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Osmotic lysis of chromaffin granules treated with the ionophores nigericin and A23187 in isotonic sucrose solution at low pH

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Bovine chromaffin granules were treated with the ionophores nigericin or A23187 in sucrose solutions with the pH varying from 4.7 to 7.0. Nigericin and A23187 induced osmotic lysis of the granules in sucrose solutions at pH values below 5.8, but not at physiological pH. This effect is explained by a progressive protonation of the acidic chromogranins induced by the ionophore-promoted exchange of internal potassium- and calcium ions for external protons. The results support the view that the interactions between catecholamines and ATP with chromogranins play a significant role in osmotic pressure reduction of the granule interior.

Introduction

Chromaffin granules are the secretory vesicles of adrenal medullary cells, which store large amounts of catecholamines (550 mM, either adrenaline or noradrenaline), nucleotides (150 mM, mainly ATP), acidic proteins called chromogranins, and opiate-like peptides (for review, see Ref. 1). Further abundant constituents are Ca^{2+} ions (20 mM) [1], K^+ ions (30–40 mM) [2], Na^+ ions (30 mM) [2], and ascorbate (22 mM) [1].

Thus, the concentration of low molecular weight constituents exceeds 700 mM. Two questions therefore arise [1]: Firstly, how can the granules be in osmotic equilibrium with the cytosol, the osmolarity of which is about 300 mosM? Secondly, by what means can a concentration of catecholamines (CA) of as much as 550 mM be maintained inside the granules, regardless of the permeability of the granule membrane to these amines?

The 'osmotic' question has been answered in different ways. Because of the stoichiometric ratio of CA to ATP of 4:1 known at that time it was originally assumed that high molecular weight aggregates of the positively charged CA and the 3–4-fold negatively charged ATP molecules would account for the lowered osmolarity inside the granules [3]. Such high molecular weight complexes were found in model solutions consisting of noradrenaline and ATP at physiological concentrations [4].

NMR measurements revealed, however, that at isotonicity and in the temperature range from −2°C to 30°C no high molecular weight complexes were present in the granules [5]. Model solutions consisting of CA and ATP at intragranular concentrations exhibited a markedly lower osmolarity than would be expected from their concentrations, although all of these molecules were in solutions as revealed by NMR [5–7]. Therefore, loose associates between CA, ATP and Ca^{2+} have been postulated in order to explain the lowered osmolarity [7,8].

Furthermore, it has been recognized that the acidic polyion chromogranin A (CGA) could interact electrostatically with Ca, ATP, and Ca^{2+} [7,9]. Ion exchanger properties for the intragranular proteins have been reported by Uvnäs and Aborg [10,11]. In a recent work, Helle et al. [12] measured the osmotic pressure exerted by model solutions consisting of CGA, CA and ATP at intragranular concentrations. They found that the osmotic activity of the low molecular weight constituents was substantially lowered due to the polyion nature of CGA. However, the capacity of CGA was calculated to be too low to account for the total osmotic pressure reduction in the granules [7,12].

Abbreviations: CA, catecholamine; CGA, chromogranin A; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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In this work, we investigated the influence of intragranular acidification on the integrity of isolated bovine chromaffin granules. The granule interior was acidified by means of the ionophores nigericin or A23187, which catalyze K^+/H^+ exchange and Ca^{2+}/H^+ exchange, respectively. Granule lysis was observed under conditions when internal K^+ or Ca^{2+} ions were exchanged for external protons. Proton influx led to the partial neutralization of acidic groups of CGA molecules, which decreased the capacity of CGA to electrostatistically interact with the catecholamines. These results support the hypothesis that CGA accounts for the osmotic inactivation of CA and probably also of ATP, at least partially.

Materials and Methods

Preparation of chromaffin granules. Bovine chromaffin granules were prepared by differential centrifugation in 0.268 M sucrose solution, 10 mM Hepes (pH 7.0) adjusted with KOH, as described [13]. The resuspended granules were centrifuged at 2000 × g for 5 min in order to sediment granule aggregates. Protein was determined by a modified biuret assay [14] after protein precipitation with 10% trichloroacetic acid, centrifugation, and resolubilization in 3% (w/v) NaOH, 2% (w/v) sodium deoxycholate. Human serum albumine was used as standard.

