Responses of the Transmembrane Potential Coupled to the ATP-evoked Catecholamine Release in Isolated Chromaffin Granules

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Abstract Using suspensions of chromaffin granules isolated from bovine adreno-medullae, the effects of Mg^{2+} ·ATP on membrane potential and catecholamine release across membranes of granules were investigated. The release of catecholamine was monitored by the measurement of changes in endogenous fluorescence of the amines, while the fluorometric method with 3, 3'-dipropylthiadicarbocyanine iodide (diS-C₃-(5)) was applied to the measurement of membrane potential changes. The catecholamine release brought about by Mg^{2+} ·ATP was found to obey Michaelis-Menten kinetics with K_m -value for Mg^{2+} of about 0.15 mM and to be inhibited by dicyclohexylcarbodiimide (DCCD), an inhibitor of membrane-bound H^+ -ATPase. Concomitantly with such an amine release, depolarization of the granule membrane was observed to occur. The magnitude of this depolarization closely paralleled with the rate of the amine release and followed a saturation kinetics of Michaelis-Menten type with K_m for Mg^{2+} of 0.21 mM and for ATP of 0.22 mM. Moreover, such a depolarization was competitively suppressed by DCCD. Based on these results, the role of the transmembrane potential change of granules in the catecholamine release was discussed.

Recently there appeared a number of reports which deal with regulatory role of membrane-bound ATPase in release from and uptake into adrenomedullar chromaffin granules of catecholamines. Radda and his colleagues (BASHFORD *et al.*, 1976; CASEY *et al.*, 1977) have found that the elements of a chemiosmotic system are present in chromaffin granules, and proposed a mechanism that catecholamine transport is coupled to the activity of membrane-bound proton pump. Experiments with some ionophores and uncouplers also showed that electroneutral H^+ influx and K^+ efflux in chromaffin granules occurred concomitantly with catecholamine release (JOHNSON and SCARPA, 1976a, b). In submitochondrial particles, chloroplasts and some bacteria, membrane-bound proton-trans-

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locating ATPase has been known to regulate H^+K^+ exchange transport (RACKER, 1976). It seems quite natural, therefore, to assume that it is also the case in the chromaffin granules and the amine release is regulated by the H^+ -ATPase activity through the ion exchange transport. But still remains unsolved the question how to couple both processes.

In order to throw light upon this problem, responses of the membrane potential of the isolated granules to $Mg^{2+} \cdot ATP$ were fluorometrically studied with a cyanine dye diS-C₃-(5). The ion exchange transport coupled with the amine release as a whole might be electroneutral as reported by JOHNSON and SCARPA (1976a, b); still remains a possibility of occurrence of a transient change in the transmembrane potential. Indeed, we found that the granules responded to ATP-induced amine release with depolarization. The present paper describes such an ATP-evoked depolarization of the isolated granules in relation to their rate of the amine release.

MATERIALS AND METHODS

Chromaffin granule preparation. The granules were prepared essentially as described by HOFFMAN *et al.* (1976). Bovine adrenal glands were obtained at a slaughter house, placed on ice, and delivered to the laboratory within 1 hr. Four to six glands were immediately subjected to preparation in the cold. After cortices were removed, the isolated medullae were combined, cut into pieces and washed carefully with ice-cold unbuffered 0.3 M sucrose. The tissue pieces were placed in about 10 ml of same solution, blended for 5 sec in a Waring Blender and then homogenized in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 500 *g* for 10 min. The supernatant was carefully sucked off with a siliconized glass pipette without disturbing the sediments and centrifuged at 20,000 *g* for 30 min to a chromaffin granule pellet. The supernatant was gently sucked off with a Pasteur pipette. The upper layer of the pellet, "fluffy layer," containing mitochondria, was removed by careful washing with 5 ml of 0.3 M sucrose. The remaining part of the pellet was dispersed in 0.3 M sucrose by repeated (20–30 times) pipetting with a wide bore pipette (1 mm in diameter), and centrifuged at 10,000 *g* for 30 min. The supernatant was gently sucked off with a Pasteur pipette and the upper layer of the pellet was again removed by gentle swirling with 5 ml of 0.3 M sucrose. The pellet was resuspended in 0.3 M sucrose by pipetting, as described above, and centrifuged at 7,000 *g* for 30 min. The resulting supernatant was once again gently sucked off with a pipette and the centrifuge tube was inverted for no more than 2 sec. The final pellet thus obtained was resuspended in 0.3 M sucrose to have a protein concentration of 4–6 mg/ml. All these procedures were carried out at 4°C. Chromaffin granules thus prepared usually contained 0.4–0.6 mg adrenaline/mg of protein. The granule sample was kept ice-cold, and used up within

