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Free concentrations of sodium, potassium and calcium in chromaffin granules

Julian R. HAIGH, Richard PARRIS and John H. PHILLIPS
Department of Biochemistry, University of Edinburgh Medical School, Hugh Robson Building, George Square, Edinburgh EH8 9XD, U.K.

We have measured the contents of Na^+ and K^+ in isolated chromaffin granules. Total contents varied between 227 and 283 nmol/mg of protein, equivalent to matrix concentrations of 53–66 mM. The value found depended on the isolation buffer used, and the ratio of the two ions reflected the composition of the buffer. We then measured the free concentration of each of these ions, and of Ca^{2+}, in the matrix, by using a null-point method with acridine-fluorescence quenching. This monitored H^+ fluxes induced by an ionophore in the presence of known concentrations of the ion in the supporting medium. In contrast with organic constituents of the matrix, which have low activity coefficients, Na^+ and K^+ were found to have activity coefficients around 0.8. Ca^{2+}, however, was strongly bound: its free concentration was only 0.03% of the total.

INTRODUCTION

It is well known that mammalian secretory granules contain bivalent cations, and there has been considerable speculation about their role. Granule membranes contain transport mechanisms for these ions, and it has been suggested that they function in granule maturation (promoting aggregation of soluble constituents such as proteins and nucleotides; Winkler, 1977) or in regulating cation homoeostasis in the secretory cell. However, two values for bivalent-cation concentrations within secretory granules from the rat illustrate how divergent are these contents: parotid granules contain 8 mM-Ca^{2+} and 5 mM-Mg^{2+} (Castle *et al.*, 1987), whereas insulinoma granules contain 120 mM-Ca^{2+} and 72 mM-Mg^{2+} (Hutton *et al.*, 1983). Much less attention has been paid to the common univalent cations such as Na^+ and K^+: these appear to be virtually excluded from parotid granules, but are present at 29 mM and 16 mM respectively in insulinoma granules.

In order to understand bioenergetic processes involving these ions, one needs to know their activity coefficients within the granule matrix. We have been interested in this for the bovine chromaffin granule, the secretory granule of the adrenal medulla, in which Ca^{2+} transport is Na^+-linked (Phillips, 1981; Krieger-Brauer & Gratzl, 1982). Many determinations have been made of Ca^{2+} and Mg^{2+} concentrations in the chromaffin-granule matrix. In a review, Njus *et al.* (1986) quote values of 17 mM and 5 mM respectively. Krieger-Brauer & Gratzl (1982) estimated the internal Na^+ concentration ($[Na^+]_i$) of isolated granules to be 47 mM. More recently, Ornberg *et al.* (1988) have found the following values for granules in cultured cells, using electron-probe microanalysis (all values in mM): Ca^{2+}, 8.8; Mg^{2+}, 5; Na^+, 0; K^+, 83. The ion content of granules isolated from intact glands was found to vary with the buffer used for isolation. In Mes/NaOH buffer the values were 7.0, 0, 29 and 19 mM respectively, whereas in Mes/KOH buffer they were 12, 0, 8 and 40 mM.

In this paper we present a method for estimating the free concentrations of Ca^{2+}, Na^+ and K^+ in isolated chromaffin granules. These granules contain approx. 120 mM nucleotides and high concentrations of glycosaminoglycans and negatively charged proteins (Winkler & Westhead, 1980), so bivalent ions are expected to be bound.

Bulenda & Gratzl (1985) estimated the free matrix concentration of Ca^{2+} ($[Ca^{2+}]_i$) in these granules to be 24 μM or 4 μM by two null-point methods using a Ca^{2+}-specific electrode to detect Ca^{2+} fluxes in the presence of the ionophore A23187. However, their granules had an unusually high internal pH (pH 6.2, whereas most authors agree on values between pH 5.5 and 5.7) and, in addition, the use of the ionophore may have affected the ion-selectivity of the electrode.

We have therefore employed a fluorimetric method using the weak base 9-amino-6-chloro-2-methoxyacridine (ACMA) to monitor intragranular pH changes in the presence of various ionophores. Salama *et al.* (1980) used somewhat similar null-point methods to investigate K^+ in chromaffin granules; they concluded that $[K^+]_i$ was about 2 mM, a value that, when compared with the analyses by Ornberg *et al.* (1988) quoted above, suggests that this ion must also have a remarkably low activity coefficient in the matrix. Our results are at variance with this conclusion.

