Adenosine 5′-Triphosphate Independent Secretion from PC12 Pheochromocytoma Cells†

Elwood E. Reynolds,‡ William P. Melega, and Bruce D. Howard*

ABSTRACT: PC12 is a clonal line of rat pheochromocytoma. The cells secrete dopamine and acetylcholine by a Ca^{2+}-dependent process. The requirement for adenosine 5′-triphosphate (ATP) in secretion from PC12 was investigated. Cellular levels of ATP were decreased by brief treatments with inhibitors of glycolysis or oxidative phosphorylation. In order to deplete ATP stores below 40% of control values during a 5-min treatment with these compounds, it was necessary to inhibit both glycolysis and oxidative phosphorylation. Depletion of ATP pools to as little as 10% of control values did not result in any inhibition of dopamine or acetylcholine release evoked by 55 mM K^+ or by carbachol. Rather, in many cases spontaneous and evoked releases were significantly increased under these conditions. The release observed in ATP-depleted cells was Ca^{2+} dependent. The data indicate that, unlike the situation with most other secretory cells, secretion from PC12 is independent of ATP. In PC12 some proteins involved in the secretion process may be altered so as to make the process independent of ATP; this may be due to the fact that PC12 is a tumor cell line. Phosphotyrosine-containing proteins, which are present in some other tumor cells, were not found in PC12.

It is well established that a rise in intracellular free Ca^{2+} triggers the secretion process that ensues upon stimulation of nerve terminals and a variety of other secretory cells (Rubin, 1970a). In recent years it has become apparent that ATP also is involved in this process. The requirement for ATP[1] in evoked secretion has been inferred from two types of experiments. Stimulated adrenal medulla, mast cells, and nerve terminals exhibit decreased secretion after treatment with metabolic inhibitors that deplete cellular stores of ATP (Beani et al., 1966; Kirshner & Smith, 1966; Rubin, 1970b; Nelson-Krause & Howard, 1978; Johansen, 1979). Chromaffin cells, whose plasma membranes have been made leaky to low molecular weight molecules, secrete catecholamines; however this secretion requires the presence of MgATP (in addition to Ca^{2+}) in the incubation buffer (Baker & Knight, 1979).

The ATP-requiring factors involved in secretion have not been identified; among suggested possibilities are contractile proteins (Berl et al., 1973) and protein kinases (Krueger et al., 1977; DeLorenzo & Freedman, 1977; Amy & Kirshner, 1981).

We have examined the effects of depletion of cellular ATP pools on the evoked release of dopamine and acetylcholine from PC12, a clonal line of rat pheochromocytoma (Greene & Tischler, 1976). Upon incubation with nerve growth factor PC12 cells extend neurites and acquire the appearance of neurons (Greene & Tischler, 1976). Both undifferentiated and nerve growth factor treated cells contain granular stores of dopamine and acetylcholine; upon exposure of the cells to depolarizing levels of K^+ or to nicotinic agonists such as carbachol, they secrete dopamine and acetylcholine in a Ca^{2+}-dependent fashion (Greene & Tischler, 1976; Greene & Rein, 1977a,b; Schubert & Klier, 1977; Rebois et al., 1980; Melega & Howard, 1981). In this paper we report that an intact store of ATP is not required for evoked secretion from PC12. We suggest that the ATP-independent step(s) of secretion may be circumvented in PC12 by virtue of their being tumor cells.

Materials and Methods

Chemicals. Antimycin A, carbachol, 2-deoxyglucose, iodoacetic acid, neostigmine methyl sulfate, oligomycin, ouabain, rotenone, sodium arsenate, and NaF were purchased from Sigma Chemical Co.; sodium azide was from Matheson Coleman and Bell, and KCN was from J. T. Baker. Nerve growth factor was purified from mouse submaxillary gland as described (Mobley et al., 1976). Phosphotyrosine was synthesized as described by Rothberg et al. (1978).

Cell Culture. The clonal rat pheochromocytoma line PC12 was obtained from Dr. D. Schubert. The cells were grown on plastic culture dishes or flasks at 37 °C under an atmosphere of 10% CO_2 and 90% air in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum and 10% horse serum. The cells were used for the various experiments 4–5 days after subculturing, just prior to becoming confluent.