60 hr after preparation.

Fluorescence measurements. I. Fluorescence of diS-C₃-(5): The fluorescent dye, 3,3'-dipropylthiadicarbocyanine iodide, diS-C₃-(5), was employed in this study. The dye was added to a known volume of solution with an adjustable pipette, the final concentration of dye was always 5.0 μ g per 3.0 ml of medium. Fluorescence measurements were performed using a spectrofluorometer (Shimadzu, Type RF502) equipped with a Hamamatsu R-446 photomultiplier tube. A 10 mm quartz cuvette was used. The medium in the cuvette was stirred with a small magnetically driven glass flea. The temperature of medium was usually controlled at 35°C by circulating water through the cuvette holder.

II. Fluorescence of catecholamine: Fluorescence of endogenous catecholamine of chromaffin granule was measured with the same spectrofluorometer with excitation at 280 nm and emission at 320 nm (STEFFEN *et al.*, 1974; JOHNSON and SCARPA, 1976b).

Measurements of catecholamines. Catecholamines were measured by two methods. For quantitative measurements of released catecholamine, granules were spun down and adrenaline was assayed by the fluorimetric trihydroxyindol method (ANTON and SAYRE, 1962), without an alumina chromatography. For kinetic measurements of catecholamine release, the endogenous fluorescence of the catecholamines was measured with the spectrofluorometer. This method was recently developed by STEFFEN *et al.* (1974) and JOHNSON and SCARPA (1976b) for catecholamine release and uptake in chromaffin granules. In this experiment, granules were added to a cuvette containing solution and the fluorescence increase was measured.

Solutions and drugs. When chromaffin granules were suspended in saline media, they behaved as an osmometer, obeying Boyle-van't Hoff's law and swelling in the media of less than 350 mOsm brought about release of catecholamines (JOHNSON and SCARPA, 1976b). Osmolarity of incubation media used in the present study was, therefore, adjusted around 370 mOsm (360–380 mOsm), which was monitored with an Advance Osmometer model 3L (Advance Instruments, Inc.). The pH was adjusted at 6.5 with 40 mM HEPES.

The fluorescent dye for potential measurement, 3,3'-dipropylthiadicarbocyanine iodide (diS-C₃-(5)) was obtained from Nippon Kanko-Shikiso Kenkyusho, Ltd., Okayama, Japan. Valinomycin, gramicidin D, ATP and other nucleotides were obtained from Sigma. X537A was a generous gift of Hoffman-La Roche, Nutley, N. J.. *N*, *N'*-dicyclohexylcarbodiimide (DCCD), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and other chemicals were of reagent grade. All the reagents were used without further purification.

Granules concentration. Concentrations of chromaffin granules in a reaction medium were expressed as μ g of protein per ml of medium. Protein was assayed with the method of LOWRY *et al.* (1951), using bovine serum albumin as a standard. Before assay, protein was precipitated with 5% (w/v) trichlo-

roacetic acid to eliminate interference due to cross-reaction of catecholamines.