THEORY

Schuldiner *et al.* (1972) demonstrated the use of acridine-dye fluorescence quenching for studying the internal pH of chloroplasts, and Salama *et al.* (1980) used this in a detailed study of the internal pH of chromaffin granules. The fluorescent weak base accumulates within the acidic matrix of the granule with quenching of fluorescence, due largely to concentration-dependent dye interaction, but also to interaction with

Abbreviations used: ACMA, 9-amino-6-chloro-2-methoxyacridine; TMA, tetramethylammonium hydroxide; $[A^+]_i$, $[A^+]_o$, free concentration of the ion A^+ either inside the granule matrix or in the supporting medium; v_i, internal volume of granules; v_o, volume of suspension medium; Q, percentage fluorescence quenching; $C_{12}E_8$, octaethyleneglycol dodecyl ether.

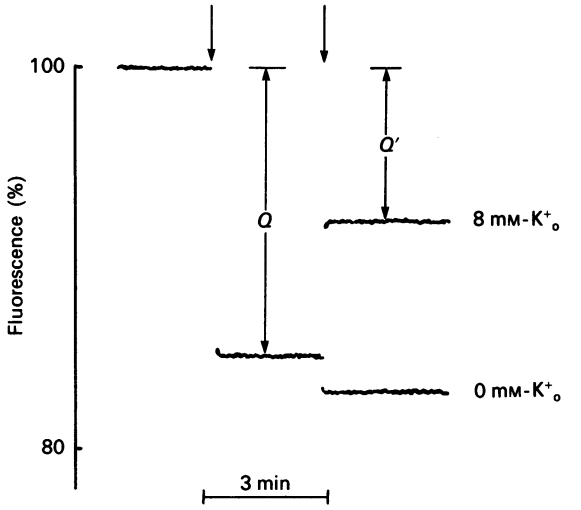

Fig. 1. ACMA fluorescence with chromaffin granules

Fluorescence (arbitrary units) was recorded continuously. At the first arrow a suspension of granules was added to give a protein concentration of 0.09 mg/ml. The percentage quenching that results is referred to as Q in the text. At the second arrow, nigericin is added. Examples are shown for medium that is free of K^+, or that contains 8 mM-K^+. The new value of percentage quenching is referred to as Q' in the text.

ATP (Salama *et al.*, 1980). ACMA is a suitable dye for this type of study.

The ionophore nigericin equilibrates K^+ and H^+ across the membrane. Protons enter or leave the granules in the presence of nigericin, depending on the concentration gradient of K^+ across the membrane, and such fluxes can be followed by using ACMA. At the null-point (no change in fluorescence quenching on adding nigericin) value of $[K^+]_o$, $[K^+]_i/[K^+]_o = [H^+]_i/[H^+]_o$, allowing $[K^+]_i$ to be calculated if pH_i is known.

A typical experiment using nigericin is shown in Fig. 1. A suspension of granules is added to a solution containing ACMA, and the fluorescence is recorded. If $pH_o > pH_i$, ACMA is accumulated in the granules and its fluorescence is quenched. Assuming that the pK_a of ACMA is the same inside the granules as it is in the suspending medium, and is high compared with pH_o, and also that fluorescence is completely quenched within the granules (and that this is unaffected by pH_i):

$$\log[Q/(100-Q)] = \Delta pH - \log(v_o/v_i) \quad (1)$$

where ΔpH is the pH difference between the matrix and the medium (outside minus inside), Q is the percentage quenching, v_i is the internal volume of the granules and v_o is the volume of the suspending medium (Schuldiner *et al.*, 1972).