Incubation of Cells in Defined Buffers. The cells were incubated at 37 °C while still attached to the plastic dishes or flasks in a cell incubation buffer consisting of 60 mM sucrose, 10 mM glucose, 130 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 25 mM Hepes, pH 7.3. Where indicated, the KCl concentration was raised to 55 mM to provide a "high-K^+" buffer to induce secretion from the cells. In this case the NaCl concentration was lowered correspondingly to maintain isoosmolarity. When glucose was omitted, sucrose was present at 70 mM.

For measurement of the K^+-induced release of acetylcholine and dopamine, the cell culture medium was aspirated from cell-containing flasks, and after the cells were washed twice with 2 mL of low K^+ incubation buffer per flask, they were preincubated in 2.5 mL of the buffer for variable times as indicated under Results. The buffer was replaced with 2.5 mL of fresh incubation buffer containing either 6 mM KCl or 55 mM KCl, and the cells were further incubated for the desired time. Test compounds were generally present during both the preincubation and incubation periods. When the experiments involved measurement of acetylcholine, the final

† From the Department of Biological Chemistry and the Brain Research Institute, School of Medicine, University of California, Los Angeles, California 90024. *Received March 16, 1982.* This work was supported by a grant from the National Institutes of Health (NS 12873).

‡ Present address: Department of Pharmacology, University of Pennsylvania, Philadelphia, PA 19104.

[1] Abbreviations: ATP, adenosine 5′-triphosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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incubation buffer contained 40 μM neostigmine. After incubation the buffer was collected and centrifuged at 750*g* for 5 min to pellet any cells dislodged during the incubation.

Determination of Dopamine. All samples for determination of dopamine were acidified to give a final concentration of 0.2 N $HClO_4$, 1 mM EGTA, and 5 mM dithiothreitol. Dopamine was measured by radioenzymatic assay or by high-performance liquid chromatography. In the radioenzymatic assay, the method of Saller & Zigmond (1977) was used for the incubation of sample, *S*-adenosyl[^{3}H]methionine, and catechol *O*-methyltransferase. The reaction was stopped and the reaction product, 3-methoxytyramine, was extracted as described by Peuler & Johnson (1977). 3-Methoxytyramine was purified by thin-layer chromatography and counted according to the method of Saller & Zigmond (1977), except that the thin-layer plates were scraped into scintillation vials containing 0.5 mL of 0.5 M sodium borate, pH 9.0. After vigorous shaking to elute the 3-methoxytyramine, the samples were mixed with 10 mL of 3a70B scintillation cocktail containing 2.5% bis(2-ethylhexyl) phosphate. Catechol *O*-methyltransferase was prepared from rat liver as described by Coyle & Henry (1973).

For the high-performance liquid chromatography, dopamine was purified on alumina, separated by liquid chromatography, and quantified by electrochemical detection (Keller et al., 1976; Kissinger, 1977; Hjemdahl et al., 1979).

Determination of Acetylcholine. Extraction and determination of acetylcholine by gas chromatography–mass spectrometry were as described by Freeman et al. (1975).

Measurement of Phospho Amino Acids. Cells were incubated for 21 h in polylysine-coated flasks in phosphate-free DMEM containing dialyzed calf serum and horse serum and [^{32}P]orthophosphate (carrier free; 0.4 mCi/mL). The cells were washed with phosphate-buffered saline and lysed in 60 mM Tris–maleate, pH 6.8, 0.3% sodium dodecyl sulfate, 0.3 mM N^{α}-(*p*-tosyl)-L-lysine chloromethyl ketone hydrochloride, and aprotinin at 100 kallikrein inhibitor units/mL. Phosphoproteins were extracted with phenol as described by Hunter & Sefton (1980), partially hydrolyzed by incubation for 2 h at 110 °C in 6 M HCl, and dried under reduced pressure to remove HCl. They were mixed with marker phosphoserine, phosphothreonine, and phosphotyrosine, and the phospho amino acids were separated on thin-layer plates by electrophoresis at pH 1.9 and at pH 3.5 as described by Hunter & Sefton (1980). The position of the marker phospho amino acids was determined with ninhydrin.

