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EVIDENCE FOR A DIVALENT CATION DEPENDENT CATECHOLAMINE STORAGE COMPLEX IN CHROMAFFIN GRANULES

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Chromaffin granules, the secretory vesicles of the adrenal medulla, are stable in isotonic sucrose solutions at room temperature; however, when low concentrations of the ionophore A23187 are added, rapid lysis ensues which is dependent on the presence of a divalent cation chelator and is prevented by the addition of either Ca^{2+} or Mg^{2+}. As little as 10 µM Ca^{2+} totally inhibit lysis of chromaffin granules by A23187, while 28 mM KCl have no effect. Lysis by A23187 at 4.7 µM is almost 100% in the presence of EDTA in isotonic sucrose in 1 h and can be suppressed by raising the osmotic strength of the medium with half maximal inhibition at 0.57 M sucrose, demonstrating that A23187 causes osmotic lysis of chromaffin granules as a consequence of the withdrawal of divalent cations from the core solution. Our results strongly suggest that divalent cations are involved in the formation of a ionic complex in the core solution which lowers its effective osmotic pressure.

Chromaffin granules, the secretory vesicles of the adrenal medullary chromaffin cells, contain high concentrations of catecholamines, ATP, divalent cations and other solutes, whose total concentration has been estimated to amount to more than 0.75 M (1-3).NMR studies have shown that all these constituents are in a soluble phase and that no separate core phase exists (4-7). Nevertheless, chromaffin granules are stable in isotonic solutions and have an only slightly hypertonic internal osmotic pressure (8,9), suggesting some kind of core complex formation. A core complex involving fast ionic interactions would be compatible with the NMR-data. I now report that under conditions which allow the selective withdrawal of divalent cations from the chromaffin granule core, where they constitute only a minor component, the granules lyse completely. This observation

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suggests the presence of a core complex in chromaffin granules for whose maintenance divalent cations are essential.

Materials and Methods

Chromaffin granules were prepared as described except that the 0.26 M sucrose solution was buffered with 25 mM Hepes pH 7.4 ('buffered sucrose') (9). A23187 was obtained from Sigma (Munich, F.R.G.) and added to the granule suspensions from a 0.5 mM stock solution in ethanol. Chromaffin granule lysis was measured by two methods which have previously been shown to correlate with each other and with the release of catecholamines and dopamine-ß-hydroxylase from the granule core (10). In the first utilizing changes of turbidity, measurements were made by tracing changes of extinction at 540 nm and 25°C in water-jacketed cuvette holders in a Zeiss PMQ 3 spectrophotometer as a function of time and medium composition, with a sample volume of 2.05 ml, a maximal turbidity of 0.5 OD and granule concentrations of 0.10-0.15 mg/ml protein. In the second method, lysis was followed by protein release. This was measured by determining the protein released after 1 h of incubation at room temperature as a function of the composition of the medium. Membranes and intact granules were separated from released soluble protein by centrifugation (30 min at 30 000 g_{max} at 5°C); concurrent samples in which granules were incubated in buffered sucrose without additions or in 10 mM Hepes served as reference points for 0% lysis (usually 8-12% of the total incubated protein) and 100% lysis (75-80% of total protein). Protein concentrations were determined according to Bradford (11). Controls in which only ethanol was added to the granule suspensions showed that in such low concentrations ethanol alone did not cause any lysis (cf. Table 1).

Results

Chromaffin granules are fairly stable at room temperature in isotonic sucrose solutions (0.26 M sucrose, 25 mM Hepes pH 7.4) in the presence of either Ca^{2+} or EDTA and only show the slow temperature dependent lysis which we previously characterised (12). However, as demonstrated in Fig. 1 by turbidimetric lysis measurements on chromaffin granule suspensions, when low concentrations of the ionophore A23187 are added, rapid lysis is observed. This lysis is dependent on the presence of a divalent cation chelator and can be totally inhibited by the addition of Ca^{2+} or Mg^{2+}; its rate is further dependent on the ionophore concentration. Table 1 reports the effect of different compositions of the incubation media on the lysis by A23187 as measured by protein release. It demonstrates that in the

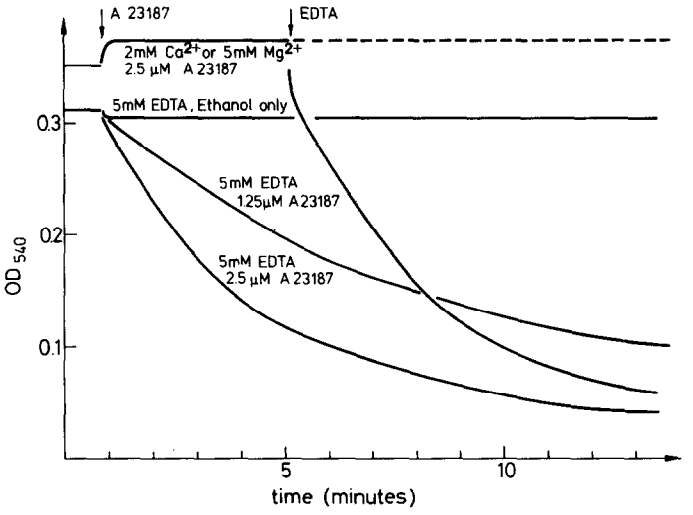

Figure 1
Lysis of chromaffin granules by A23187 as followed by the suspension's turbidity at 540 nm and at 25°C. In the presence of either 2 mM Ca^{2+} or 5 mM Mg^{2+} A23187 only causes further aggregation of the granules (which is also responsible for the higher starting turbidity of the samples); in the presence of 5 mM EDTA, however, rapid lysis is observed whose rate is dependent on the ionophore concentration. The inhibition of lysis by Ca^{2+} or Mg^{2+}, on the other hand can be reversed by adding a surplus of chelator.