Lysis determination. Lysis of the granules was determined by a fluorimetric CA release assay [13,15]. Inside the granules, CA fluorescence is quenched. When the CAs are diluted upon lysis, their fluorescence intensity increases. 0% lysis refers to the fluorescence intensity of an aliquot of granules in isotonic sucrose whereas 100% lysis refers to the intensity in 10 mM Hepes for a given pH. Measurements were carried out in a Perkin-Elmer MPF 4B fluorescence spectrometer using wavelengths of 285 nm (excitation) and 320 nm (emission), 50 µl granules were added from a stock suspension into 2 ml of solution in a quartz cuvette to give a final protein concentration of 0.06 mg/ml protein. The cuvette holder was thermostated at 14°C; the suspension was magnetically stirred.

Ionophores. Nigericin (Calbiochem) was dissolved in ethanol at 1.35 mM; A23187 (Calbiochem) was dissolved in ethanol/dimethylformamide (2 : 1, v/v) at 1.9 mM. 5 µl of either ionophore were added to the granule suspension in the glass cuvette to give a final concentration of 3.4 µM (nigericin) or 4.7 µM (A23187). The solvent concentration was 0.25% and caused not more than 1% CA release after 30 min; its influence was neglected. Experiments were repeated twice with different granule preparations, which gave similar results except that the stability of granules in sucrose solution at low pH varied. Therefore, results are shown for one granule preparation only, which showed the highest release of the control at pH 4.7.

Results

Osmotic stability of granules treated with the K^+ ionophore nigericin in K^+-containing solutions

In order to test the action of nigericin as a K^+/H^+ exchanger, chromaffin granules were incubated with nigericin in isotonic potassium-containing solutions at pH 7.0, i.e. at low external proton concentration. The results are shown in Fig. 1. Curve 1 and 2 show CA release of control granules in 160 mM K-gluconate, 10 mM Hepes, and in 160 mM KCl, 10 mM Hepes, respectively. The granules were stable in K-gluconate, but 27% of CA were released after 30 min in KCl solution. Addition of 3.4 µM nigericin induced rapid lysis of the granules in both, K-gluconate (curve 1′) and KCl (curve 2′) solutions. The total release was higher in KCl than in K-gluconate solution. However, the difference curve between nigericin-induced release and release of the control is approximately the same for both solutions (not shown). Nigericin did not directly disturb the membrane as was tested by incubation of granules in isotonic buffered sucrose solution at pH 7 (adjusted with NaOH). Addition of 3.4 µM nigericin caused 4% CA release after 30 min, only.

The relatively high release of granules incubated in KCl solution is probably due to the simultaneous permeation of K^+ and Cl^- ions down their electrochemical gradients into the granules, leading to osmotic lysis. The granule membrane is known to be slightly permeable for Cl^- ions, but less permeable for K^+ ions [16,17]. Inwardly-directed movement of Cl^- ions is probably

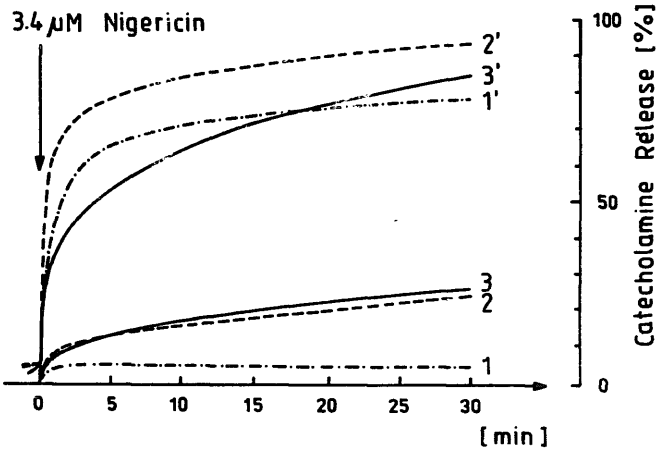

Fig. 1. Kinetics of nigericin-induced lysis of chromaffin granules in terms of catecholamine release. Granules (0.06 mg/ml) were incubated in either 160 mM potassium gluconate, 10 mM Hepes, pH 7.0 (curves 1, 1′), 160 mM KCl, 10 mM Hepes, pH 7.0 (curves 2, 2′) or 268 mM sucrose, 10 mM Hepes, pH 4.7 (curves 3, 3′). Curves 1–3 refer to control granules; curves 1′–3′ refer to granules that were treated with 3.4 µM nigericin at $t = 0$.