Determination of ATP and Protein. The cells were mixed with 0.2 N $HClO_4$, 1 mM EGTA, and 5 mM dithiothreitol and frozen at −20 °C. After thawing, the amount of ATP in the $HClO_4$ extract was determined by the firefly luciferin–luceferase assay of Strehler & Totter (1954), using a scintillation counter to measure emitted light (Stanley & Williams, 1962). Protein was measured by the method of Lowry et al. (1951).

Results

Inhibition of Energy Metabolism in PC12. Table I shows the effects of a 20-min treatment with various inhibitors of energy metabolism on the level of ATP in PC12 cells. Arsenate, NaF, iodoacetate, and deoxyglucose are inhibitors of glycolysis while KCN, rotenone, azide, antimycin A, and oligomycin are inhibitors of oxidative phosphorylation. ATP levels did not drop after incubation of cells in glucose-free buffer in the absence of an inhibitor. Exposure of the cells for 20 min to an inhibitor of glycolysis in glucose-free medium resulted in at most a 50% depletion of ATP stores. The inhibitors of oxidative phosphorylation also had little effect on the level of ATP when glycolysis was not inhibited.

Table I: Effect of Metabolic Inhibitors on the Cellular Level of ATP

treatment[a]	ATP level[b] with glucose	ATP level[b] without glucose
control	100 ± 3	99 ± 3
arsenate, 1 mM	N.D.	98 ± 17
NaF, 1 mM	N.D.	91 ± 2
iodoacetate, 0.2 mM	N.D.	57 ± 2
deoxyglucose, 5 mM	N.D.	50 ± 2
KCN, 0.5 mM	85 ± 5	66 ± 5
KCN, 0.5 mM, + deoxyglucose, 5 mM	N.D.	14 ± 1
rotenone, 1 μM	99 ± 1	33 ± 2
rotenone, 1 μM, + iodoacetate, 0.2 mM	N.D.	3 ± 0
rotenone, 1 μM, + deoxyglucose, 5 mM	N.D.	39 ± 3
azide, 0.5 mM	91 ± 3	38 ± 2
azide, 0.5 mM, + iodoacetate, 0.2 mM	N.D.	13 ± 1
azide, 0.5 mM, + deoxyglucose, 5 mM	N.D.	22 ± 1
antimycin A, 100 nM	93 ± 2	20 ± 3
antimycin A, 100 nM, + iodoacetate, 0.2 mM	N.D.	7 ± 1
antimycin A, 100 nM, + deoxyglucose, 5 mM	N.D.	10 ± 1
oligomycin, 0.2 μg/mL	92 ± 5	28 ± 3
oligomycin, 0.2 μg/mL, + deoxyglucose, 5 mM	N.D.	8 ± 2

[a] Cells were incubated for 20 min as described under Materials and Methods in buffer containing the indicated drugs and either 10 mM glucose or no glucose. The cells were washed twice with ice-cold buffer and harvested for measurement of ATP. [b] The results are expressed as the percentage of ATP in control cells (incubated in glucose-containing buffer), which had 24–26 nmol of ATP/mg of protein. The values are means ± range for duplicate incubations. N.D., not determined.

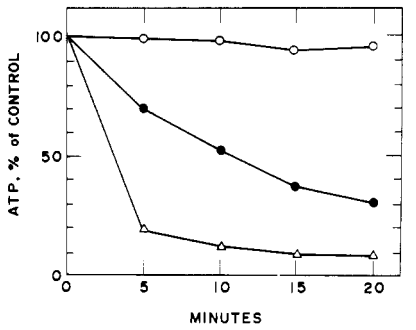

FIGURE 1: Effect of antimycin A and iodoacetate on cellular ATP. Cells were incubated as described under Materials and Methods in a buffer containing 100 nM antimycin A, and 10 mM glucose (○), 0.2 mM iodoacetate, and no glucose (●), or 100 nM antimycin A, 0.2 mM iodoacetate, and no glucose (△). After the incubation the cells were washed twice with ice-cold buffer and harvested for the measurement of ATP. The results are expressed as the percent of ATP in control cells (incubated in glucose-containing buffer), which had 27.3 ± 2 nmol of ATP/mg of protein.

To achieve a substantial depletion of ATP after a brief treatment, it was necessary to inhibit both glycolysis and oxidative phosphorylation. When cells were treated with both types of inhibitors in glucose-free medium, the ATP level dropped to less than 40% of the control value and with some treatments to less than 10% of the control value.