presence of 5 mM EDTA 4.7 µM A23187 causes almost total lysis of chromaffin granules in 1 h in isotonic sucrose solutions, while in sucrose solutions without additions the lysis is significantly

TABLE 1
Chromaffin granule lysis by A23187 as measured by protein release as a function of the composition of the medium

Additions (µM)[1]					% Lysis ± SD (n)
EDTA	Ca^{2+}	Mg^{2+}	A23187	KCl	
5000	-	-	-	-	0 (8)
-	2000	-	-	-	0 (8)
5000	-	-	4.7	-	95 ± 9 (8).
-	2000	-	4.7	-	2 ± 3 (8)
-	10	-	4.7	-	2 ± 1 (3)
-	-	-	4.7	-	22 ± 6 (3)
-	-	3000	4.7	-	1 ± 2 (4)
5000	-	-	4.7	28000	91 ± 8 (3)
-	2000	-	4.7	28000	5 ± 3 (3)

1) To suspension medium containing 0.26 M sucrose, 25 mM Hepes pH 7.4 and 0.9% ethanol.

reduced, presumably by contaminating endogenous Ca^{2+}. As little as 10 μM exogenous Ca^{2+} or 3 mM Mg^{2+} can completely inhibit lysis. On the other hand, the monovalent ion K^{+} has no effect on the lysis reaction. It may be concluded that lysis of chromaffin granules by the ionophore A23187 is dependent on conditions which cause movement of Ca^{2+} or Mg^{2+} out of the granules. This would suggest that lysis by A23187 is osmotic lysis caused by an increase in the osmotic pressure of the core solution as a consequence of the dissociation of a divalent-cation-stabilised core complex and is not caused by a direct effect of A23187 on the membrane. To test this point I investigated whether lysis could also be prevented by increasing the osmotic pressure of the suspension medium. In Fig. 2, where percent lysis is plotted as a function of the sucrose concentration, it is demonstrated that increasing the osmotic strength indeed suppresses lysis with 50% inhibition at ca 0.57 M sucrose, 25 mM Hepes, 5 mM EDTA.

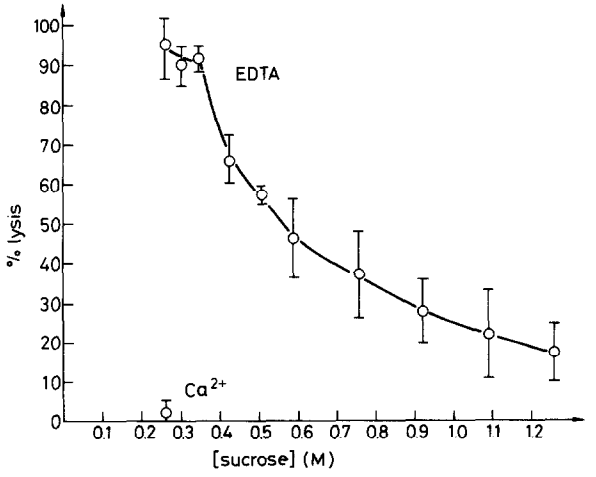

Figure 2
The suppression of chromaffin granule lysis in the presence of 4.7 μM A23187 and 5 mM EDTA by increasing the osmotic strength of the medium. The ordinate gives % lysis as measured by protein release, the abszisse the molarity of the sucrose in the incubation medium which always additionally contained 25 mM Hepes and 5 mM EDTA. Points and bars represent means ± SD from 3 experiments.

Discussion

The divalent-cation-selective ionophore A232187 is frequently used to stimulate cells by importing Ca^{2+} into them (3). The experiments reported here demonstrate that in the presence of A23187 chromaffin granules are lysed osmotically exclusively under conditions under which the ionophore causes depletion of Ca^{2+} or Mg^{2+} from the granules. Our results suggest that the previously observed high concentrations of divalent cations in the granule core (2) are involved in binding the catecholamines and ATP into an osmotically inactive form. The extremely low concentrations of Ca^{2+} sufficient to suppress lysis indicate a very low Ca^{2+} activity in the granule core in spite of its high total core concentration. The same might be true for the other core constituents so that the effective concentration gradients across the granule membrane for catecholamines and ATP might be lower than the total concentration gradient, making it much less expensive in terms of energy for the cell to accumulate and maintain high concentrations of catecholamines and ATP in the granule core.

Several *in vitro* model studies (reviewed in [14]) have both suggested (15-18) and excluded (19,20) a vital role for divalent cations in the formation of the chromaffin granule core solution. This report provides the first experimental demonstration that the depletion of divalent cations from the granule core leads to lysis. In the course of previous work with the ionophore X537A, which transports both mono- and divalent cations including catecholamines across lipid membranes, it has both been reported (21) and disputed (22) that A23187 releases catecholamines from chromaffin granules, although there seems to be general agreement that A23187 only transports divalent cations (23). We have demonstrated that selective conditions are necessary to achieve

lysis and catecholamine release with A23187 and therefore the conflicting reports might be based in the different incubation procedures.

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