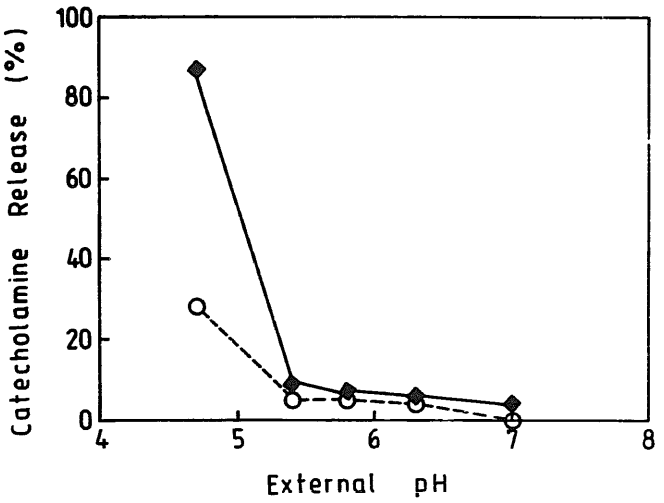

Fig. 2. Nigericin-induced catecholamine release of chromaffin granules as a function of external pH. Cumulative release after a 30 min incubation period in 268 mM buffered sucrose is shown. ○, control granules without nigericin; ◆, granules that were treated with 3.4 μM nigericin at $t = 0$.

limited by the low K^+ permeability. The gluconate anion is impermeable (cf. Ref. 18), therefore, only few K^+ ions can enter the granules, which is not sufficient to cause osmotic lysis. Further influx of K^+ ions is inhibited by the generation of a positive diffusion potential.

When nigericin is present in K^+-containing solutions at pH 7, it incorporates into the granule membrane and exchanges internal protons for external K^+ ions. Assuming an intragranular pH of 5.6 [19], the internal proton concentration is 24 times larger than the external proton concentration at pH 7. The internal concentration of K^+ of about 40 mM [2] is 4-times lower than the external concentration (160 mM). Protons are osmotically inactivate because they do not exist as single particles, but are bound, e.g. to water molecules or to proteins. Nigericin therefore exchanges osmotically inactive internal protons for osmotically active external K^+ ions down the electrochemical gradients of the two ion species, and thus causes lysis of the granules.

In another experiment the electrochemical gradients of H^+ and K^+ were reversed using K^+-free sucrose solutions with varying pH (adjusted with NaOH or HCl). CA release was measured for a period of 30 min in the absence (control) or presence of 3.4 μM nigericin. Results are shown in Fig. 2, in which cumulative release after 30 min is drawn as a function of the external pH. On decreasing the pH from 7.0 to 5.3 the release of both, controls and nigericin-treated granules, increased slightly. Release in the presence of nigericin was 2–4% higher than in its absence. When the pH was lowered to 4.7, however, release of nigericin-treated granules amounted to 88% after 30 min. Release of control granules did also increase at this low pH; it varied from 10–28% between different granule preparations.

The kinetics of CA release at pH 4.7 is shown in curves 3 and 3′ of Fig. 1. It deviates from the release kinetics of nigericin-treated granules in K^+ containing solutions at pH 7 (curves 1′, 2′) indicating that a different mechanism is responsible for granule lysis in sucrose media at low pH.

Osmotic stability of granules treated with the Ca^{2+} ionophore A23187

Experiments that could have tested the action of A23187 as an ionophore at high external Ca^{2+} concentration and high pH were not performed since chromaffin granules are known to aggregate and fuse at millimolar Ca^{2+} concentrations [20]. The effect of A23187 on chromaffin granules suspended in buffered sucrose solutions was investigated as a function of external pH. In order to chelate contaminating Ca^{2+} ions, parallel experiments were carried out in which the solutions contained 1 mM EDTA. At pH 7, there was a negligible CA release after 30 min of granules that were or were not treated with A23187. When the external pH