RESULTS

1. Dye fluorescence and estimation of transmembrane potential

On adding small amount of granules, the intensity of fluorescence of diS-C₃-(5) dissolved in NaCl or choline Cl media attained a constant level within a few minutes. When the dye concentration was fixed (usually 5 μ g per 3 ml), this steady level of fluorescence was same for both media, but strongly depended upon K⁺ concentrations (Figs. 1 and 3). Moreover, addition of valinomycin, an ionophore known to induce a marked increase in K⁺ permeability in chromaffin granules (KITABGI and PERLMAN, 1975), brought about further decreases in the fluorescence; the lower the K⁺ concentration the greater such a decrease in fluorescence intensity (*F*). In the presence of ionophore, *F* varied log-linearly with external K⁺ concentration, (K)_o (Fig. 1). The relationship between *F* and log (K)_o in the presence of gramicidin, an ionophore which makes biological and artificial membrane permeable to K⁺ and Na⁺ but not to choline⁺, was also found to be linear over most of the range studied in choline⁺-K⁺ media (Fig. 1). Such results were quite similar with those obtained on a number of different cell preparations (for review see COHEN and SALZBERG, 1978), indicating that increases

Fig. 1. The influence of the extragranular potassium concentration on the steady level of fluorescence intensity of the dye diS-C₃-(5) in suspensions of intact bovine chromaffin granules (19 μ g protein/ml) in the absence (○) and presence of 1.0 μ g/ml gramicidin (●) or 0.2 μ g/ml valinomycin (▲). The media were made by substituting 160 mM KCl for a portion of 160 mM choline Cl solution to give the (K⁺)_o shown on the figure.

in F reflect depolarization of membrane potential.

Applying essentially the same procedure as that employed for other preparations, therefore, the magnitude of changes in the membrane potential was estimated from the fluorescence changes; assuming that the log-linear changes in F with respect to $(K^+)_{\circ}$ in the presence of ionophores reflect the Nernst equation for the reversible K^+ electrode. Thus the broken lines in Figs. 1 and 3 were drawn to transform the fluorescence scale into the potential one. This procedure is sufficient to approximately estimate the potential change in mV. It is necessary for estimating the absolute value of membrane potential to determine the fluorescence intensity at 0 mV. For this purpose, we tentatively used the F measured on the suspension of lysed granules; the intact granules were suspended in hyposmotic media (20 mM HEPES of pH 6.5 alone) to rupture granules and then the suspensions were brought to 380 mOsm by adding choline Cl solution. The value of F obtained on this suspension was taken as that corresponding to the transmembrane potential of 0 mV. This approximation was supported by the observation that when gramicidin was added to the intact chromaffin

Fig. 2. Effect of Mg²⁺ and ATP on the fluorescence intensity of diS-C₃(5) in suspensions of intact chromaffin granules (25 μ g protein/ml). The medium was 160 mM NaCl. At time marked with arrows, Mg²⁺ and ATP were added with an adjustable fine syringe to give the final concentration shown on the figure.

Fig. 3. Effect of $(K^+)_o$ on the Mg^{2+} -ATP-evoked fluorescence changes of diS-C₈-(5) in suspension of intact chromaffin granules ($16 \mu\text{g}$ protein/ml). The media were made by substituting 160 mM KCl for a portion of 160 mM NaCl solution to give the $(K^+)_o$ shown on the figure.

granules in $Na^+ + K^+$ medium F increased identical to the value obtained with lysed granules. Furthermore, the attained level of F on addition of gramicidin was independent of Na^+ and K^+ concentrations of medium. These are the results to be expected if gramicidin makes chromaffin granule membrane indiscriminately permeable to Na^+ and K^+ and depolarizes granules to 0 mV as shown with electroplax membranes (PODLESKI and CHANGEAUX, 1969). With this method the membrane potential of granules was estimated to be around -45 mV (inside negative) in 1 mM $(K^+)_o$, and $+15$ mV in 100 mM $(K^+)_o$ (Fig. 1).

2. Membrane depolarization evoked by $Mg \cdot ATP$

In the presence of Mg^{2+} , but not in its absence, ATP brought about increase in the fluorescence, namely depolarization (Fig. 2). As shown in Fig. 3, the magnitude of depolarization strongly depended on $(K^+)_o$; around 72 mV in 1 mM $(K^+)_o$ but about 20 mV in 100 mM $(K^+)_o$.