On addition of nigericin to the medium (Fig. 1), there will be a movement of K^+ ions and a compensatory flux of protons as equilibrium is established. This is reflected by an increase or decrease of ACMA quenching: in the case illustrated in Fig. 1, quenching is decreased in the presence of K^+_o, with Q' as the new percentage of quenching at equilibrium. At this point:

$$[K^+]_i/[K^+]_o = [H^+]_i/[H^+]_o = [ACMA]_i/[ACMA]_o \quad (2)$$

and, by analogy with eqn. (1),

$$\log[Q'/(100-Q')] = -\log[K^+]_o + \log[K^+]_i - \log(v_o/v_i) \quad (3)$$

The volume ratio is a constant. For small variations in $[K^+]_o$ there will be a small variation in $[K^+]_i$, but this will be approximately proportional to $[K^+]_o$, so that a plot of $\log[Q'/(100-Q')]$ against $\log[K^+]_o$ should be a straight line. A null point may be defined as that value of $[K^+]_o$ that produces no change in ACMA quenching on addition of the ionophore. Here $Q = Q'$, and pH_i is unchanged. Knowing pH_i, pH_o and $[K^+]_o$, $[K^+]_i$ can be calculated.

Eqn. (3) can be generalized for any ionophore that is specific for protons and for a single cation, M^{x+}:

$$\log[Q'/(100-Q')] = -\{\log[M^{x+}]_o - \log[M^{x+}]_i\}/x - \log(v_o/v_i) \quad (4)$$

Whether a plot against $\log[M^{x+}]_o$ gives a straight line depends on how far the internal concentration of the ion is buffered.

In this work we have used monensin for Na^+ and A23187 for Ca^{2+} in addition to nigericin for K^+. We determined the null point for K^+ using nigericin and then added this concentration of K^+ to media used in experiments with monensin in order to minimize K^+ fluxes during study of Na^+. Mg^{2+} fluxes did not interfere with studies on Ca^{2+} when A23187 was used (see below).

EXPERIMENTAL

Materials

Bovine adrenal glands were trimmed in the local slaughterhouse and cooled on ice within 60 min of slaughter. They were transported to the laboratory and chromaffin granules were prepared immediately. [^{14}C]Methylamine was obtained from Amersham International, Amersham, Bucks., U.K. Biochemicals were in general from Sigma Chemical Co., Poole, Dorset, U.K., except that A23187 was from Eli Lilly, and ACMA was a gift from Dr. R. Kraayenhof (Vrije University, Amsterdam, The Netherlands). $C_{12}E_8$ was obtained from Kouyoh Chemical Co., Tokyo, Japan.

Monensin and A23187 were used as 0.5 mM stock solutions in ethanol, and nigericin as a 10 μM stock in ethanol. Hepes and Mes were generally prepared as 1 M solutions and adjusted to the required pH with NaOH, KOH or tetramethylammonium hydroxide (TMA). The pH values given were measured on solutions used in the experiments. Ca^{2+} buffers were prepared in media containing 5 mM-EGTA/TMA, pH 6.5. Concentrations of free Ca^{2+} were calculated by using a microcomputer program (Dawson, 1982) based on the method of Storer & Cornish-Bowden (1976).

Preparation of granules

Washed granules were prepared as described by Phillips (1974): dissected medullae were minced and homogenized in 0.3 M-sucrose containing 10 mM-Hepes/TMA, pH 7.0. This was replaced by Hepes/NaOH or Hepes/KOH where indicated. Homogenates were centrifuged as previously described, except that centrifugation at 27000 *g* was performed three times with vigorous removal of overlying mitochondria by swirling with buffered sucrose before each wash. This process greatly decreases granule yield, but markedly increases purity.

Granules were then suspended in buffered sucrose at 20–35 mg/ml. Protein was determined by the method of Bradford (1976), and mitochondrial contamination was assessed by assaying cytochrome *c* oxidase (EC 1.9.3.1) as described by Apps & Schatz (1979). All experiments were performed within a few hours of preparing the granules.

Analysis of granules

Granule suspension (1 ml) was centrifuged at 356000 *g* for 5 min at 4 °C in a TLA-100.2 rotor of the Beckman TL-100 ultracentrifuge. The supernatant was discarded and the pellet resuspended in 0.3–0.5 ml of aq. 0.5% (w/v) $C_{12}E_8$ by homogenizing in a hand-held homogenizer. Lysed granules were vortex-mixed for 2 min and stored at −20 °C. For flame-photometric analysis samples were thawed, re-centrifuged in the TLA-100.2 rotor, and the supernatant was carefully removed. In some cases the lysis procedure was repeated and the second supernatant was also recovered for analysis; no significant quantity of K^+ or Na^+ was detected, however. K^+ and Na^+ were analysed in an IL 543 [Instrumentation Laboratory (U.K.) Ltd., Altrincham, Cheshire, U.K.] flame photometer with 3 M-$LiNO_3$ as an internal standard. Each sample was analysed in triplicate.