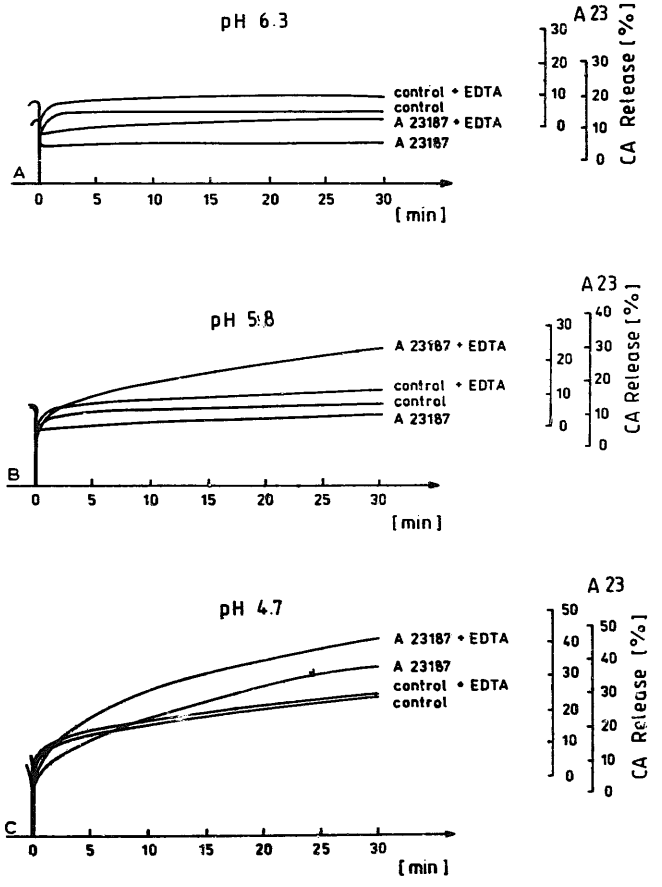

Fig. 3. Kinetics of catecholamine release of chromaffin granules induced by the ionophore A23187 at three different external pH values. Granules (0.06 mg protein/ml) were incubated in 268 mM sucrose, 10 mM Hepes with or without 1 mM EDTA at different pH (controls). A23187 was added to two samples at $t = 0$ to give a final concentration of 4.7 μM. As A23187 itself absorbed fluorescence light, a second scale for catecholamine release is given for A23187-containing samples.

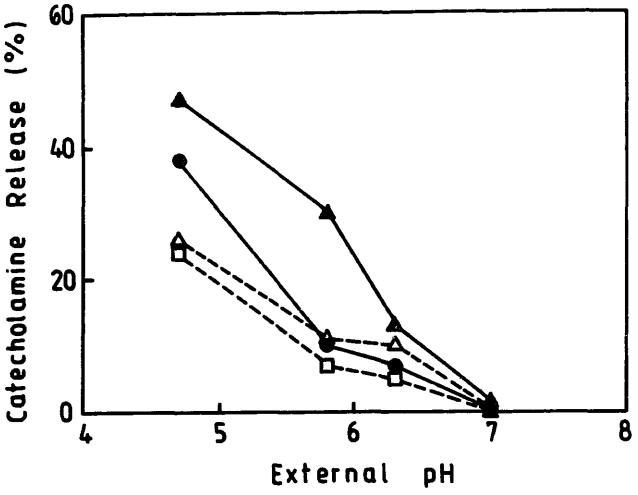

Fig. 4. A23187-induced catecholamine release of chromaffin granules as a function of external pH. Cumulative release after a 30 min incubation period in 268 mM buffered sucrose is shown. □, controls without EDTA; △, controls containing 1 mM EDTA; ●, 4.7 μM A23187, no EDTA; ▲, 4.7 μM A23187, 1 mM EDTA.

was lowered, release of both, controls and A23187-treated granules, increased. This increase was higher in the presence of EDTA, respectively. The time course of release is shown in Fig. 3 for pH 6.3, 5.8, and 4.7. As A23187 itself absorbed fluorescence light, two different scales for CA release, one for control experiments and one for A23187 experiments, are given in Fig. 3. The decrease in fluorescence intensity was found to be proportional to A23187 concentration and only slightly dependent on pH.

In Fig. 4, CA release after 30 min is shown as a function of pH on increasing the external proton concentration, A23187 caused progressive release of CA. The addition of EDTA, which elevated the electrochemical gradient for Ca^{2+} across the granule membrane by Ca^{2+} chelation, increased release of control granules only slightly (by 0–5% of total CA), but that of A23187-treated granules markedly (by 20% of total CA at pH 5.8 and 9% at pH 4.7).