Nucleotides other than ATP, for instance AMP, cAMP and GTP were all without effect of such a remarkable depolarizing action. It might be said that $Mg^{2+} \cdot ATP$ is specifically responsible for the depolarization observed here.

As shown in Fig. 4, the increment of fluorescence, ΔF , (*i.e.* magnitude of depolarization) brought about by $Mg^{2+} \cdot ATP$ in low K^+ media increased hyperbolically with increasing ATP and Mg^{2+} concentrations. Indeed, the double reciprocal plots, $1/\Delta F$ vs. $1/ATP$ or $1/Mg^{2+}$ were linear (Fig. 5) and the dissociation constants, K_m , estimated therefrom, were 0.22 and 0.21 mM for ATP and Mg^{2+} ,

Fig. 4. Influence of ATP concentration on fluorescence change (ΔF) of diS-C₈-(5) in suspension of intact chromaffin granules (25 μ g protein/ml). The media contained 160 mM NaCl and 0.1 mM (○), 1.0 mM (●) or 0 mM Mg²⁺ (dashed line).

Fig. 5. *Left*: Double-reciprocal plots for fluorescence changes (ΔF) with ATP. Data are shown in Fig. 4. The media contained 0.1 mM (○) or 1.0 mM Mg (●). The intercept at the abscissa of the extrapolated lines gives the value of negative inverse of K_m for ATP. *Right*: Double-reciprocal plots for fluorescence changes with Mg²⁺. The media contained 160 mM NaCl and 0.1 mM (○) or 0.2 mM ATP (●). The intercept at the abscissa of the extrapolated lines gives the value of negative inverse of K_m for Mg²⁺.

Table 1. Dependence of Mg^{2+} -ATP-evoked depolarization on temperature.

Temperature ($^{\circ}C$)	$\Delta F(a.u.)^*$	Depolarization (mV)**
35	58	72
25	48	59
15	34	42
10	20	25
5	8	10

The enhancement of fluorescence of dye upon the addition of 1.0 mM Mg^{2+} and 0.2 mM ATP was measured at various temperature in suspensions of intact chromaffin granules (25 μg protein/ml). The media contained 160 mM NaCl.

* The scale of fluorescence change is given in Fig. 3.

** By assuming that the fluorescence change in 10-fold increase in $(K^+)_{\circ}$ under the presence of valinomycin corresponds to a potential change $2.3 \times RT/F$, the Mg^{2+} -ATP-evoked fluorescence change is estimated in mV. A result for $35^{\circ}C$ is given in Fig. 3.

Fig. 6. Influence of chloride concentration on the Mg^{2+} -ATP-evoked fluorescence change of diS-C₃(5) in suspensions of intact chromaffin granules (18 μg protein/ml). The media were made by substituting 110 mM Na_2SO_4 for 160 mM NaCl to give the Cl^- concentration shown on the figure.

respectively. The values coincided well with those obtained from Mg^{2+} -ATP evoked release of catecholamine from chromaffin granules (HOFFMAN *et al.*, 1976). Such a fact strongly points to a close correlation of the amine release with the membrane depolarization in granules. In fact, the present study proved, as described below, that this is the case. In this connection, it should be noticed in Fig. 5b that $1/\Delta F-1/Mg^{2+}$ relation at high Mg^{2+} concentration shows deviation from linearity, which has, as described below in detail (*cf.* Fig. 10), a close relation to inhibition of amine release by high Mg^{2+} (HOFFMAN *et al.*, 1976).

The magnitude of depolarization brought about by Mg^{2+} -ATP was dependent on temperature, but its dependency was found to be far more greater than

that expected from the thermodynamic factor RT/F (Table 1). This finding suggests participation of a carrier or carriers to ionic mechanisms of ATP-induced depolarization.

The effects of anions on the release of catecholamine from and the activity of ATPase in chromaffin granules were discussed by TAUGNER (1972) and CASEY *et al.* (1976). They found that replacement of Cl⁻ with SO₄²⁻ does not inhibit ATPase activity but does suppress the Mg²⁺·ATP-induced amine release. As shown in Fig. 6, the membrane potential in the absence of ATP remained unaltered despite varying external Na⁺ and Cl⁻ concentrations, where the osmolarity of medium remained constant, but the magnitude of Mg²⁺·ATP-evoked depolarization decreased with decreasing Cl⁻. Such a finding suggests that change of permeability or distribution of Cl⁻ across the granule membrane contributes to Mg²⁺·ATP-evoked depolarization and amine release.