[^{14}C]Methylamine accumulation was measured by the membrane-filtration method described by Apps *et al.* (1980), by using 2.5 μM-methylamine (0.15 μCi/ml) and granules (0.5 mg/ml) in 0.3 M-sucrose solutions buffered with 10 mM-Mes/TMA (the medium used for granule preparation) at 25 °C. For all calculations we have used an exchangeable water space inside the granules of 4.3 μl/mg, as found by Pollard *et al.* (1976) and Phillips *et al.* (1977).

Fluorescence experiments

All experiments were performed with granules at 0.07–0.10 mg/ml in a Perkin–Elmer 3000 fluorimeter, with excitation at 420 nm (slit-width 5 nm) and emission at 525 nm (slit-width 10 nm). Samples (2 ml) were investigated in a chamber thermostatically maintained at 25 °C, and results were recorded continuously. All measurements were made in duplicate or triplicate.

Fluorescence quenching as a function of pH was measured in media containing 0.3 M-sucrose/0.1 mM-EGTA/30 mM-Mes or Hepes/0.75 μM-ACMA. The pH, adjusted with TMA, was measured immediately after each experiment.

A similar medium was used for determining ion null points, except that the buffer was always 10 mM-Mes/TMA, pH 6.5. For measurements on K^+, the medium contained 1 mM-Na_2SO_4 plus K_2SO_4 in the range 0.5–4.0 mM. Nigericin was used at 25 nM. For measurements on Na^+, the medium was supplemented with K_2SO_4 to give a concentration of K^+ previously determined to be the K^+ null point for each batch of granules. Na_2SO_4 was then added in the range 0.75–4.0 mM. Monensin was used at 1.25 μM. The media for Ca^{2+} determinations contained EGTA/Ca buffers, with EGTA at 5 mM. After addition of the required concentration of $CaCl_2$, the pH was re-adjusted to 6.5 with HCl or TMA. A23187 was used at 1.25 μM; a slight correction was necessary for its fluorescence. In some experiments EDTA/Mg buffers were used; $CaCl_2$ was also added to give a free concentration of Ca^{2+} equal to the null point previously determined for the batch of granules.

All values of Q (see Fig. 1) determined on a batch of granules were averaged, using readings taken over 3 min for each value; this value of Q was then used for determination of the null-point value of the ion under investigation. Readings were taken for a further 3 min after ionophore addition, steady traces being obtained after 0.5–1 min.

RESULTS

Granule purity

Chromaffin granules may be purified by density-gradient centrifugation. However, this makes them fragile, so, for the purpose of these measurements, pellets of granules were simply washed vigorously with buffered sucrose to remove overlying mitochondria, which were discarded. Mitochondrial contamination of the granule preparations was assessed by using cytochrome oxidase; it was found to be equivalent to 4.5±0.80% (S.E.M., for five preparations of granules) of the protein content.

Internal pH of chromaffin granules

We used [^{14}C]methylamine to determine the internal pH of the freshly isolated chromaffin granules, measuring its accumulation at a variety of external pH values. Our results (not shown) were identical with those of Johnson & Scarpa (1976), giving a value for pH_i of 5.53±0.07 (S.E.M., $n = 4$). The measured ΔpH was proportional to pH_o, giving a line with slope 1.0 over the pH range investigated (pH 6.5–9.0), confirming the high buffering capacity of the matrix and the low proton permeability of the membrane.