Discussion

Action of ionophores.

The ionophore nigericin catalyzes electroneutral exchange of protons for monovalent cations with a high specificity for K^+ [16,19,21]. Experiments in which chromaffin granules suspended in K^+-containing solutions at pH 7 were treated with nigericin have been performed [16,17,19,22,23]. In all of these experiments, nigericin induced lysis of the granules in dependence on the external K^+ concentration. Lysis was interpreted to be caused by an increase in intragranular osmotic pressure, since buffered (osmotically inactive) protons had been exchanged for osmotically active K^+ ions [23].

We confirmed these results using KCl and potassium gluconate. For the first time, we present data on nigericin-induced lysis of chromaffin granules in sucrose solutions at low pH. Assuming the intragranular pH to be 5.6 [19], the internal proton concentration (2.5 μM) is 25, 5, 1.6, 0.63, 0.13 times the external proton concentration at pH 7, 6.3, 5.8, 5.4, and 4.7, respectively. The external concentration of potassium was estimated as follows; Chromaffin granules were isolated in buffered sucrose containing 2 mM K^+ due to pH adjustment by KOH. The presence of K^+ ions in the isolation medium has been reported to best maintain intragranular potassium, the concentration of which is 40 mM [2]. Assuming further that leakage of 10% of total CA might have occurred during isolation and storage of the granules, the external K^+ concentration in the cuvette was not higher than 50 μM. Thus, the internal K^+ concentration was 800-times larger than the external one. Lysis of the granules occurred when the external pH was decresed below 5.4, i.e. when the proton gradient became opposite to the K^+ gradient (Fig. 2).

Ionophore A23187 exchanges two protons for one divalent cation, with the highest specificity for Ca^{2+} ions [24,25]. In the absence of EDTA, A23187 caused significant release only at pH 4.7, under conditions when the external proton concentration exceeded the internal one. In the presence of 1 mM EDTA, lysis of chromaffin granules already started at pH 5.8. The external Ca^{2+} concentration was estimated to be less than 10 μM. Thus it was 2000-times smaller than the internal Ca^{2+} concentration, which amounts to 20 mM [1]. Addition of 1 mM EDTA caused chelation of external Ca^{2+}, resulting in an infinitely large Ca^{2+} gradient. Under these conditions, external protons were probably transported uphill into the granules at pH 5.8 due to the large Ca^{2+} gradient, and caused lysis.

Südhof [26] observed A23187-induced granule lysis at 25°C in isotonic buffered sucrose, 5 mM EDTA, already at pH 7. A vital role of calcium ions for granule core integrity was deduced from these experiments. We were not able to confirm these results. Even at 25°C, no significant CA release occurred. Our observations are, however, consistent with those of Johnson and Scarpa [27].

The treatment of chromaffin granules with either nigericin or A23187 induced lysis when either internal K^+ or internal Ca^{2+} ions were exchanged for external protons down the electrochemical gradients of these ions. A significant amount of internal Ca^{2+} ions is bound to CGA [28,29], therefore, the osmotic activity of internal Ca^{2+} ions is presumably low. K^+ ions, however, are not known to be specifically bound to any of the core constituents, thus they possess osmotic activity. This means that the exchange of internal, at least partially osmotically active metal ions for external, osmoti-

cally inactive protons caused osmotic lysis. This apparent contradiction can only be explained if one assumes that intragranular acidification led to the protonation of functional groups of constituents that were involved in CA storage. Moreover, a cooperative mechanisms for CA storage must be postulated, since the substitution of one K^+ ion or one Ca^{2+} ion by a positively charged amine in the granule matrix would not account for an increase in osmolarity.