3. Effect of DCCD on ATP-evoked depolarization

Dicyclohexylcarbodiimide (DCCD) is known to covalently bind with membrane H⁺-ATPase, resulting in inhibition of active proton transport in submitochondrial particles and some bacteria (RACKER, 1976); furthermore it is also a potent inactivator of Mg²⁺-dependent ATPase in the chromaffin granule membrane (BASHFORD *et al.*, 1976). This drug never affected the so-called resting potential of chromaffin granules, nor altered its depolarization response to high (K⁺)_o, however, DCCD competitively inhibited the depolarization response to

Fig. 7. *Left:* Effect of DCCD on the Mg²⁺·ATP-evoked fluorescence change of diS-C₈-(5) in suspensions of intact chromaffin granules (21 μg protein/ml). The media contained 160 mM NaCl and the concentrations of DCCD indicated. The fluorescence changes of diS-C₈-(5) on addition of ATP were then measured. *Right:* Double-reciprocal plots for fluorescence changes (ΔF) with ATP under several concentrations of DCCD. Data are shown in left.

$\text{Mg}^{2+} \cdot \text{ATP}$ (Fig. 7). In contrast the addition of oligomycin, a potent inhibitor of H^+ -ATPase in mitochondria, had, unlike DCCD, no significant effect upon $\text{Mg} \cdot \text{ATP}$ -evoked depolarization. This finding was consistent with those of BASHFORD *et al.* (1976), who found that oligomycin was without effect on the DCCD-sensitive ATPase activity in chromaffin granules. Thus it is very likely that ATP induces depolarization through interaction with DCCD-sensitive sites in membrane, most probably with a DCCD-sensitive H^+ -ATPase. These results correspond with those of CASEY *et al.* (1977), who observed active proton uptake which followed the addition of ATP in chromaffin granules was abolished by a mitochondrial uncoupler S-13.

4. Changes in internal K^+ evoked by $\text{Mg}^{2+} \cdot \text{ATP}$

JOHNSON and SCARPA (1976a, b) found that K^+ - H^+ exchange across the membrane of chromaffin granules induced by the ionophore nigericin or by valinomycin and the uncoupler FCCP. It is conceivable that exchange of H^+ with K^+ may be involved in generation of the depolarization response to $\text{Mg}^{2+} \cdot \text{ATP}$. To test such a possibility, we attempted to indirectly estimate changes in intragranular K^+ concentration by measuring the K^+ equilibrium potential before and after the onset of $\text{Mg}^{2+} \cdot \text{ATP}$ -evoked depolarization response. For this purpose, we used valinomycin which produces highly selective increase in K^+ permeability in biological and artificial membranes, but which hardly affects the H^+ permeability in choline Cl media (JOHNSON and SCARPA, 1976a, b). As shown in Fig. 1, valinomycin induced hyperpolarization and the fluorescence of dye in its presence log-linearly varied for $(\text{K}^+)_o$ of 10–100 mM, reflecting the K^+ equilibrium potential. Selecting an appropriate external K^+ concentra-

Fig. 8. Time course of the effects of valinomycin ($0.2 \mu\text{g/ml}$) on the $\text{Mg}^{2+} \cdot \text{ATP}$ -evoked fluorescence change of diS- C_8 -(5) in suspensions of intact chromaffin granules ($17 \mu\text{g protein/ml}$). The media contained 160 mM choline Cl and 1.0 mM MgCl_2 . After the fluorescence attained a steady level, 0.2 mM ATP was added to the medium (○). Valinomycin was added at the time indicated (●). Note that the transient minimum fluorescence intensity (indicated by arrow) shifts upward as the ATP-evoked response proceeds.