We then measured the quenching of ACMA fluorescence by granules in solutions at a variety of pH values (pH 5.85–7.72). Results have been plotted according to eqn. (1) in Fig. 2. A good straight line is obtained, with a slope of 0.85 and a y intercept of −1.53. The latter is equal to $-\log(v_o/v_i)$, giving a notional value for v_o/v_i of

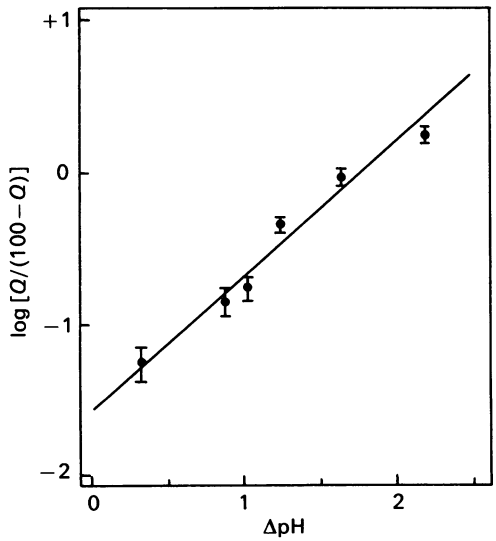

Fig. 2. ACMA fluorescence quenching by chromaffin granules

Chromaffin granules (0.10 mg/ml) were added to media buffered at various pH values, and ACMA fluorescence quenching (Q) was measured. Values are means±S.E.M. for triplicate determinations.

Table 1. Na^+ and K^+ contents of chromaffin granules

Chromaffin granules were prepared in the isolation buffer shown, and Na^+ and K^+ contents were measured by flame photometry. Internal concentrations were calculated by using an internal volume of 4.3 μl/mg (Phillips *et al.*, 1977). Values given are means ± S.E.M. for triplicate determinations on the numbers of granule preparations shown (*n*).

Isolation buffer...	Hepes/NaOH		Hepes/KOH		Hepes/TMA	
	Na^+	K^+	Na^+	K^+	Na^+	K^+
Ion content (nmol/mg)	156 ± 11.5	127 ± 7.8	75 ± 10.5	179 ± 12.3	99 ± 10.6	128 ± 6.9
n	6	6	5	6	5	5
Internal concn. (mM)	36.2	29.5	17.4	41.6	23.0	29.7
Sum of $[Na^+]_i + [K^+]_i$ (mM)	65.7		59.0		52.7	

33. The actual value of this ratio is approx. 2300. Both slope and intercept deviate from ideal values to about the same extent as has been found in other systems of biological membrane vesicles. As discussed by Warnock *et al.* (1982), the deviation can probably be ascribed to binding of the fluorescent probe to the membranes.

In all subsequent experiments with ACMA, media were buffered to pH 6.5, and ΔpH was assumed to be 1.00. This gives around 20% quenching of fluorescence at the protein concentrations used.

Ion contents of granules

The results of Ornberg *et al.* (1988) suggested that the ion content of granules is highly dependent on the method of isolation, and that univalent ions in particular may be expected to exchange with ions in the isolation medium. Indeed, granules in cultured cells were found to be devoid of Na^+, but to have a high content of K^+.

In our work, trimmed adrenal glands have been placed on ice within 1 h of slaughter: there is inevitably, therefore, a long period when such re-equilibration can occur. We then prepared granules in the laboratory in ice-cold sucrose media buffered with Hepes neutralized with NaOH, KOH or TMA. After the granules had been washed free of buffer, their Na^+ and K^+ contents were determined by flame photometry (Table 1). The K^+ content was identical in granules isolated in Hepes/NaOH and Hepes/TMA, and slightly higher when Hepes/KOH was used. Similarly, Na^+ contents were nearly the same in granules isolated in Hepes/KOH or Hepes/TMA, but were approximately double these values when Hepes/NaOH was used. The combined total concentration of these univalent cations was 50–60 mM, similar to the values of Ornberg *et al.* (1988).

Such ion redistribution is not unexpected. Our results give no information about the ion content *in vivo*. We used granules prepared in media buffered with Hepes/TMA in subsequent experiments.

Free concentrations of ions in granules

The null point for K^+ was determined by using nigericin. A typical experiment is shown in Fig. 3(*a*), with the results plotted according to eqn. (3). A good straight line is obtained over this range of $[K^+]_o$ (1.5–6.0 mM), with a slope of 0.91 (theoretical value 1.0), suggesting rather little change in $[K^+]_i$ over this range. Quenching after ionophore addition in this experiment gave values for Q' of 7.4% to 22.5%; the null point is found by averaging values for Q and determining the corresponding value of $[K^+]_o$, as shown in Fig. 3(*a*) (2.45 mM in this case).

