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Phosphorylation of Membrane Components of Adrenal Chromaffin Granules by Adenosine Triphosphate

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SUMMARY

Adenosine triphosphate acts on chromaffin granules of the adrenal medulla to produce structural changes and release of catecholamines, endogenous ATP, and soluble protein. When ATP exerts this action on chromaffin granules, it is hydrolyzed by the Mg^{++}-dependent ATPase present in the granule membrane, and part of the P_i liberated from ATP under the action of the granule ATPase is transferred to the chromaffin granule membrane. This transphosphorylation from ATP is Mg^{++}-dependent and temperature-sensitive; substitution of Mn^{++} for equimolar concentrations of Mg^{++} potentiates the effect, but Ca^{++} has no significant effect on the transphosphorylation from ATP. This phosphorylation is also blocked by inhibitors of the Mg^{++}-dependent mechanism of catecholamine release, such as *N*-ethylmaleimide and gramicidin. Ouabain and atractyloside, which were ineffective in blocking ATP-evoked catecholamine release, did not inhibit the membrane phosphorylation.

The membrane phosphorylation was due to an active transfer of phosphate from ATP to the chromaffin granule membranes, rather than to binding of the nucleotide to the chromaffin granule structures or to the secondary incorporation of the P_i freed into the incubation medium.

The similarities among ATPase activity, ATP-evoked catecholamine release, and phosphorylation of the membrane of chromaffin granules by ATP are discussed. This membrane phosphorylation is, to our knowledge, the first demonstration of a phenomenon of this nature in a granule that stores or releases hormones.

INTRODUCTION

We have previously demonstrated that ATP, in the presence of Mg^{++}, releases catecholamines, endogenous ATP, and soluble protein from chromaffin granules of the adrenal medulla (1, 2). This process was associated with structural modifications, as revealed by light-scattering changes in granule suspensions (3, 4). In addition, studies on the action of ATP on secretory granules isolated from other tissues have recently shown that this phenomenon is not restricted to chromaffin granules; ATP induces release of the contents of isolated zymogen granules (5), vasopressin-containing granules (6), and synaptic vesicles (7).

Since ATP is hydrolyzed by the granule ATPase during its action on chromaffin granules (1, 3), it was desirable to see whether the terminal phosphate group of ATP liberated by this reaction would be incorporated into chromaffin granule structures, as occurs in other organelles (8–13).

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This paper reports findings on the transphosphorylation from ATP to chromaffin granule membranes, and in addition demonstrates that this phosphorylation is blocked by inhibitors of the MgATP-dependent mechanism of catecholamine release.

METHODS

Bovine adrenal glands obtained from a slaughterhouse were kept on ice, and the medullae were separated from the cortices. Each medulla was homogenized in 5 volumes of ice-cold 0.27 M sucrose (pH 7.0). A low-speed sediment was removed by centrifugation at 800 × *g* for 10 min, and the supernatant fraction was recentrifuged at 20,000 × *g* for 20 min. The pellet thus obtained was resuspended in 0.27 M sucrose, layered on a sucrose–Ficoll–D_2O gradient (14), and centrifuged at 100,000 × *g* for 60 min. The final pellet contained chromaffin granules that were almost free from other organelles. This method has the additional advantage of isolating granules under isotonic conditions, so that they are suitable for release studies *in vitro* (14). The granule pellet was suspended in ice-cold 0.3 M sucrose, and aliquots (2.0 mg of protein) of this suspension were used in the experiments reported below. Incubations were conducted in centrifuge tubes for various periods of time at 30° in a total volume of 1.0 ml. The standard incubation medium contained KCl, 160 mM; NaCl, 5 mM; *N*-tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid buffer (pH 7.0), 10 mM; and $MgCl_2$, 0.5 mM; this was varied in some experiments as described below. [γ-^{32}P]ATP (specific activity, 1.8 mCi/μmole), synthesized in our laboratory according to a modification by Post and Sen (15) of the method of Glynn and Chappell (16), was added to the medium to give a concentration of 0.25, 0.5, 1.0, or 2.0 mM; in some experiments ATP-8-^{14}C (specific activity, 47 mCi/mmole) or orthophosphate-^{32}P (carrier-free) at the same final concentration was used instead of [γ-^{32}P]ATP. The addition of 10% (final concentration) ice-cold trichloracetic acid containing 0.2 mM K_2HPO_4 and 0.2 mM ATP terminated the incubations. The tubes were cooled in an ice-water bath for a few minutes and centrifuged in the cold (4°) at 20,000 × *g* for 10 min, and the pellets thus obtained were washed three times with 10% trichloracetic acid and resuspended in 88% formic acid. Radioactivity in the formic acid suspension was determined by adding an aliquot to scintillation vials containing 1.0 ml of "NCS" (Nuclear-Chicago) and 14.0 ml of the mixture previously described (17, 18). ATPase activity was measured by treating aliquots of the 20,000 × *g* supernatant fluids with isobutyl alcohol–benzene (1:1 by volume) and extracting the P_i released from [γ-^{32}P]ATP as a phosphomolybdic acid complex (17, 18).

In all experiments zero time samples were prepared by adding the radioactive compounds after treating the incubation medium containing the granule aliquots with trichloracetic acid (10%, final concentration). Radioactivity in the precipitates and supernatant fractions was determined as indicated above.

The ^{32}P and ATP-8-^{14}C radioactivity was measured with counting efficiencies of 90% and 72%, respectively; the results were corrected to 100% efficiency and expressed in nanomoles or picomoles of P_i or ATP per milligram of granule protein, calculated on the basis of the specific activities of the added ^{32}P, [γ-^{32}P]ATP, or ATP-8-^{14}C. In some experiments, lipids were extracted from the trichloracetic acid-treated pellets with chloroform–methanol (2:1 by volume) containing 0.25% HCl (19). Protein was determined by a modified biuret method (20), and catecholamines were assayed by the trihydroxyindole fluorometric method (21). Results are expressed as means ± standard errors.