Figure 1 shows the time course of the effect of antimycin A and iodoacetate on ATP levels. A drop in ATP to less than 20% of control values could be observed with a 5-min treatment.

In subsequent studies we chose to deplete ATP stores by treatment with antimycin A in glucose-free buffer or by

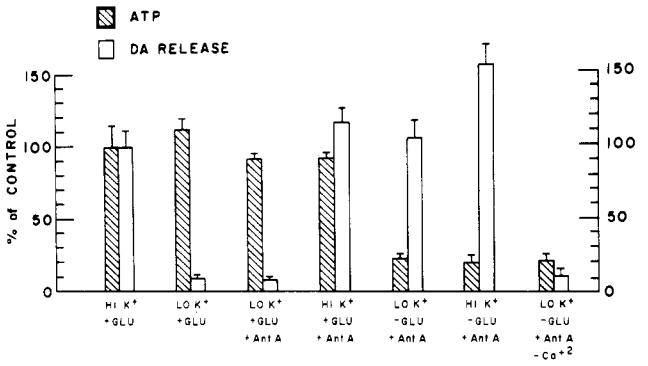

FIGURE 2: Effect of antimycin A on dopamine release. Cells were incubated for 5 min in low-K^+ buffer (LO K^+) with or without glucose (GLU) and 100 nM antimycin A (Ant A), washed, and incubated for an additional 5 min in the same buffer except that in some cases the K^+ concentration was 55 mM (HI K^+). As indicated, in one case Ca^{2+} was omitted from the buffer. The results are expressed as the percentage of ATP and dopamine release for control cells incubated in HI K^+, GLU-containing buffer during the second 5-min period. Control cells contained 22–26 nmol of ATP/mg of protein and released 28–34% of their dopamine upon exposure to the HI K^+ buffer. The values are means for triplicate incubations and the error bars represent the standard deviation: (hatched bars) ATP; (open bars) dopamine release.

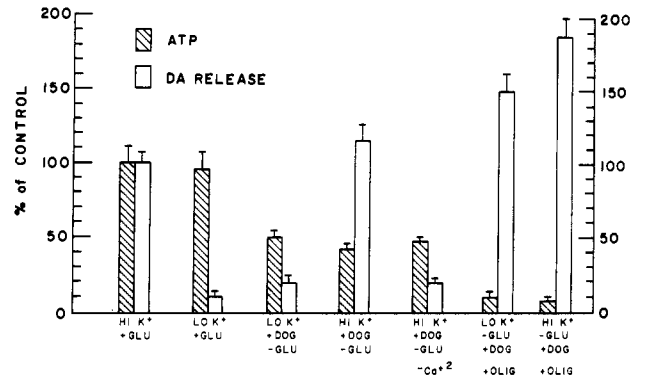

FIGURE 3: Effect of deoxyglucose and oligomycin on dopamine release. Cells were incubated for 10 min in low-K^+ buffer (LO K^+) with or without glucose (GLU), 5 mM deoxyglucose (DOG), and 0.2 μg/mL oligomycin (OLIG), washed, and incubated for an additional 5 min in the same buffer except that in some cases the K^+ concentration was 55 mM (HI K^+). As indicated, in one case Ca^{2+} was omitted from the buffer. The results are expressed as the percentage of ATP and dopamine release for control cells incubated in HI K^+, GLU-containing buffer during the second 5-min period. Control cells contained 17–24 nmol of ATP/mg of protein and released 27–34% of their dopamine upon exposure to the HI K^+ buffer. The values are means for triplicate incubations and the error bars represent the standard deviation: (hatched bars) ATP; (open bars) dopamine release.

treatment with a combination of oligomycin and deoxyglucose in glucose-free medium. Under the conditions described in Table I, these treatments did not irreversibly damage the cells. The treated cells were able to exclude the vital stain trypan blue, and they multiplied normally after washing to remove the inhibitors and reincubation in normal growth medium.