Steady fluorescence traces were obtained, with stabilization within about 0.5 min of adding the ionophore. The media, however, contained 2 mM-Na^+. This is approximately the null point for Na^+. If this was omitted, there was a steady acidification of the granule interior, owing to Na^+ efflux catalysed by the Na^+/H^+ antiporter of the granule membrane (Haigh & Phillips, 1989).

Similar experiments were then performed for Na^+ by using monensin (Fig. 3*b*). In this case media contained the null-point concentration of K^+, in order to decrease K^+ fluxes. Again, a straight line is obtained, with a slope of 0.88 over the range of $[Na^+]_o$ of 1.5–6 mM. In the experiment shown the null point is found at 1.47 mM-Na^+.

Finally we investigated Ca^{2+}, using A23187. External Ca^{2+} was buffered using EGTA. In the experiment shown in Fig. 3(*c*) the null point was found at 56 nM-Ca^{2+}. In experiments with Ca^{2+} a wider range of values of $[Ca^{2+}]_o$ was used than with the univalent ions, and the line obtained had a slope (0.27 in Fig. 3*c*) that deviated more from the predicted value of 0.5, in spite of Ca^{2+}-buffering inside the granule, which is expected to decrease variation in $[Ca^{2+}]_i$. Nevertheless, the use of the null-point method enables us to obtain a value for $[Ca^{2+}]_o$ at which no movement of protons is detected.

We considered whether Mg^{2+} might contribute to the proton movements observed on addition of A23187. We replaced the EGTA/Ca buffers with EDTA buffers in which Mg^{2+} was included to give a free Mg^{2+} concentration in the range 20–120 nM in the presence of 63 nM free Ca^{2+}; no fluorescence change was found (results not shown), and we conclude (as did Bulenda & Gratzl, 1985) that Mg^{2+} movements are negligible under these conditions.

The results of these determinations are given in Table 2. Null points are listed, with the range of slopes found in experiments with different batches of granules. The calculated free concentrations of these ions within the granule matrix are also presented.

DISCUSSION

We have described a simple method for determining the free ion concentration inside organelles. We have