Chemicals were obtained from the following sources: ATP and Tris buffer, Sigma Chemical Company; sodium acetate–acetic acid buffer and EDTA, Fisher Scientific Company; orthophosphate-^{32}P, New England Nuclear Corporation; ATP-8-^{14}C, Schwarz BioResearch, Inc.; "NCS", Nuclear-Chicago Corporation; *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid buffer, Calbiochem; *N*-ethylmaleimide, Eastman Organic Chemicals; ouabain, Nutritional Biochemicals Corporation; gramici-

TABLE 1

Mg^{++}-dependent ATPase activity and transphosphorylation from ATP into chromaffin granules and mitochondria

Chromaffin granules and mitochondria preparations were incubated for 10 min at 30° in a standard incubation medium (pH 7.0). Mitochondrial aliquots were prepared from the layer of the sucrose–Ficoll–D_2O gradient which corresponded to the highest specific activity of two different enzyme markers used (14). When EDTA or *N*-ethylmaleimide was used, it was added to the medium 5 min prior to the addition of [γ-^{32}P]ATP (specific activity, 0.77 μCi/μmole). The incubations were terminated by the addition of 10% trichloracetic acid, and the tubes were centrifuged at 20,000 × *g* for 10 min. The ATPase activity of the supernatant fractions and the ^{32}P incorporated into the trichloracetic acid-insoluble fraction were determined as indicated in METHODS. Values are the means ± standard errors of 4–12 separate observations.

Preparation	Additions				ATPase		Transphosphorylation from ATP	
	Mg^{++} (0.5 mM)	[γ-^{32}P] ATP (0.5 mM)	EDTA (2.0 mM)	*N*-Ethyl-maleimide (0.2 mM)	Activity	Inhibition relative to Mg^{++} control	Activity	Inhibition relative to Mg^{++} control
					nmoles P_i/mg protein	%	*pmoles P_i/mg protein*	%
Chromaffin granules	+	+	−	−	193 ± 13		260 ± 26	
	−	+	+	−	25 ± 2	87	14 ± 6	94
	+	+	−	+	59 ± 6	69	86 ± 4	67
Mitochondria	+	+	−	−	454 ± 64		433 ± 63	
	−	+	+	−	48 ± 13	89	44 ± 7	90
	+	+	−	+	304 ± 40	33	316 ± 27	27

din (mol wt 1800), Mann Research Laboratories; and atractyloside (mol wt 880), a generous gift from Dr. R. Santi, University of Padova, Italy.

RESULTS

The presence of ATPase in chromaffin granules was first demonstrated by Hillarp in 1958 (22), and, although the activity of the enzyme has been measured in studies of catecholamine uptake (23, 24) or release (1), a full account of the characteristics of the enzyme has not appeared in the literature.

Since ATP causes some conformational change in the membranes of isolated chromaffin granules so that they release their soluble contents (1–4), and because of the correlation of this effect of ATP with the ATPase activity of the granule membrane (1, 3), it was decided to examine this ATP-dependent process further to see whether terminal phosphate liberated from ATP by the action of the ATPase would be incorporated into granule structures.

Mg^{++}-dependent ATPase activity of chromaffin granules and phosphorylation of granule structures by [γ-^{32}P]ATP. Chromaffin granules incubated in the presence of 0.5 mM Mg^{++} and 0.5 mM ATP had an ATPase activity of 15.9 ± 1.2 nmoles of P_i per milligram of total granule protein per minute (45 observations), which is close to the value reported by other authors (25). The Mg^{++}-dependent ATPase of the adrenal medullary mitochondria had 2.35 times the activity of the chromaffin granules when it was tested under similar conditions (Table 1). When Mg^{++} was omitted and 2.0 mM EDTA was included in the incubation medium, both the granular and the mitochondrial ATPases were inhibited to the same extent (Table 1). In contrast, 0.2 mM *N*-ethylmaleimide added to the incubation medium before ATP reduced the ATPase activity of the chromaffin granules more effectively than that of the mitochondria (Table 1).

When chromaffin granules were incubated

with 0.5 mM [γ-^{32}P]ATP in the presence of 0.5 mM Mg^{++}, part of the ^{32}P released from the [γ-^{32}P]ATP during the reaction was incorporated into chromaffin granule structures (Table 1). Moreover, as with the ATPase activity, the omission of Mg^{++} (in the presence of 2.0 mM EDTA) or the addition of *N*-ethylmaleimide (0.2 mM) produced a decrease in the phosphorylation of the granule structures (Table 1). When adrenal mitochondria were incubated under similar conditions, it was observed that, as with the ATPase activity, *N*-ethylmaleimide produced much less inhibition of mitochondrial phosphorylation than of granule phosphorylation (Table 1).

Localization of Mg^{++}-dependent ATPase and site of phosphorylation in chromaffin granule structures. Intact chromaffin granules, as well as their membranes and soluble proteins (chromogranins), were incubated in the presence of Mg^{++} and [γ-^{32}P]ATP. These experiments showed that the ATPase activity, as well as the site of phosphorylation, was localized in the chromaffin granule membranes (Fig. 1A and B). The specific activity of the enzyme in the membranes and the phosphorylation of these structures by ATP was 3–4 times greater than in intact granules when the results were expressed per milligram of protein. This difference was due to the fact that soluble proteins accounted in these experiments for 62.9% of the total granule proteins. Membranes and intact granules hydrolyzed the same absolute amount of ATP; in addition, the total transphosphorylation from ATP was the same in both granule membranes and intact granules. No phosphorylation or ATPase activity was found in the soluble proteins (Fig. 1). Since the majority of the present experiments used intact granules, so that conditions were the same as those under which catecholamine release had been studied previously (1–4), the results in the following sections are expressed per milligram of total granule protein.

