of 15 mM $K^+$ exhibits almost the identical value as found with 0.1 mM $Mg^{2+}$ present (170 $\mu$M) whereas $Ca^{2+}$ binding to the chromaffin vesicle content proteins exhibit a $K_d$ of 340 $\mu$M in the presence of 15 mM $Na^+$, which is to be compared to that found with 0.5 mM $Mg^{2+}$ 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 $Ca^{2+}$ 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, $Ca^{2+}$ 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 neurohypophyseal vesicles (Poisner & Douglas, 1968; Gratzl et al., 1980). Thus, the matrix proteins may be even more important for storage and binding of $Ca^{2+}$ in these vesicles than in chromaffin vesicles.

$Ca^{2+}$ binding within other subcellular structures participating in the control of cytoplasmic $Ca^{2+}$ 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 $Ca^{2+}$ binding properties of chromaffin vesicle matrix proteins can be compared with those of calsequestrin, a well-known $Ca^{2+}$ binding protein present in the lumen of the sarcoplasmic reticulum. Calsequestrin binds nearly 1000 nmol of $Ca^{2+}$/mg of protein, and Mac Lennan and Wong (1971) measured a dissociation constant for the $Ca^{2+}$–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 $Ca^{2+}$–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). $Mg^{2+}$ decreases $Ca^{2+}$ binding affinity of both types of proteins (Mac Lennan & Wong, 1971; Ikemoto et al., 1973).

The same influence of the monovalent ion $K^+$ 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 $Ca^{2+}$ binding proteins (Campbell et al., 1983). Since the binding of the dye is not affected by low concentrations of $Ca^{2+}$ (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 $Ca^{2+}$ (Caday & Steiner, 1985). The low amount of protein required for the dramatic change in the dye specrum opens a new possibility for a simple and efficient assay for chromogranins.

ACKNOWLEDGMENTS

We thank I. Lind and P. Welk for excellent technical assistance and B. Mader for the preparation of the manuscript.

We thank Prof. Simon, Zürich, for the membrane used for the $Ca^{2+}$ electrode. We also thank the local slaughterhouse for providing the bovine adrenal glands.

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

## REFERENCES

Bashford, L. C., Casey, R. P., Radda, G. K., & Gillian, R. A. (1976) *Neuroscience (Oxford) 1*, 399–412.

Borowitz, J. L., Fuwa, K., & Weiner, N. (1965) *Nature (London) 205*, 42–43.

Bulenda, D., & Gratzl, M. (1985) *Biochemistry 24*, 7760–7765.

Caday, C. G., & Steiner, R. F. (1985) *J. Biol. Chem. 260*, 5985–5990.

Campbell, K. P., Mac Lennan, D. H., & Jorgensen, A. O. (1983) *J. Biol. Chem. 258*, 11267–11273.

Chiu, V. C. K., & Haynes, D. H. (1977) *Biophys. J. 18*, 3–22.

Cohn, D. V., Zangerle, R., Fischer-Colbrie, R., Chu, L. L. H., Elting, J. J., Hamilton, J. W., & Winkler, H. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6056–6059.

Ehrhart, M., Grube, D., Aunis, D., & Gratzl, M. (1986) *J. Histochem. Cytochem.* (in press).

Gratzl, M. (1984) *Anal. Biochem. 142*, 148–152.

Gratzl, M., Krieger-Brauer, H., & Ekerdt, R. (1981) *Biochim. Biophys. Acta 649*, 355–366.

Grinstein, S., Furuya, W., Van der Meulen, J., & Hancock, R. G. V. (1983) *J. Biol. Chem. 258*, 14774–14777.

Hansford, R. G., & Castro, F. (1982) *J. Bioenerg. Biomembr. 14*, 361–376.

Herman, L., Sato, T., & Hales, C. N. (1973) *J. Ultrastruct. Res. 42*, 298–311.

Ikemoto, N., Bhatnagar, G. M., Nagy, B., & Gergely, J. (1972) *J. Biol. Chem. 247*, 7835–7837.