Secretion from ATP-Depleted PC12 Cells. Figures 2–4 show the effect of depleting ATP stores on the spontaneous and evoked release of dopamine and acetylcholine from PC12 cells. In each of these figures the levels of ATP and release are expressed as a percentage of those obtained when control cells were preincubated in buffer containing glucose and 6 mM K^+ and then exposed to the same buffer but containing 55 mM K^+ to evoke release of dopamine and acetylcholine. When cells were treated with antimycin A in the presence of glucose, there was little change in cellular levels of ATP or in the amount

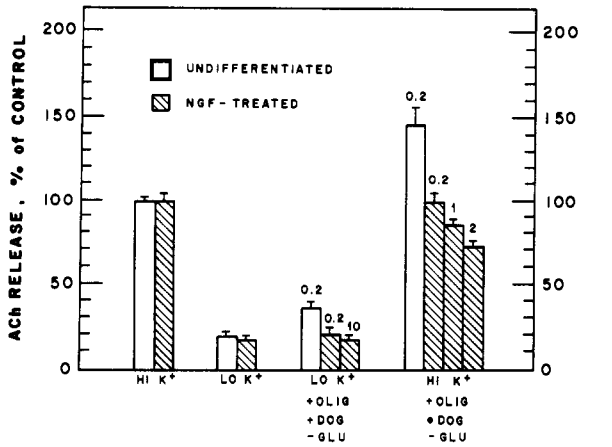

FIGURE 4: Effect of deoxyglucose and oligomycin on acetylcholine release. Cells were incubated for 10 min in low-K^+ buffer (LO K^+) with or without glucose (GLU), 10 mM deoxyglucose (DOG), and 0.2–10 μg/mL oligomycin (OLIG), washed, and incubated for an additional 5 min in the same buffer except that in some cases the K^+ concentration was 55 mM (HI K^+). The results are expressed as the percentage of acetylcholine release for control cells incubated in HI K^+, GLU-containing buffer during the second 5-min period. Control undifferentiated cells secreted 7% of their acetylcholine upon exposure to the HI K^+ buffer. Open bars indicate acetylcholine release from undifferentiated cells. The experiment was repeated with cells that had been induced to extend neurites by treatment with nerve growth factor for 14 days. Acetylcholine release from such cells is indicated by hatched bars. The numbers above the bars indicate the concentration of oligomycin (μg/mL) in the incubation buffer. Control nerve growth factor treated cells released 9% of their acetylcholine upon exposure to the HI K^+ buffer. The values are means for duplicate incubations and the error bars represent the range. The ATP level in undifferentiated cells exposed to deoxyglucose and 1 μg/mL oligomycin was 9% of the control level; the ATP level in nerve growth factor treated cells exposed to deoxyglucose and 1 μg/mL oligomycin was 34% of the control level.

of K^+-evoked or spontaneous release of dopamine (Figure 2). However, treatment with antimycin A in glucose-free buffer caused a marked depletion of cellular ATP; under this condition the spontaneous release of dopamine increased to a level that was comparable to that evoked by 55 mM K^+ in control cells. When Ca^{2+} was omitted from the incubation buffer, the spontaneous release of dopamine from treated cells was no greater than the spontaneous release from control cells. Therefore, the abnormally increased spontaneous release of dopamine from treated cells is Ca^{2+} dependent.

Figure 2 also shows that antimycin A induced depletion of ATP stores causes an increase (relative to untreated cells) in the amount of dopamine released after exposure to 55 mM K^+. The antimycin A treatment did not alter the total level of dopamine (total of dopamine in the cell and medium at the end of the incubation).

Figure 3 shows how treatment with deoxyglucose or a combination of deoxyglucose and oligomycin affected dopamine release. Deoxyglucose-induced reduction of ATP stores to less than half of control levels resulted in no reduction in the K^+-evoked release of dopamine; the K^+-evoked release under this condition was Ca^{2+} dependent as is release from control cells. When ATP was depleted to a greater extend by treatment with a combination of deoxyglucose and oligomycin, both the spontaneous release and the K^+-evoked release of dopamine were well above control values. As shown in Table II, dopamine release from ATP-depleted cells can also be evoked by carbachol.