Binding of ATP-8-^{14}C and incorporation of orthophosphate-^{32}P. To test the possibility that the radioactivity measured was due to the binding of ATP to granule membranes rather than to the incorporation of ^{32}P

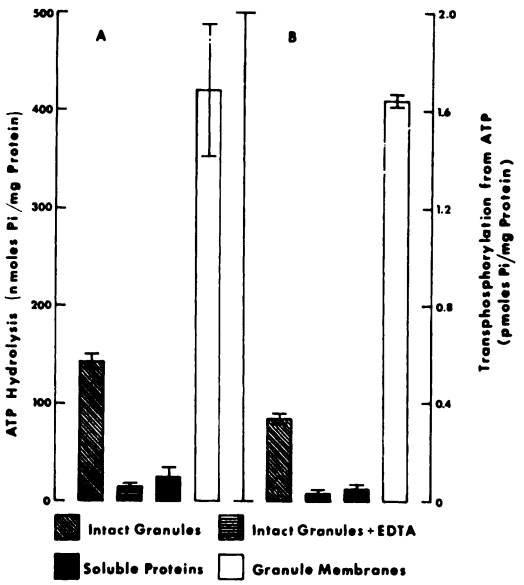

FIG. 1. *Localization of Mg^{++}-dependent ATPase and site of phosphorylation in chromaffin granule structures*

Chromaffin granules were lysed in distilled water, and membranes were separated from the soluble constituents by centrifugation at 100,000 × *g* for 30 min. Aliquots of the various fractions were incubated for 10 min at 30° in a standard medium (pH 7.0) containing 0.5 mM Mg^{++} and 0.5 mM [γ-^{32}P]ATP (specific activity, 0.56 μCi/μmole). When EDTA (2.0 mM) was present, Mg^{++} was omitted from the incubation medium. Chromogranins (granule soluble proteins) accounted for 62.9% of the total chromaffin granule protein. ATPase activities of the different granule preparations are depicted in part A, and transphosphorylation from ATP to the same chromaffin granule preparations is represented in part B. ATPase activity and P_i incorporation were determined as described in METHODS. Each bar represents the mean ± standard error of three separate observations.

derived from [γ-^{32}P]ATP, granules were incubated for 20 min in the presence of 0.5 mM Mg^{++} and 0.5 mM ATP-8-^{14}C. The specific activity of the ATP-8-^{14}C allowed determination of the binding of 0.5 pmole/mg of protein (0.09% of the observed P_i incorporation). Upon termination of the reactions by trichloracetic acid (see METHODS), 1.70 ± 0.06 pmoles/mg of protein of bound ATP were found under these conditions. This value, which represents 0.31%

TABLE 2

$^{32}P_i$ incorporation into aliquots of chromaffin granule membranes incubated successively in the same $^{32}P_i$-containing medium

Granule preparations (0.95 mg of protein) were incubated for 15 min in 1.5 ml of standard incubation medium (pH 7.0) containing either 0.5 mM Mg^{++} or 2.0 mM EDTA. Experiments were carried out at either 30° or 2°. At the end of incubation 1, the tubes were centrifuged for 10 min at 20,000 × *g* and the supernatant fractions were transferred to empty tubes, in which incubation 2 was carried out with the addition of new aliquots of granules. This procedure was repeated through incubations 3 and 4. The pellets were treated with 10% trichloracetic acid (final concentration) and prepared for counting as described in METHODS. Series a, b, and c in all four experiments represent the same respective supernatant fractions, which were carried successively from incubation 1 through incubation 4. The decrease in the concentration of phosphate in the medium indicated in the table was due to the dilution effect caused by the addition of new granule aliquots in each successive incubation. Each series of experiments was carried out in triplicate except for series c, which represent single values.

Sequential incubations	Conditions		^{32}P concentration	^{32}P specific activity	^{32}P incorporation	Radioactivity bound to total protein as percentage of total counts in medium
	Addition	Temperature				
			mM	*dpm × 10^{-3}/ μmole P_i*	*dpm/mg protein*	%
1st (a)	Mg^{++}	30°	1.0	1490	5341 ± 356	0.3585
(b)	EDTA	30	1.0	1394	5016 ± 170	0.3597
(c)	Mg^{++}	2	1.0		6118	
2nd (a)	Mg^{++}	30	0.97	1294	144 ± 36	0.0111
(b)	EDTA	30	0.97	1343	100 ± 35	0.0074
(c)	Mg^{++}	2	0.97		18	
3rd (a)	Mg^{++}	30	0.94	1359	33 ± 9	0.0024
(b)	EDTA	30	0.94	1332	22 ± 4	0.0016
(c)	Mg^{++}	2	0.94		0	
4th (a)	Mg^{++}	30	0.91	1385	41 ± 3	0.0029
(b)	EDTA	30	0.91	1381	28 ± 2	0.0020
(c)	Mg^{++}	2	0.91		8	

of the total incorporation (540 ± 35 pmoles/mg of protein), was similar to that obtained either when Mg^{++} was omitted (2.0 mM EDTA present) or when ATP-8-^{14}C was added to granules incubated at 2°.

During the hydrolysis of [γ-^{32}P]ATP by the granule ATPase, ^{32}P is continuously released into the incubation medium. To determine whether the labeling observed in the presence of [γ-^{32}P]ATP was due to the incorporation into the membranes of the ^{32}P freed in solution rather than to an active mechanism of incorporation due to the transfer of the ^{32}P from [γ-^{32}P]ATP to the granule membranes, successive incubations were carried out in the presence of ^{32}P as described by Skou and Hilberg (12).

Aliquots of the chromaffin granule membrane preparations were incubated as described in the legend to Table 2. This table shows that the membrane labeling observed after the first incubation period was 37 times greater than that observed after the second, and the latter was 4 times higher than the labeling found after the third incubation period, although there was no change in the specific activity of the ^{32}P throughout all the incubations. Table 2 also shows that after the first incubation the labeling was the same in the presence or absence of Mg^{++}