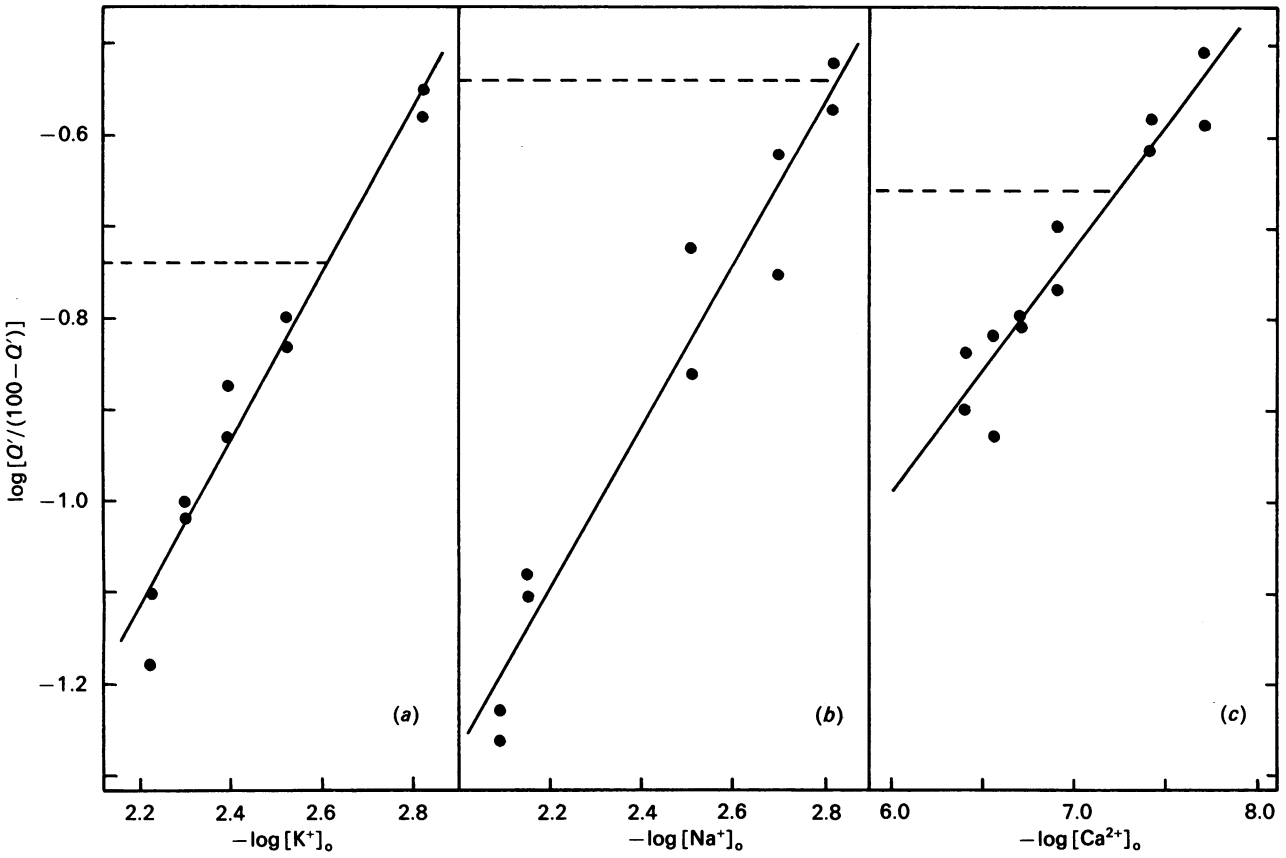

Fig. 3. Null-point determination for K^+, Na^+ and Ca^{2+}

(*a*) Quenching of ACMA fluorescence (*Q'*) by granules (0.09 mg/ml) was measured in the presence of various concentrations of K^+, with all readings made in duplicate. The mean value of quenching before ionophore addition (*Q*) was 15.4±0.6% (±S.E.M., *n* = 10). This is indicated by the broken line on the Figure. (*b*) As (*a*), except that $[Na^+]_o$ was varied. Granule concentration was 0.07 mg/ml, and the mean value of *Q* was 22.5±1.0% (*n* = 10). (*c*) As (*a*), except that $[Ca^{2+}]_o$ was varied. Granule concentration was 0.07 mg/ml, and the mean value of *Q* was 18.2±1.1% (*n* = 14). All concentrations are molar.

Table 2. Free ion concentrations of the chromaffin-granule matrix

Chromaffin granules were prepared in Hepes/TMA. Null points were determined as described in Fig. 3. Results are means±S.E.M. for the numbers of granule preparations shown (*n*). Slopes are given for plots as in Fig. 3. The free ion concentration is calculated by using ΔpH = 1.0.

	Na^+	K^+	Ca^{2+}
Null-point concn.	1.88±0.17 mM	2.28±0.13 mM	0.056±0.0064 μM
n	4	5	4
Range of slopes	0.82–0.90	0.72–0.91	0.24–0.35
Free internal concn.	18.8 mM	22.8 mM	5.62 μM

found that ACMA gives particularly rapid response times, and the use of the null-point method enables many of the assumptions made in the derivation of eqn. (4) to be ignored.

A rather similar method has been described by Grinstein *et al.* (1983). They applied it to platelet α-granules, which contain 32 mM-Ca^{2+} and 172 mM-Mg^{2+}, so that they developed a method that enabled the free concentrations of both bivalent ions to be determined (values of 12 μM and 326 μM respectively were found). Russell (1984) has monitored membrane-potential changes of pituitary neurosecretory vesicles fluorimetrically, inducing changes by varying $[K^+]_o$ in the presence of valinomycin. In this case a null point gave a value for $[K^+]_i$ of 0.75 mM. Application of a similar method to chromaffin granules gave a value of 2 mM (Salama *et al.*, 1980).

A preparation of organelles made by subcellular fractionation is never pure; we purified granules as completely as was possible while providing intact granules rapidly. Mitochondrial contamination can be assessed accurately, by using purified mitochondria as a standard. This was found to be relatively low, and we calculate that

mitochondrial K^+ (assayed by Ornberg *et al.*, 1988) contributed only 2 or 3% to the measured content of the granule preparation (Table 1). We therefore ignored ACMA movements induced by addition of nigericin to, and the resultant acidification of, these organelles.