Structure of the granule core

For a long time it has been assumed that interactions between the positively charged CA and the negatively charged ATP molecules lower the osmotic pressure inside the granules (for review, see Ref. 1). In model solutions containing 500 mM noradrenaline, 150 mM ATP, and 40 mM Ca^{2+}, the formation of high molecular weight aggregates was shown to occur below 10°C [4]. However, NMR measurements revealed that even at low temperatures, high molecular weight CA-ATP complexes are absent from the chromaffin granule phase [5,30]. Granot and Rosenheck [31] showed using ^{1}H-NMR that ATP and CA form small complexes at a molar ratio of 1:2 or 1:3. ^{1}H-NMR measurements of isolated granules as well as model solutions by Daniels et al. [32] revealed that the tumbling rates of ATP and CAs were considerably lowered as compared with the unhindered motion of these molecules; therefore, low molecular weight complexes of ATP and CAs were assumed. A series of detailed ^{1}H- and ^{13}C-NMR studies of Sharp and co-workers indicated, however, that 97% of the CA was freely diffusible in the granules and that no CA-ATP-complexes were present [6,7]. These authors suggested that dynamic associates between CA and ATP molecules, being in rapid exchange with molecules in solution, account for osmotic pressure lowering [6,7]. Model solutions of CAs and ATP at intragranular concentrations indeed exhibit a high degree of osmotic nonideality [7,8].

There are, however, several facts that contradict the picture of osmotic lowering solely by CA–ATP interaction: Firstly, NMR studies revealed that the rotational correlation time of ATP molecules is 4-times longer than that of CA molecules [7,9]. Also, considerable deviations from the 4:1 stoichiometry of CA/ATP have been reported. Granules seem to be heterogeneous with respect to their CA/ATP ratio, with values ranging from 4 to 13 [33–35]. These findings exclude that all of the CAs could simultaneously interact with ATP electrostatically.

Secondly, release experiments using isolated granules superfused with isotonic KCl solution indicated the existence of two CA storage pools inside the granules [10,11]. The small pool 1 was ascribed to ATP-free CA molecules in free solution, which is in equilibrium with pool 2. Pool 2 was suggested to correspond to the acidic protein matrix, in which the COO^- groups from acidic side chains are ionically linked to CA molecules and the NH_3^+ groups of basic amino acids are linked to ATP. $COO^- \cdots NH_3^+$ salt bridges in the protein matrix should be forced successively open on increasing the CA concentration, leading to a cooperative rise in ionic binding sites for CA and ATP [10,11].

Thirdly, micro-osmometric measurements revealed that in model solutions consisting of purified chromogranins and salts, the acidic proteins substantially lowered the osmotic pressure that would be exerted by the salt due to ionic interactions. ATP was especially active in this osmotic pressure lowering [12].

Capacity of the chromogranins for osmotic pressure reduction

Chromaffin granules contain unusually acidic proteins of unknown function(s), the chromogranins A, B, and C. Chromogranin A is the most abundant species of this family, comprising 80% of the soluble protein [36]. Previous calculations of the storage capacity of CGA were based on an apparent molecular mass of 75 kDa [7,12], a value which had been obtained from SDS gel electrophoresis. Sequence analyses of CGA cDNA revealed, however, that CGA comprises 431 amino acids with a total molecular mass of 48 kDa [37]. The discrepancy between the two values were attributed to an unusual migration behaviour of CGA in SDS gels [37,38] due to its high negative net charge and its random coil structure [7,39].

In the following calculations we use a molecular weight of 50 kDa which takes account of post-translational modifications. Besides the large amount of acidic amino acids, CGA contains 9.17 μM sialic acid/100 mg [40], which corresponds to 5 molecules sialic acid/molecule CGA. It also contains phosphate groups; after correction of the data from Settleman et al. [41] one arrives at 3 phosphorylated serine residues per molecule. Table I shows the number of potential charged groups per CGA molecule, their pK values, the degree of ionization of these groups, and the concentration of negative and positive charged at intragranular pH (5.6) and at pH 4.7. For the latter data, the concentration of CGA used in crucial. This concentration was calculated to range from 2.8 to 3.6 mM [36,42]. For the calculations in Table I, a value of 2.8 mM was used. The pK values are those of secondary charged groups of single amino acids. Although the pK values of the respective acid or basic groups may be different within the protein, this deviation is small. When the isoelectric point of CGA is calculated from these pK values, one arrives at a pI of 4.4, a value which is in the range of experimentally determined pI values (4.4–5) [38,43,44].

From Table I follows that there are 305 mM negative charges, 195 mM positive charges and 110 mM net negative charges in total CGA at the intragranular pH

TABLE I

Number of potential charged groups of chromogranin A, degree of ionization and charge concentration at pH 5.6 and pH 4.7

Amino acid content was taken from Ref. 37, phosphate content from 41, and sialic acid content from [40]. The pK values are those for acid or basic groups of the single amino acids. An intragranular CGA concentration of 2.8 mM was assumed [36,42].