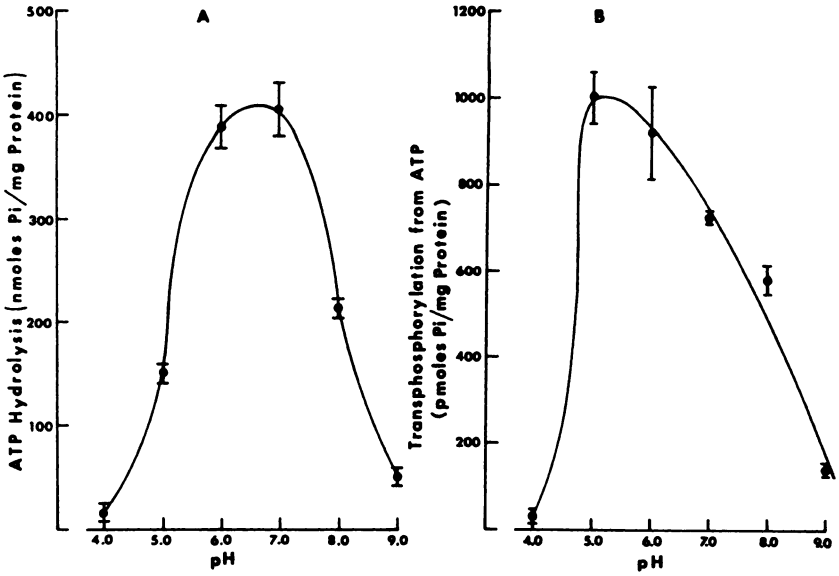

FIG. 2. *Effect of pH on Mg^{++}-dependent ATPase activity and transphosphorylation from ATP to chromaffin granule membranes*

Chromaffin granule membranes were incubated for 10 min at 30° in a standard medium containing various buffers (40 mM): acetate–acetic acid (pH 4–5), *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (pH 6–7), and Tris (pH 8–9). The incubation medium contained 0.5 mM Mg^{++} and 0.5 mM [γ-^{32}P]ATP (specific activity, 0.25 μCi/μmole). ATPase activity is depicted in part A, and transphosphorylation from ATP in part B. Points are the means ± standard errors of three separate experiments.

or when incubation was performed at 2°. The amount of labeling became constant after the third and fourth incubations, and in both the labeling was affected by Mg^{++} and temperature (Table 2). Furthermore, the amount of radioactivity removed by the first incubation was 0.36% of the total radioactivity in the medium, whereas the second, third, and fourth incubations removed 0.01, 0.002, and 0.003% of the total counts, respectively.

The results suggest that ^{32}P was contaminated to the extent of 0.3–0.36% (expressed as disintegrations per minute) with another isotope which was bound during the incubations to chromaffin granule membranes. Similar levels of contamination in commercial ^{32}P preparations were found by other workers (12). It is unlikely that this artifact occurred in the experiments in which [γ-^{32}P]ATP was used as the substrate, since Skou and Hilberg (12) have demonstrated that the contaminant could be eluted together with the $^{32}P_i$ peak from the Dowex column used in the purification of [γ-^{32}P]ATP (12). The [γ-^{32}P]ATP used in the present experiments was prepared in our laboratory, and the purity of the ATP eluted from the Dowex column was determined by two different methods (17, 26). In addition, under our experimental conditions, any contaminant that could have been present in the [γ-^{32}P]ATP fraction was subtracted from the experimental values in the form of a zero time value. If the ^{32}P incorporation in incubations 3 and 4 (Table 2) is expressed in picomoles of P_i per milligram of protein, the values obtained are 6.6 and 7.7, which represent 1.3 and 1.5% of the incorporation of 508.2 ± 18.7 pmoles of P_i per milligram of protein obtained when the membrane preparations were incubated for the same period of time in the presence of 1.0 mM [γ-^{32}P]ATP.

The results presented in this section appear to rule out the possibility that the phosphorylation of the granule membranes by [γ-^{32}P]ATP was due either to the binding

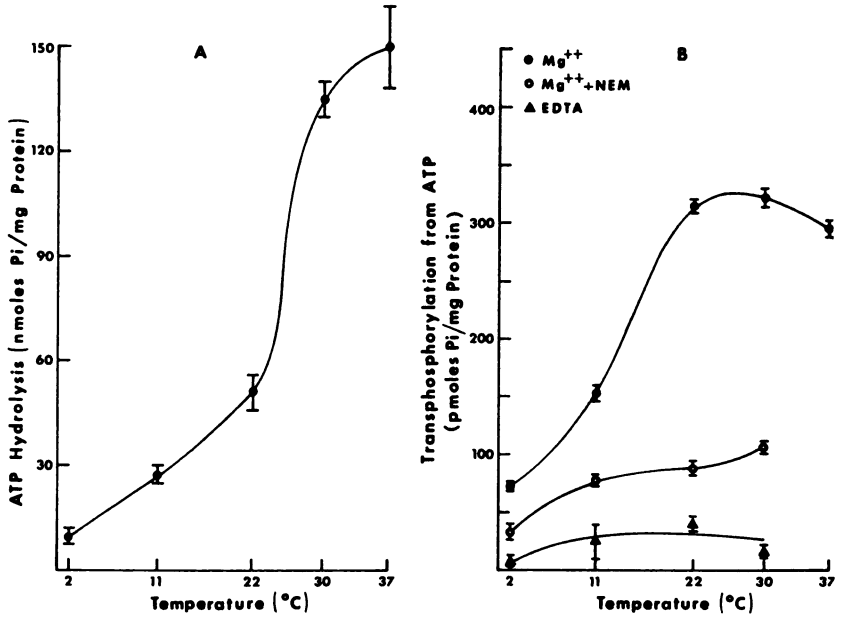

FIG. 3. *Effect of temperature on Mg^{++}-dependent ATPase activity and transphosphorylation from ATP to chromaffin granule membranes*

Chromaffin granules were incubated for 10 min, at the temperatures indicated along the abscissae, in a standard medium (pH 7.0) containing 0.5 mM Mg^{++} and 0.5 mM [γ-^{32}P]ATP (specific activity, 0.56 μCi/μmole). *N*-Ethylmaleimide (NEM) was added to the incubation medium to a final concentration of 0.2 mM 5 min prior to the addition of ATP. When EDTA (2.0 mM) was used, Mg^{++} was omitted from the incubation medium. The incubations were terminated by the addition of 10% trichloracetic acid (final concentration), and the ATPase activity (A) and transphosphorylation from ATP (B) were determined as indicated in METHODS. Points are the means ± standard errors of three separate observations.

of this nucleotide or to the incorporation of free ^{32}P into the membranes.