We have also examined the effect of depleting ATP stores on the release of acetylcholine from PC12 cells. The results are presented in Figure 4. For this study cells were treated

Table II: Effect of Iodoacetate on Carbachol-Induced Release of Dopamine

treatment[a]	ATP[b]	dopamine release[c]
control	26 ± 1	1.0 ± 0.3
carbachol	28 ± 3	11.4 ± 0.3
iodoacetate	19 ± 1	1.2 ± 0.2
iodoacetate + carbachol	11 ± 1	18.2 ± 1.1

[a] Cells were preincubated for 10 min in the presence or absence of 0.2 mM iodoacetate, washed, and incubated for an additional 5 min in the same buffer except that 1 mM carbachol was present as indicated. Glucose was omitted from iodoacetate-containing buffers. [b] Nanomoles per milligram of protein. [c] Dopamine release is expressed as the percentage of total dopamine in the cells and medium at the end of the incubation period. Values are means ± standard deviation for triplicate incubations.

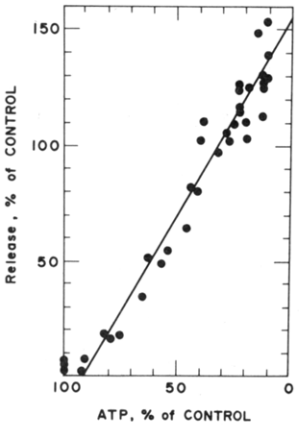

FIGURE 5: Relationship between cellular ATP and spontaneous release of dopamine. Cells were depleted of their ATP to the indicated extent by treatment with the various inhibitors listed in Table I. The spontaneous release (release in low-K^+ buffer) is expressed as the percentage of release evoked by 55 mM K^+ for control cells not treated with an inhibitor. The values, which are compiled from other experiments described in this paper and from some experiments not otherwise reported, are the means for two to three incubations.

with deoxyglucose and varying concentrations of oligomycin. This combination of inhibitors did not decrease acetylcholine release from undifferentiated PC12 cells; in fact there was an increase in both the spontaneous and K^+-evoked releases of acetylcholine from these cells. We obtained somewhat different results with PC12 cells that had been induced to differentiate into neuronlike cells by treatment with nerve growth factor. When the oligomycin concentration was 1 or 2 μg/mL, there was a slight inhibition of the K^+-evoked release of acetylcholine from the differentiated cells. The spontaneous and K^+-evoked releases were not affected when oligomycin was at 0.2 μg/mL. Oligomycin at 1 μg/mL caused the level of ATP in nerve growth factor treated cells to fall to 34% of the control value. We did not measure ATP levels after treatment with the other concentrations of oligomycin.

Spontaneous Release from ATP-Depleted Cells. An analysis of the spontaneous release of dopamine from PC12 cells depleted of ATP by various means shows that the amount of spontaneous release varied inversely with the ATP level (Figure 5). The increase in spontaneous release does not appear to be due to depolarization of the cells secondary to inactivation of the Na^+ pump. Treatment of the cells for 10 min with 1 mM ouabain, an inhibitor of the (Na^+/K^+)-ATPase, produced only a doubling of the amount of spontaneous release of dopamine, far less than that observed when ATP levels were less than 50% of control. More likely the increased spontaneous (and evoked) release of neurotransmitter from ATP-depleted cells is due to a decreased ability to extrude or sequester Ca^{2+} from the cytosol of such cells. We have not fully characterized Ca^{2+} metabolism in ATP-depleted cells, but we have found that after treatment for 5 min with oligomycin in glucose-free buffer and then exposure to 55 mM K^+, the cells took up from the medium less than half of the $^{45}Ca^{2+}$ taken up by control cells. A similar phenomenon was observed and more extensively studied in hybrid neuroblastoma–glioma cells and attributed to a decreased sequestration of Ca^{2+} by organelles of the ATP-depleted cells (Kürzinger et al., 1980).

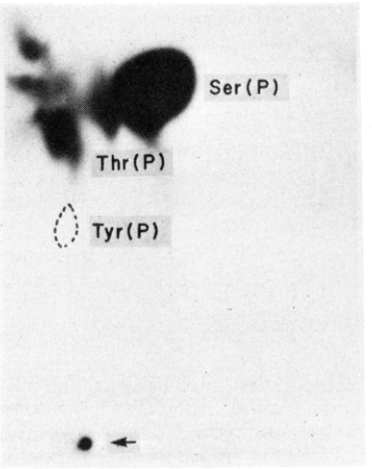

FIGURE 6: Autoradiogram of phospho amino acids of PC12 proteins. As described under Materials and Methods, cell proteins were labeled with [^{32}P]orthophosphate, extracted, partially hydrolyzed, and separated on thin-layer plates by two-dimensional electrophoresis. A total of 980000 cpm was applied; the film was exposed for 96 h without an intensifying screen. The origin (arrow) and the position of the marker phospho amino acids are indicated.