tion, therefore, changes in the fluorescence level in the presence of the ionophore reflects those in internal K⁺ concentration, (K⁺)_i. An example of such experiments is presented in Fig. 8. Addition of the ionophore brought about instantaneous decrease in the fluorescence (hyperpolarization) to a new level, corresponding to the K⁺ equilibrium potential determined by the ratio (K⁺)_o/(K⁺)_i. After the onset of ATP-induced depolarization, a biphasic change in the fluorescence was induced by addition of valinomycin; a rapid decrease and a slow increase. The transient minimum level in fluorescence (indicated with arrows in the figure), corresponding to the K⁺-equilibrium potential, gradually increased as the ATP-depolarization response proceeded; the difference in the K⁺ equilibrium potential between before and 3.5 min after the onset of ATP-depolarization was estimated to be about 60 mV in choline Cl medium. The volume of chromaffin granules in suspension was so small that (K⁺)_o can be safely regarded as constant. Thus the change of K⁺ equilibrium potential of 60 mV indicates (K⁺)_i to be reduced to about 1/10 at 3.5 min after the onset of the ATP-depolarization response. While we did not estimate the volume changes of chromaffin granules, these should not affect the qualitative observation of intragranular K⁺ change since 10-fold increase in volume would be required to account for the observed decrease in (K⁺)_i. Thus it might be concluded that Mg²⁺·ATP brings about release of intragranular K⁺ concomitantly with generation of membrane depolarization.

Fig. 9. Time course of the catecholamine release from intact chromaffin granules (25 μ g protein/ml) after addition of various concentrations of Mg²⁺. The media contained 160 mM NaCl and 0.2 mM ATP. Catecholamine release was directly monitored by measuring the fluorescence increase of endogenous amines (excited at 280 nm and emission at 320 nm).

Fig. 10. *Left*: Effect of Mg^{2+} on catecholamine release from intact chromaffin granules (25 μ g protein/ml). Experimental conditions as in Fig. 9. By computing the fluorescence increase induced by X537A as full release of catecholamines from granules, per cent release of catecholamines was estimated with Mg^{2+} -induced fluorescence enhancement for initial 6 min. *Right*: Double-reciprocal plot for relative rate of catecholamine release with Mg^{2+} . Data are shown in left.

5. Mg^{2+} ·ATP-dependent catecholamine release

Catecholamine release was directly followed up by monitoring the endogenous amine of suspended chromaffin granules by fluorescence measurement. To avoid disturbances due to absorption of light near 280 nm by ATP, this series of experiments was conducted in the presence of a fixed amount of ATP in the suspending media. Thus the amine release was evoked by adding Mg ion. An example of such experiments is illustrated in Fig. 9.

As already shown by JOHNSON and SCARPA (1974), an ionophore X537A produced very rapid efflux of whole catecholamine in the granules independently of whether or not ATP and/or Mg^{2+} are present. Furthermore, X537A induced full release of catecholamine in the presence of DCCD. Therefore, we always used such a catecholamine release brought about by X537A as the reference for estimating the rate of amine release evoked by Mg^{2+} ·ATP.

Compared to the X537A-induced amine release, Mg^{2+} ·ATP-dependent release was slow. The rate of release depended upon Mg^{2+} concentration but remained constant, at a fixed Mg^{2+} concentration, at least for initial 10 min, and then slowed down. Under the optimal conditions, Mg^{2+} ·ATP-dependent amine release was completed within 15 min. All these results were consistent with those obtained with the direct measurement of released adrenaline (HOFFMAN *et al.*, 1976), a fact providing a support for our fluorometric method.

Fig. 11. Effect of DCCD on Mg²⁺·ATP-evoked catecholamine release from intact chromaffin granules (18 µg protein/ml). The media contained 160 mM NaCl and 0.2 mM ATP. The experiment was performed as described in Fig. 9.