Effect of pH on Mg^{++}-dependent ATPase activity and transphosphorylation from ATP to chromaffin granule membranes. It has been shown that the ATP-induced release of catecholamines from isolated chromaffin granules is dependent on pH (2). Since the spontaneous release of catecholamines increased, although the ATP-induced release decreased, as the pH was raised above 7.0 (2), it was desirable to study the effect of changes in pH on the ATPase activity and membrane phosphorylation. Figure 2A shows that the granule ATPase had an optimal pH for activity between 6.0 and 7.0. The optimal conditions for transphosphorylation from ATP to granule membranes occurred between pH 5.0 and 6.0, after which there was a gradual decrease in the transphosphorylation as the pH was raised to 9.0 (Fig. 2B).

Effect of temperature on Mg^{++}-dependent ATPase activity and transphosphorylation from ATP to chromaffin granule membranes. The highest ATPase activity was observed at temperatures between 30° and 37°, although the enzyme is capable of hydrolyzing ATP in the presence of Mg^{++} at temperatures as low as 2° (Fig. 3A).

The effect of different temperatures on the phosphorylation of chromaffin granule membranes was also tested. In the presence of 0.5 mM Mg^{++} the phosphorylation was almost the same at 22°, 30°, or 37°. Reducing the incubation temperature to 11° diminished the phosphorylation to half that observed at 22°. At 2° the membrane phosphorylation, although significantly higher than the control value, was reduced to one-fourth the value obtained at 22° (Fig. 3B). In the presence of 0.2 mM *N*-ethylmaleimide phosphorylation was significantly inhibited at all the temperatures tested (Fig. 3B), but

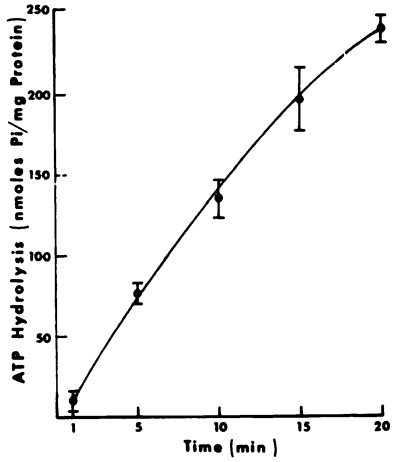

FIG. 4. *Time course of ATPase activity of chromaffin granules*

Chromaffin granules were incubated at 30° for different periods of time, as indicated on the abscissa, in a standard medium (pH 7.0) containing 0.5 mM Mg^{++} and 0.5 mM [γ-^{32}P]ATP (specific activity, 0.47 μCi/μmole). Addition of ice-cold 10% trichloracetic acid terminated the reactions. ATPase activity was assayed as indicated in METHODS. Each point represents the mean ± standard error of four separate observations.

was almost the same at 11° or 22° in the presence of this reagent.

Since previous studies on the ATP-evoked catecholamine release were carried out at 30° (1–4), this temperature was chosen for the phosphorylation studies.

Time course of ATPase activity and transphosphorylation from ATP to chromaffin granule membranes. Figure 4 shows the time course of the ATPase activity of the chromaffin granules at 30° and pH 7.0. The same figure also shows that the ATP cleavage progresses nearly linearly with time.

Figure 5 (upper curve) shows the time course of ^{32}P incorporation into the chromaffin granule membranes (trichloracetic acid-insoluble fraction); the lower curve in the same figure shows the values obtained after extracting the lipids. The two were different, and indicate that at least two different membrane components were phosphorylated.

In general, incubation was conducted with 0.5 mM ATP, since this concentration produces 60–85% release of bound amines (1, 3). This amount of ATP is not completely hydrolyzed by the ATPase during 20 min of incubation, and it was therefore decided to test the effect of a smaller concentration of ATP (0.2 μM). The results are shown in Fig. 6; as ATP disappeared, membrane labeling increased and reached a maximum after 15 min of incubation, when only 16% of the ATP remained intact. These results indicate a good correlation between membrane phosphorylation and the ATPase activity of chromaffin granule membranes.

Effect of ions on ATPase activity and transphosphorylation from ATP to chromaffin granule membranes. The effects of different ions on the ATPase activity of chromaffin granules were also tested. In the presence of Ca^{++} the ATPase activity was reduced, but it was increased when Mn^{++} was substituted for Mg^{++} (Fig. 7A). The activity of the enzyme was not significantly different when tested in a standard incubation medium containing either 160 mM KCl or 160 mM NaCl. Similarly, the ATP-evoked release of catecholamines from chromaffin granules occurred to an equal extent under these conditions (1).

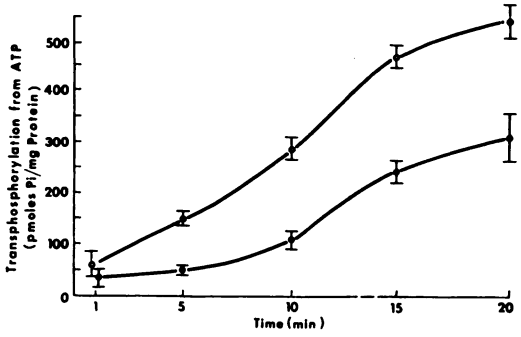

FIG. 5. *Time course of transphosphorylation from ATP to chromaffin granule membranes*

Chromaffin granules were incubated in a standard medium (pH 7.0) at 30° for different periods of time in the presence of 0.5 mM Mg^{++} and 0.5 mM [γ-^{32}P]ATP (specific activity, 0.48 μCi/μmole). Curves show the transphosphorylation from ATP to trichloracetic acid-insoluble fractions before (○) and after (●) lipid extraction. The difference between the two curves represents the transphosphorylation from ATP to phospholipids. Points are the means ± standard errors of four separate observations.