Amino acid/ charged group	Number/CGA molecule	pK *	Charged form (%)		Charge concentration (mM)	
			pH 5.6	pH 4.7	pH 5.6	pH 4.7
Aspartic acid	22	3.7	98.8	90.9	61	56
Glutamic acid	63	4.3	95.2	71.5	221	166
Serine-P_1	3	6.5	11.2	1.6	1	0
		2.5	99.9	99.4	8	8
Sialic acid	5	< 4	97.6	83.4	14	12
Lysine	33	10.5	100.0	100.0	92	92
Arginine	32	12.5	100.0	100.0	90	90
Histidine	6	6.1	76.0	96.2	13	16
Negative charges					305	242
Positive charges					195	198
Net negative charges					110	44

5.6. In principle, CGA could 'bind' about half of the CA and all of the ATP by electrostatic interactions. This bound fraction is osmotically inactive [7,12,45]. The other half of CA could then interact with the remaining charges of ATP, which also decreases the osmotic activity of the CA molecules [7,8]. If the intragranular pH is lowered to 4.7, 63 mM negative charges of CGA will be neutralized by protonation, and will not interact with positively charged CA. A rise of 63 mM in osmotically active concentration is sufficient to cause significant lysis (40% CA release) [13,46].

It might be argued that the protonation of ATP is also affected on lowering the internal pH, such that the decrease in ATP-CA interaction is responsible for the increase in internal osmolarity. Assuming a pK value of 6.5 for the last hydroxyl group of ATP and an internal concentration of 150 mM, only 14 mM negative charges are neutralized on decreasing the pH from 5.6 to 4.7. This increase in osmolarity, however, would induce only minor lysis (less than 5% CA release) [13,46].

The function(s) of the chromogranins are not known as yet. Chromogranins have been found in many endocrine tissues and in brain [36,38,47]. They are not confined to amine-storing vesicles, but are also present in secretory vesicles that store peptide hormones. Recently it has been found that CGA-derived peptides formed after exocytosis exert a feedback control on chromaffin cell secretion, suggesting the role of a prohormone for CGA [48]. However, this need not to be the only function of CGA. The concentration of CGA in chromaffin granules is much higher than that of chromogranins in other tissues, e.g. it is 1000 times larger than that in brain [47]. Thus, CGA in chromaffin granules could specifically act as a condensing protein that enables the granules to store unusual large amounts of catecholamines and ATP.

References

1 Winkler, H. and Westhead, E. (1980) Neuroscience 5, 1803–1823.
2 Ornberg, R.L., Kuijpers, G.A.J. and Leapman, R.D. (1988) J. Biol. Chem. 263, 1488–1493.
3 Hillarp, N.-A. (1958) Acta Physiol. Scand. 42, 321–332.
4 Berneis, K.H., Pletscher, A. and Da Prada, M. (1970) Br. J. Pharmac. 39, 382–389.
5 Sen, R. and Sharp, R.R. (1981) Biochem. J. 195, 329–332.
6 Sen, R., Sharp, R.R., Domino, L.E. and Domino, E.F. (1979) Biochim. Biophys. Acta 587, 75–88.
7 Sen, R. and Scharp, R.R. (1982) Biochim. Biophys. Acta 721, 70–82.
8 Kopell, W.N. and Westhead, E.W. (1982) J. Biol. Chem. 257, 5707–5710.
9 Sharp, R.R. and Richards, E.P. (1977) Biochim. Biophys. Acta 497, 260–271.
10 Uvnäs, B. and Aborg, C.-H. (1980) Acta Physiol. Scand. 109, 345–354.
11 Uvnäs, B. and Aborg, C.-H. (1988) Acta Physiol. Scand. 132, 297–311.
12 Helle, K.B., Reed, R.K., Pihl, K.E. and Serck-Hanssen, G. (1985) Acta Physiol. Scand. 123, 21–33.
13 Engel, J., Donath, E., Ermakov, Y.A., Meyer, H.W. and Richter, W. (1989) Biochim. Biophys. Acta 985, 11–119.
14 Casey, R.P., Njus, D., Radda, G.K. and Sehr, P.A. (1976) Biochem. J. 158, 583–588.
15 Steffen, H., Da Prada, M. and Pletscher, A. (1974) Biochim. Biophys. Acta 338, 561–571.
16 Johnson, R.G. and Scarpa, A. (1976) J. Gen. Physiol. 68, 601–631.
17 Phillips, J.H. (1977) Biochem. J. 168, 289–297.
18 Perlman, R.L. (1976) Biochem. Pharmacol. 25, 1035–1038.
19 Johnson, R.G. and Scarpa, A. (1976) J. Biol. Chem 251, 2189–2191.
20 Ekerdt, R., Dahl, G. and Gratzl, M. (1981) Biochim. Biophys. Acta 646, 10–22.