The granules may contain a relatively high proportion of adrenal lysosomes (perhaps 25%; see Phillips, 1973), but the extent of contamination of the granules in terms of percentage of their protein cannot be assessed accurately in the absence of a preparation of 'pure' lysosomal material. The adrenal content of lysosomal protein is low compared with its massive content of granule protein (Smith & Winkler, 1966), and we have, again, ignored this. The small extent of ACMA quenching by lysosomes in the suspension will itself be little affected by ionophore addition; the consequence would be a slight decrease in the change in quench that is observed. Such errors contribute to the non-ideality of the responses that is seen in Fig. 3 and recorded in Table 2.

Our value for the free Ca^{2+} concentration (5.6 μM) agrees closely with one of the values given by Bulenda & Gratzl (1985), a value they obtained when the pH gradient of the granules was collapsed by using NH_4Cl. Chromaffin granules contain about 60% of the Ca^{2+} of the adrenal medulla, 85% of this being released together with catecholamines and nucleotides by granule lysis (Phillips *et al.*, 1977). Atomic absorption analyses of granules give values for the Ca^{2+} content of 77–100 nmol/mg (Borowitz, 1967; Phillips *et al.*, 1977) or a nominal internal concentration of around 20 mM. The concentration of free Ca^{2+} in the matrix is therefore about 0.03% of the total content.

We were unable to detect Mg^{2+} movements in our experiments with chromaffin granules, when using A23187. We had expected to find a lower free $[Mg^{2+}]_i$ than $[Ca^{2+}]_i$, but expected to be able to monitor Mg^{2+} fluxes induced by A23187 if we set $[Ca^{2+}]_o$ equal to the null value. However, we note that, although Ornberg *et al.* (1988) found that granules in cultured chromaffin cells contained 5 mM-Mg^{2+}, isolated granules had lost this; this is inconsistent with previous analyses of highly purified granules (Phillips *et al.*, 1977), but consequently some query must remain over the Mg^{2+} status of chromaffin granules.

Comparing our values for the free Na^+ and K^+ concentrations (Table 2) with the total contents given in Table 1 gives activity coefficients for these ions of about 0.8, assuming that all the ion is free in the granule matrix.

The composition of the matrix of the bovine chromaffin granule has been reviewed by many authors. Winkler (1976) and Winkler & Westhead (1980) have made detailed compilations of analytical data. The granules are not homogeneous, varying in their catecholamine:nucleotide ratio and in their catecholamine content; these are different also for granules from adrenaline- and noradrenaline-containing cells. However, the work of Kopell & Westhead (1982) shows that, whatever the detailed composition, within the matrix the behaviour of catecholamine and nucleotide molecules is far from ideal. Solutions of approx. 0.5 M-adrenaline in the presence of 0.16 M-ATP had an osmotic pressure of 250 mosM, and this value varies rather little with changes in ATP concentration above this value. However, subsequent addition of Na^+, K^+, Cl^- and even Ca^{2+} to such mixtures increases the osmotic pressure in a way that is close to ideal. The relatively high activity coefficients that we found for Na^+ and K^+ are consistent with this. The very low value for the free Ca^{2+} concentration is indicative of most of the Ca^{2+} being bound to acidic components inside the granule, such as chromogranins (Reiffen & Gratzl, 1986), glycosaminoglycans and the interior of the granule membranes (Phillips, 1981), since the results of Kopell & Westhead (1982) suggest that little is bound to ATP. It has nevertheless been reported that selective Ca^{2+} depletion of granules destabilizes them and is followed by osmotic lysis (Südhof, 1983), re-emphasizing the need for more detailed investigations of the intragranular location of this ion.

Granules are very abundant in chromaffin cells (Phillips, 1982) and therefore constitute an important reservoir for Ca^{2+}. In contrast with Ca^{2+}, changes in $[Na^+]_i$ and $[K^+]_i$ would have a major effect on granule osmotic pressure, and it is important to discover how the content of these ions is regulated *in vivo* in order to determine their role in the bioenergetic processes of these secretory granules.

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