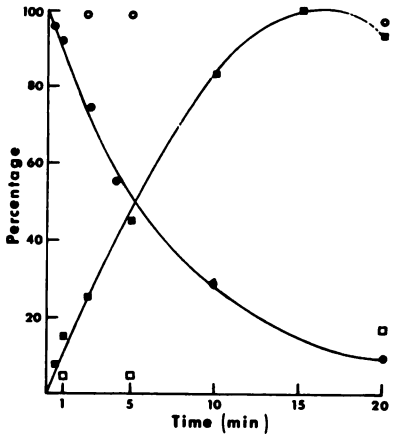

FIG. 6. *Relationship between complete hydrolysis of ATP and transphosphorylation from ATP to chromaffin granule membranes*

Incubations were carried out for the indicated periods of time at 30° in a standard medium (pH 7.0) containing 0.5 mM Mg^{++} (●, ■) and 0.2 μM [γ-^{32}P]ATP (specific activity, 1.55 μCi/μmole). When 2.0 mM EDTA (○, □) was present, Mg^{++} was omitted from the medium. All values are duplicate means except for EDTA data, which represent single values. ATPase activity (●, ○) is expressed as the percentage of unhydrolyzed ATP remaining in the medium, whereas transphosphorylation from ATP to granule membranes (■, □) is expressed as a percentage of maximal labeling under these conditions (0.577 pmole of P_i per milligram of protein at 15 min).

Ca^{++} did not produce any significant transphosphorylation above the values obtained in the presence of EDTA (Fig. 7B). Mg^{++} (0.5 mM) was effective, and replacement of this divalent cation by equimolar concentrations of Mn^{++} increased the transphosphorylation from ATP to chromaffin granule membranes (Fig. 7B). This increase in transphosphorylation was observed at all the concentrations tested (Table 3). The effect of monovalent cations was also studied; like the ATPase activity, phosphorylation was not significantly different when tested in 160 mM KCl or in 160 mM NaCl.

Effect of varied Mg^{++} and ATP concentrations on Mg^{++}-dependent ATPase activity and transphosphorylation from ATP to chromaffin granule membranes. It has also been shown that ATP-induced release of catecholamines from isolated chromaffin granules is dependent upon the concentration of ATP, and that release increases linearly with a logarithmic rise in ATP concentration (1). In the present experiments, increasing the ATP concentration in the medium yielded a linear increase in the ATPase activity of the chromaffin granule preparations. Four ATP concentrations were tested (0.25, 0.5, 1.0, and 2.0 mM), and the activity rose from 5.59 ± 0.21 (at 0.25 mM ATP) to 25.24 ± 0.58 nmoles of P_i per milligram of protein per minute (at 2.0 mM ATP). This linear increase in the enzyme activity upon ATP addition was observed in the presence of three different concentrations of Mg^{++} (0.5, 1.0, and 2.0 mM). By contrast, when the ATP concentration was kept constant, there was no difference in the ATPase activity when granules were incubated in

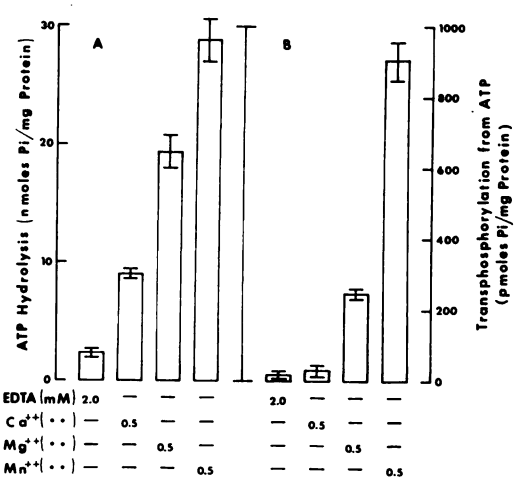

FIG. 7. *Effect of divalent cations on ATPase activity and transphosphorylation from ATP to chromaffin granule membranes*

Chromaffin granules were incubated for 20 min in a standard medium (pH 7.0) in the absence (with EDTA present) or presence of Ca^{++}, Mg^{++}, or Mn^{++}, at the final concentrations indicated. [γ-^{32}P]ATP (specific activity, 0.75 μCi/μmole) was present at a concentration of 0.5 mM. The ATPase activities of the preparations are depicted in part A, and the transphosphorylation from ATP to the chromaffin granule membranes is represented in part B. ATPase activity and transphosphorylation from ATP were determined as described in METHODS. Each bar represents the mean ± standard error of three to five separate observations.

TABLE 3

Effects of different concentrations of Mg^{++} and Mn^{++} on transphosphorylation from ATP to chromaffin granule membranes

Chromaffin granules were incubated for 10 min at 30° in a standard incubation medium (pH 7.0) containing [γ-^{32}P]ATP (specific activity, 0.75 μCi/μmole) in the absence (with EDTA present) or presence of three different concentrations of Mg^{++} or Mn^{++}. P_i incorporation was determined as indicated in METHODS. The results represent the means ± standard errors of three to five separate experiments.

Experiment	Additions				Transphosphorylation from ATP
	Mg^{++}	Mn^{++}	[γ-^{32}P] ATP	EDTA	
	mM	*mM*	*mM*	*mM*	*pmoles P_i/mg protein*
1			0.5	2.0	7 ± 3
2	0.25		0.5		64 ± 10
3	0.5		0.5		125 ± 2
4	2.0		0.5		418 ± 26
5		0.25	0.5		270 ± 19
6		0.5	0.5		449 ± 21
7		2.0	0.5		702 ± 55

0.5, 1.0, or 2.0 mM Mg^{++}. This finding is also in agreement with previously reported studies which showed that release of catecholamines in the presence of 0.5 mM ATP was the same when tested in the presence of 0.5 or 5.0 mM Mg^{++} (1).

Different concentrations of Mg^{++} and ATP were also tested for their effects on membrane phosphorylation. The results of these experiments (Fig. 8) show that if the ATP concentration remains constant, the membrane phosphorylation increases with increasing Mg^{++} concentrations. On the other hand, if the Mg^{++} concentration is kept constant, the phosphorylation of the membrane increases with increasing ATP concentrations until the concentration of ATP in the medium is equal to that of Mg^{++}. In other words, the phosphorylation of the membranes increases until the molar ratio of ATP to Mg^{++} is 1:1 (Fig. 8). The 1:1 ratio is indicated in Fig. 8 by a filled circle (●) for all concentrations of Mg^{++} and ATP used. Molar ratios of ATP to Mg^{++} of 2:1, 4:1, and 8:1 did not increase membrane phosphorylation above the values obtained with molar ratios of 1:1, for any of the Mg^{++} concentrations used. On the other hand, decreasing the molar ratios of ATP to Mg^{++} to 1:2, 1:4, and 1:8 decreased the membrane phosphorylation (Fig. 8) for all the Mg^{++} concentrations that were tested.