The spontaneous catecholamine release (occurring without Mg²⁺ addition) was quite small and slow, and may result from endogenous Mg²⁺ of chromaffin granules (around 0.04 µmol/mg of protein, SMITH and WINKLER, 1967). The rate of amine release, expressed as the relative amount of amine released for initial 6 min, apparently increased with increasing Mg²⁺ concentration up to 1.0 mM, but responded with a substantial decrease to further increase in Mg²⁺ (Fig. 10 left). Such a dual effect of Mg²⁺ in the amine release was also noted by HOFFMAN *et al.* (1976).

The linear part of the double-reciprocal plot presented in Fig. 10 right provides K_m of around 0.15 mM for Mg²⁺ in range of low concentrations, a value is consistent with that derived from the depolarization response to Mg²⁺ in the presence of ATP (Fig. 5). Deviation from the linear double-reciprocal plot observed in high Mg²⁺ range is, as already noted in the previous section, also consistent with the results obtained on the Mg²⁺·ATP-evoked depolarization. Moreover, DCCD was quite effective in inhibiting the amine release evoked by Mg²⁺·ATP. As shown in Fig. 11, addition of an ample amount of DCCD immediately stopped liberation of catecholamine activated by prior addition of ATP and Mg²⁺. All these findings strongly indicate a close correlation of the membrane depolarization with the catecholamine-releasing process.

DISCUSSION

The main findings of the present study are that the fluorometric method

with a cyanine dye diS-C₃-(5) was also effective to follow up changes in the membrane potential of isolated chromaffin granules, and that the membrane depolarization proved to occur in the presence of Mg²⁺·ATP, the magnitude of which closely paralleled the rate of catecholamine release from the granules.

As shown in Figs. 1 and 3, responses of the fluorescence intensity of diS-C₃-(5) in granule suspensions to external cations (K⁺, Na⁺ and choline⁺) and ionophores (valinomycin and gramicidin) were essentially the same as those observed in a number of different preparations (*cf.* COHEN and SALZBERG, 1978). Such findings strongly support that fluorometric method of estimating membrane potential changes is also applicable to chromaffin granules. Because of some ambiguities in determining the fluorescence intensity corresponding to zero membrane potential, there remains some doubt as to the absolute value of the membrane potential. The value of K⁺ concentration within the chromaffin cells of adrenal medulla has not yet been measured, but it is expected to be so high as in other cells, probably in the magnitude of 50–100 mM. Allowing some ambiguities, the membrane potential of granules *in situ* is expected not far from zero from the results presented in Figs. 1 and 3. On the other hand, the membrane potential of granules in low K⁺ media is expected to be some ten mV inside negative. POLLARD *et al.* (1976), using the distribution of the permeant anion thiocyanate to measure membrane potential of the chromaffin granules, have shown virtually identical value of –45 mV of granule membrane potential in 0.3 M sucrose, which gives support for the validity of the present fluorometrical measurements of granule membrane potential.

For K⁺ concentrations higher than 20 mM, the relation between F and $\log(K^+)_{\circ}$ was identical with that in the presence of valinomycin (Figs. 1 and 3), which indicates that granule membrane potentials change responding to changes of K⁺ as a potassium electrode at high K⁺ medium. While K⁺ concentrations less than 20 mM, F in Fig. 3 is not linear with $\log(K^+)_{\circ}$, which suggests that granule membrane potentials depend on Na⁺ and Cl[–]. It is unlikely that chloride played a significant role in this effect because F was almost independent of replacement of Cl[–] by SO₄^{2–} (Fig. 6). Thus it is suggested from our results that chromaffin granules have membrane potentials which approximate the K⁺ diffusion potential, as is the case of the resting membrane potentials in most nerve cells ($P_K \gg P_{Na}, P_{Cl}$).