Ikemoto, N., Nagy, B., & Bhatnagar, G. M. (1973) *J. Biol. Chem. 249*, 2357–2365.

Johnson, R. G., & Scarpa, A. (1976) *J. Gen. Physiol. 68*, 601–631.

Joseph, S. K., Coll, K. E., Copper, R. H., Marks, J. S., & Williamson, I. R. (1983) *J. Biol. Chem. 258*, 731–741.

Kiang, W.-L., Krusius, T., Finne, J., Margolis, R. U., & Margolis, R. K. (1982) *J. Biol. Chem. 257*, 1651–1659.

Krieger-Brauer, H., & Gratzl, M. (1981) *FEBS Lett. 133*, 244–246.

Krieger-Brauer, H., & Gratzl, M. (1982) *Biochim. Biophys. Acta 691*, 61–70.

Krieger-Brauer, H., & Gratzl, M. (1983) *J. Neurochem. 41*, 1269–1276.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–684.

Leitner, J. W., Sussman, K. E., Vatter, A. E., & Schneider, F. M. (1975) *Endocrinology (Philadelphia) 96*, 662–677.

Lloyd, R. V., & Wilson, B. S. (1983) *Science (Washington, D. C.) 222*, 628–630.

Lowry, O. H., Rosebrough, N. J., Farr, S. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Mac Lennan, D. H. (1974) *J. Biol. Chem. 249*, 980–984.

Mac Lennan, D. H., & Wong, P. T. S. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1231–1235.

Mani, R. S., Shelling, J. G., Sykes, B. D., & Kay, C. M. (1983) *Biochemistry 22*, 1734–1740.

Mohan, M. S., & Rechnitz, G. A. (1972) *J. Am. Chem. Soc. 94*, 1714–1716.

O'Connor, D. T., & Frigon, R. P. (1984) *J. Biol. Chem. 259*, 3237–3247.

O'Connor, D. T., Burton, D., & Deftos, L. J. (1983) *Life Sci. 33*, 1657–1663.

Ostwald, T. J., & Mac Lennan, D. H. (1974) *J. Biol. Chem. 249*, 974–979.

Philipps, J. H. (1981) *Biochem. J. 200*, 99–107.

Phillips, J. H., Allison, Y. P., & Morris, S. J. (1977) *Neuroscience (Oxford) 2*, 147–152.

Poisner, A. M., & Douglas, W. W. (1968) *Mol. Pharmacol. 4*, 531–539.

Pollard, H. B., Zinder, O., Hoffmann, P. G., & Nikodejevic, O. (1976) *J. Biol. Chem. 251*, 4544–4550.

Ravazzola, M. (1976) *Endocrinology (Philadelphia) 98*, 950–953.

Reiffen, F. U., & Gratzl, M. (1986) *FEBS Lett. 195*, 327–330.

Reinhart, P. H., Van De Pol, E., Taylor, W. M., & Bygrave, F. L. (1984) *Biochem. J. 218*, 415–420.

Saermark, T., Thorn, N. A., & Gratzl, M. (1983a) *Cell Calcium 4*, 151–179.

Saermark, T., Krieger-Brauer, H., Thorn, N. A., & Gratzl, M. (1983b) *Biochim. Biophys. Acta 727*, 239–245.

Simon, W., Amman, D., Oehme, M., & Marf, W. E., (1978) *Ann. N.Y. Acad. Sci. 307*, 52–70.

Stoeckel, M. E., Hindelang-Gertner, C., Deilmann, H. D., Porte, A., & Stutinsky, F. (1975) *Cell Tissue Res. 157*, 307–322.

Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.

Winkler, H., & Carmichael, S. (1982) in *The Secretory Granule (poisner*, A. M., & Trifaro, J. M., Eds.) Vol. 1, pp 3–79, Elsevier Biomedical Press, Amsterdam, New York, Oxford.

Yount, R. G., Babcock, D., Ballantyne, W., & Ojala, D. (1971) *Biochemistry 10*, 2484–2489.