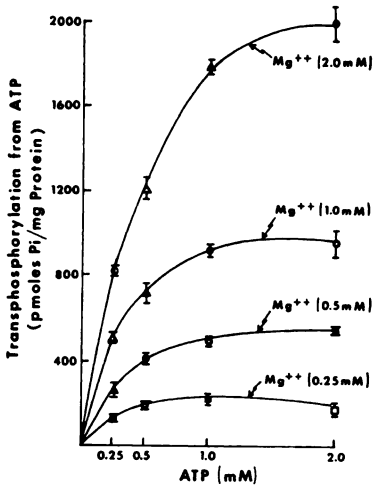

FIG. 8. *Effect of varied Mg^{++} and ATP concentrations on transphosphorylation from ATP to chromaffin granule membranes*

Chromaffin granules were incubated for 15 min at 30° in a standard medium (pH 7.0) in the presence of four different Mg^{++} concentrations, as indicated by the different curves. The [γ-^{32}P] ATP concentrations used were 0.25, 0.5, 1.0, and 2.0 mM, with respective specific activities of 2.18, 1.09, 0.54, and 0.27 μCi/μmole. Transphosphorylation from ATP was determined as indicated in METHODS. Each point represents the mean ± standard error of three separate observations. The different ratios of ATP to Mg^{++} (mole for mole) used are indicated by the following symbols: ◐, 1:8; △, 1:4; ▲, 1:2; ●, 1:1; ○, 2:1; ■, 4:1; □, 8:1.

Effects of ouabain, atractyloside, and gramicidin on release of catecholamines, ATPase activity, and transphosphorylation from ATP to chromaffin granule membranes. It was previously reported that ouabain (10 μM) did not inhibit catecholamine release from granules by ATP (3). The lack of effect of this agent on the ATPase activity and membrane phosphorylation of chromaffin granules is shown in Table 4. Atractyloside (0.1–100 μM), which inhibits the translocation of nucleotides in mitochondria (27),

TABLE 4

Effect of ouabain, atractyloside, and gramicidin on ATP-evoked catecholamine release, ATPase activity, and transphosphorylation from ATP to chromaffin granule membranes

Chromaffin granules were incubated for 20 min at 30° in an incubation medium containing NaCl, 160 mM; KCl, 5 mM; *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid buffer (pH 7.0), 10 mM; $MgCl_2$, 0.5 mM; and [γ-^{32}P]ATP (specific activity, 0.75 μCi/μmole), 0.5 mM. The various compounds tested were incubated for 10 min with the granules prior to the addition of ATP. Catecholamines, ATPase activity, and P_i incorporation were determined as described in METHODS. Results are the means ± standard errors of three to five separate experiments. The control values are the amount of catecholamines released, ATPase activity, and P_i incorporation in granules incubated under the same conditions without the addition of the agents. The molecular weights of atractyloside and gramicidin used to calculate the molarities were taken as 880 and 1800, respectively.

Agent	Catecholamine release	ATPase activity	Transphosphorylation from ATP
	% *control*	% *control*	% *control*
Ouabain, 10 μM	100[a]	100 ± 2	107 ± 6
Atractyloside, 10 μM	99 ± 7	104 ± 6	104 ± 8
Gramicidin, 10 μM	60 ± 7	79 ± 8	67 ± 3

[a] Catecholamine release was not determined in this experiment, since we have previously shown that this concentration of ouabain did not inhibit the ATP-evoked release of catecholamines (3).

did not inhibit either ATP-evoked catecholamine release or ATPase activity and membrane phosphorylation of the chromaffin granules (Table 4). However, gramicidin (10 μM) produced significant inhibition of all three ATP-dependent responses (Table 4).

Stability of phosphorylated components of chromaffin granule membranes. Cleavage of the phosphorylated membrane components by hydroxylamine has been interpreted to provide evidence for the existence of an acyl-phosphate linkage (10, 13). Therefore, phosphorylated membranes which had previously been denatured by treatment with trichloracetic acid were incubated in 20 mM acetate buffer (pH 5.4) for 30 min in the presence or absence of 0.5 M hydroxylamine. No increase in the cleavage of ^{32}P bound to denatured membranes was observed upon the addition of hydroxylamine. Furthermore, the phosphorylated membranes were very stable when incubated in buffers at various pH values (pH 2, 4, 6, 8, and 10). These findings indicate that membrane phosphorylation may be related to the labeling of stable sites.

DISCUSSION

Previous work has related the presence of ATPase in chromaffin granules with their ability to take up amines from the incubation medium (23, 24). However, we have recently demonstrated that ATP acts on isolated chromaffin granules to cause the release of their soluble constituents (1–4). This effect of ATP was correlated with its hydrolysis by the chromaffin granule ATPase (1). From these studies it was concluded that ATP produced some conformational change in the granule membranes, leading to the release of catecholamines, endogenous ATP, and soluble protein (1–4). In the present paper the above results have been extended to show that part of the P_i liberated from ATP under the action of the ATPase is incorporated into chromaffin granule membranes.

The ATPase activity was found to be localized in the granule membrane, and this agrees with Hillarp's observations (22). The specific activity of the enzyme in membrane preparations was 3 times higher than in intact granules, and this difference was due entirely to the fact that membrane proteins represented about one-third of the total granule proteins. The specific activity of the ATPase in the membrane preparations was close to that reported previously by Banks (28). In addition, the present results show that the ATPase activity of the chromaffin granules increased with increments in the ATP concentration from 0.25 to 2.0 mM. In contrast to this concentration-dependent effect of ATP, Mg^{++} at the different concentrations tested (0.5, 1.0, and 2.0

mM) was found to support the ATPase activity to the same extent. These observations are well correlated with previous findings on catecholamine release from isolated granules, in which the release evoked by ATP rose with increasing ATP concentrations, but the release was the same either in 0.5 or 5.0 mM Mg^{++} (1).