The present study clearly demonstrates that Mg²⁺·ATP brought about depolarization of the chromaffin granule membrane concomitantly with the catecholamine release therefrom. Our studies on the kinetics of both responses (Figs. 7 and 11) and of inhibitory action of DCCD thereon (Figs. 8 and 12) showed that the magnitude of depolarization paralleled the rate of amine release. It suggests that Mg²⁺·ATP activates a DCCD-sensitive carrier (s) to give rise to electrogenic ion transport accompanied by the amine liberation. DCCD, a potent inhibitor of Mg²⁺-dependent ATPase associated with

the chromaffin granule membrane (BASHFORD *et al.*, 1976), was reported to inhibit H⁺ accumulation in submitochondrial vesicles through binding with membrane H⁺-ATPase (RACKER, 1976). CASEY *et al.* (1976) provided evidence that only if a permeant anion was present could Mg²⁺·ATP induce an active H⁺ transport. As shown in Fig. 4, the magnitude of the Mg²⁺·ATP-evoked depolarization was decreased with increasing impermeant anion (sulfate) concentration in media. In view of these results, therefore, there is no doubt that a membrane H⁺-ATPase plays some important role in generating the depolarization response to Mg²⁺·ATP. Our indirect estimation of changes in intragranular K⁺ concentration (Fig. 9) suggests a possibility that the ATP-evoked depolarization brings about significant release of internal K⁺. Furthermore admitting the hypothesis that the membrane H⁺-ATPase plays a key role in generating the membrane depolarization, it is indeed impossible to attribute the inside positive potentials to increases in H⁺ and Cl⁻ permeability alone, since the intragranular concentrations of Cl⁻ is smaller than that of medium and pH_i < pH_o. (pH_i=5.5 (JOHNSON and SCARPA, 1976b), (Cl⁻)_i=15–40 mM (PAZOLES and POLLARD, 1978), (Cl⁻)_o=160 mM and pH_o=6.5). Thus the depolarization may occur at least through regulation of H⁺-influx and K⁺-efflux through granule membrane. Additional evidence for this was given by combination of K⁺-ionophore and uncoupler. In choline Cl medium, valinomycin induced hyperpolarization of granules (Fig. 1) without release of catecholamine, while further addition of FCCP, which makes granule membrane freely permeable to H⁺ (JOHNSON and SCARPA, 1976b), caused depolarization and release (data not shown).

PAZOLES and POLLARD (1978) have investigated in detail the role of Cl⁻ on Mg²⁺·ATP-evoked release of catecholamine from isolated chromaffin granules and found that release is a function of Cl⁻ concentration. This is fundamentally compatible with the present result that the magnitude of depolarization was dependent on Cl⁻ (Fig. 6).

The time courses of depolarization and catecholamine release was quite different; the former was rapid, completed within 1 min and sustained stable over 10 min, while the latter was a slow process lasting over 15 min (Figs. 2, 9 and 10). These results suggest that Mg²⁺·ATP induced depolarization of granules may actually be preconditions for catecholamine release therefrom. The physiological significance of depolarization of granules is not fully elucidated, but it was suggested that depolarization promotes the transport of K⁺, H⁺ and Cl⁻ across the granule membrane to result in release of catecholamine.

Concerning the mechanism of H⁺ transport-linked catecholamine release evoked by Mg²⁺·ATP, only a speculation is possible at the present stage of the investigations. Chromaffin granules contain a very high amount of catecholamines and ATP (HILLARP, 1958), and a storage complex of the amines, ATP and Mg has been postulated (for review see WINKLER and SMITH, 1975). Indeed,

a recent NMR study suggested the ionic bonds between positively charged N of adrenaline and the negatively charged phosphates of nucleotides (DANIELS *et al.*, 1978). Thus it is not so unlikely that ATP-driven H^+ influx possibly modifies such an ionic bond to liberate the amines, resulting in passive efflux of the amines and ATP out of the granules. The rapid catecholamine release from the chromaffin granules observed under external pH lower than 6.0 (HILLARP and NILSON, 1954) appears to support such a speculation.

It is insufficient to assign a physiological role at cellular level to the present results, but there is an attractive hypothesis concerning the role of granule Mg^{2+} -ATPase on secretory process through exocytosis (PAZOLES and POLLARD, 1978). Recently, BAKER and KNIGHT (1978) have demonstrated that ATP is necessary for release of catecholamine through exocytosis in adrenal medulla cells. We feel that granule ATPase is one of the main factors which regulate the actual mechanism of release from cells, but further work is necessary to see whether granule ATPase is operating during release *in vivo*.

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