21 Zoratti, M., Favoron, M., Petrobon, D. and Petronilli, V. (1984) Biochim. Biophys. Acta 767, 231–239.
22 Pollard, H.B., Shindo, H., Creutz, C.E., Pazoles, C. and Cohen, J.S. (1978) J. Biol. Chem. 254, 1170–1177.
23 Pazoles, C.J., Creutz, C.E., Ramu, A. and Pollard, H.B. (1980) J. Biol. Chem. 255, 7863–7869.
24 Reed, P.W. and Lardy, H.A. (1972) J. Biol. Chem. 247, 6970–6977.
25 Pfeiffer, D.R. and Lardy, H.A. (1976) Biochemistry 15, 935–943.
26 Südhof, T.C. (1983) Biochem. Biophys. Res. Commun. 116, 663–668.
27 Johnson, R.G. and Scarpa, A. (1974) FEBS Lett. 47, 117–121.
28 Bulenda, D. and Gratzl, M. (1985) Biochemistry 24, 7760–7765.
29 Reiffen, F.U. and Gratzl, M. (1986) FEBS Lett. 195, 327–330.
30 Costa, J.L., Sokoloski, E.A. and Morris, S.J. (1984) Res. Commun. Chem. Pathol. Pharmacol. 45, 389–398.
31 Granot, J. and Rosenheck, K. (1978) FEBS Lett. 95, 45–48.
32 Daniels, A.J., Williams, R.J.P. and Wright, P.E. (1978) Neuroscience 3, 537–585.
33 Van Dyke, K., Robinson, R., Urquilla, P., Smith, D., Taylor, M., Trush, M. and Wilson, M. (1977) Pharmacology 15, 377–391.
34 Terland, O., Flatmark, T. and Kryvi, H. (1979) Biochim. Biophys. Acta 553, 460–468.
35 Bolstadt, G., Helle, K.B. and Serck-Hanssen, G. (1980) J. Auton. Nerv. Syst. 2, 337–354.
36 Fischer, Colbrie, R., Hagn, C. and Schober, M. (1987) Ann. N.Y. Acad. Sci. USA 493, 120–134.
37 Grimes, M., Iacangelo, A., Eiden, L.E., Godfrey, B. and Herbert, E. (1987) Ann. NY Acad. Sci. USA 493, 351–378.
38 Simon, J.-P. and Aunis, D. (1989) Biochem. J 262, 1–13.
39 Smith, A.D. and Winkler, H. (1967) Biochem. J. 103, 483–492.
40 Kiang, W.-L., Krusius, T., Finne, J., Margolis, R.U. and Margolis, R.K. (1982) J. Biol. Chem. 257, 1651–1659.
41 Settleman, J., Fonseca, R., Nolan, J. and Hogue-Angeletti, R. (1985) J. Biol. Chem. 260, 1645–1651.
42 Phillips, J.H., Allison, Y.P. and Morris, S.J. (1977) Neuroscience 20, 147–152.
43 Corcoran, J.J., Lattanzio, Jr., F.A., Rubin, R.W. and Pressman, B.C. (1982) Biochim. Biophys. Acta 707, 226–235.
44 Apps, D.K., Phillips, J.H. and Purves, F.C. (1985) Neuroscience 16, 477–487.
45 Oosawa, F. (1971) Polyelectrolytes, Marcel Dekker, New York.
46 Südhof, T.C. and Morris, S.J. (1983) Biochem. Biophys. Acta 730, 207–216.
47 O'Connor, D.T. and Frigon, R.P. (1984) J. Biol. Chem. 259, 3237–3247.
48 Simon, J.-P., Bader, M.-F. and Aunis, D. (1988) Proc. Natl. Acad. Sci. USA 85, 1712–1716.