The specific activity of the Mg^{++}-dependent ATPase of adrenal mitochondria was also measured, and it was found that this mitochondrial activity was 2.35 times higher than that of the chromaffin granules. This value is somewhere between the values of 2.29 and 4.0 that can be calculated from the results reported by Taugner and Hasselbach (25) and Banks (28). The results also show that the optimal pH for the enzyme activity was 6.5, and that the enzyme is active at 2°.

In agreement with previous observations (23, 28), Ca^{++} was found to be a poor cofactor for ATPase activity in chromaffin granules. We reported previously that Ca^{++} was not a necessary requirement for the ATP-evoked catecholamine release (1, 3). On the contrary, Mn^{++} was even more effective than Mg^{++} in supporting ATPase activity. This observation also agrees with our previous finding that Mn^{++} was more effective than Mg^{++} in potentiating the ATP-induced release of catecholamines (1). The results presented in this paper also show that the ATPase activity of the chromaffin granules was the same in media containing 160 mM KCl or 160 mM NaCl, and that ouabain did not inhibit the activity of the chromaffin granule ATPase. These findings also parallel the previous observations on the ATP-evoked catecholamine release (1, 3). The lack of inhibition observed in the presence of atractyloside might indicate that the ATPase is localized on the external surface of the granule membrane, and in contrast to mitochondria (27), granular ATP need not be translocated first in order to act on the ATPase. This is, of course, a theoretical assumption, since there is no proof yet that the granular ATPase is localized externally. The inhibition of the ATPase activity observed in the presence of gramicidin cannot be explained by any of the known properties of this compound (29), but gramicidin also blocked the ATP-induced catecholamine release. Chromaffin granule ATPase is inhibited by *N*-ethylmaleimide, which indicates that sulfhydryl groups are important for ATPase activity. Adrenal mitochondrial ATPase is not inhibited to this extent by a similar concentration of *N*-ethylmaleimide (25).

The most striking feature of the present results is that a fraction of the terminal phosphate hydrolyzed from ATP is transferred to granule membranes (protein and lipids). Since the granule preparations used could be slightly contaminated with mitochondria [1–4% of the protein present in the granule fraction was previously shown to be of mitochondrial origin (14)], aliquots from the mitochondrial fraction of the gradient were incubated under the same experimental conditions. The transphosphorylation from [γ-^{32}P]ATP to the acid-insoluble fraction in the granules was 60–90% of that into mitochondria, and therefore the granule preparations would have to contain at least 60% of mitochondria to account for the observed phosphorylation.

The radioactivity measured in the granules that were incubated with [γ-^{32}P]ATP was not due to the binding of ATP to granule structures, because ATP-8-^{14}C labeling was only 0.3% of the transphosphorylation from [γ-^{32}P]ATP. In addition, the results show that this small binding was the same in the presence of EDTA or at 2°.

Another possibility was that the radioactivity measured in the granule membranes was due to the incorporation of ^{32}P freed in solution rather than to an active mechanism of transfer. The results presented in this paper show that, when precautions are taken during the experiments with ^{32}P [commercial ^{32}P can be contaminated with other isotopes (12), to an extent of 0.36% in our case], no more than 1.5% of the transphosphorylation can be attributed to the ^{32}P free in the incubation medium. As with ATP-evoked catecholamine release and ATPase activity, this labeling was an Mg^{++}-dependent process, and Ca^{++} was not effective, whereas Mn^{++} was found to be more potent than Mg^{++} at equimolar

concentrations. The labeling of the membranes increased with increasing concentrations of Mg^{++}, Mn^{++}, or ATP. But for any chosen Mg^{++} concentration, the phosphorylation increased with the increase in ATP concentration until the molar ratio of ATP to Mg^{++} in the incubation medium was 1:1. No further increase in phosphorylation was observed with higher ratios. It thus appears that the membrane phosphorylation is dependent upon the formation of an Mg^{++}-ATP (1:1) complex.

In parallel with the results for ATPase activity, ouabain and atractyloside were ineffective in blocking membrane phosphorylation, whereas gramicidin was found to inhibit phosphorylation. Gramicidin stimulates the rate of re-entry of Na^+, K^+, and H^+ in mitochondria (29). Thus, the mitochondrial proton pump can cause accumulation of alkali metal ions when gramicidin is present (29). The mechanism of action of this drug on chromaffin granules is unknown. However, gramicidin was able to inhibit the three ATP-dependent processes, catecholamine release, ATPase activity, and membrane phosphorylation, to a similar extent.

The transphosphorylation from ATP to granule membranes was a pH- and temperature-dependent phenomenon and, as with the localization of ATPase activity, the chromaffin granule membrane was the phosphorylated structure. Three different enzymes are known to be present in chromaffin granule membranes: cytochrome *b*-559 (30), dopamine β-hydroxylase (31), and ATPase (1, 22–25, 28). It seems likely that the last enzyme, which in other tissues is a lipoprotein (32, 33), is phosphorylated, as occurs, for example, in mitochondria (11). However, unlike other tissues (11, 34), the chromaffin granule ATPase has not yet been isolated in a soluble form or even in a relatively pure particulate form, and this isolation must be accomplished before more definitive conclusions can be reached about phosphorylation of the enzyme. The results obtained after treatment of the phosphorylated membranes with hydroxylamine or after incubation of membranes in buffers at various pH values indicate that the phosphorylation is possibly related to the labeling of stable sites in the membrane. These findings, taken together with the fact that the membrane phosphorylation increases (instead of remaining constant) as the ATP cleavage progresses linearly with time, suggest the possibility that this membrane phosphorylation is not due to the formation of a phosphorylated "intermediate." However, it is clear that the phosphorylation of the chromaffin granule membrane is dependent on the same divalent cations, and is inhibited in the same manner, as the granule ATPase and the ATP-evoked catecholamine release process, but further work is necessary to establish whether the three ATP-dependent processes are interrelated.

The results reported in this paper on membrane phosphorylation by ATP constitute, to our knowledge, the first demonstration of such a change in granules that store and release hormones.

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