Phospho Amino Acids of PC12 Proteins. The fact that evoked release of dopamine or acetylcholine from PC12 cells is not inhibited when ATP levels are reduced to a low level suggested that in PC12 some protein involved in the release process may be altered so as to make the process independent of ATP. Because PC12 is a tumor cell, one possibility appealing to us was that this protein might be phosphorylated at tyrosine residues. It is now known that, as a consequence of an unusual protein kinase, many tumor cells contain proteins with phosphotyrosine residues whereas phosphorylated residues of most other proteins are serine and threonine (Collett & Erickson, 1978; Hunter & Sefton, 1980). To determine whether PC12 cells contain similarly modified proteins, we grew the cells in [^{32}P]orthophosphate and looked for [^{32}P]-phosphotyrosine in a partial hydrolysate of a protein extract. Phosphotyrosine could not be detected in these protein extracts under the conditions used (Figure 6).

Discussion

PC12 apparently can produce sufficient ATP for its needs either by glycolysis or by oxidative phosphorylation. Thus, to obtain substantial depletion of ATP in a brief period of time, it is necessary to inhibit both glycolysis and oxidative phosphorylation in PC12. In this regard PC12 resembles normal adrenal medullary cells where it is also necessary to inhibit both pathways to observe a decrease in ATP levels (Kirshner & Smith, 1966).

PC12 differs from other well-characterized secretory systems (including the adrenal medulla) in that secretion from PC12 does not require intact stores of ATP. This could reflect a difference in the size of the various cellular pools of ATP. In adrenal medullary cells much of the ATP is sequestered in the granules that store catecholamines. By comparison, PC12 cells

contain fewer storage granules (Rebois et al., 1980) and therefore may have a cytoplasmic ATP store that is larger than that of adrenal medullary and other secretory cells; if the PC12 cytoplasmic pool of ATP were 5–10 times larger, drug-treated PC12 cells could be depleted to 10% of control ATP levels and still have a cytoplasmic ATP pool comparable to that of other secretory cells. Such a situation seems unlikely although the size of the cytoplasmic pool of ATP in most secretory cells, including PC12, is not known.

The following explanations of the apparent ATP independence of secretion from PC12 should also be considered: (1) The K_m for ATP of the ATP-requiring release factor in PC12 may be so low that this factor binds ATP normally even when ATP levels in treated cells are 10% of control levels. (2) The ATP-requiring release factor may be present in a PC12 cell compartment, in which the ATP level was unaffected by the metabolic inhibitors and which is too small to affect changes in total cell ATP levels. (3) Evoked release may be ATP independent in PC12 cells because the ATP-requiring steps(s) may have already been accomplished or this step may be circumvented in PC12 cells. Each of these explanations implies that the ATP-requiring release factor in PC12 cells differs from that of other secretory systems. However, only the third explanation seems reasonable, and indeed, recent studies of tumor cells make this explanation very appealing. It is now known that many tumor cells differ from normal cells in the modification of certain proteins. In PC12 cells, a protein that is modified as a consequence of the cells being tumor cells could make the secretion process ATP independent. For example, a protein that is activated by Ca^{2+}-dependent phosphorylation during the release process in nontumor cells (Krueger et al., 1977; DeLorenzo & Freedman, 1977; Amy & Kirshner, 1981) may be activated by virtue of some unusual modification in PC12. According to this scheme, the ATP-independent release in PC12 is still Ca^{2+} dependent because Ca^{2+} is also required for other steps in the secretion process. It will be of interest to determine whether during secretion from PC12 cells there occurs phosphorylation of proteins similar to those phosphorylated in nontumor secretory cells (Kruger et al., 1977; DeLorenzo & Freedman, 1977; Amy & Kirshner, 1981). Such studies are in progress.

The one type of protein modification that has been identified in some tumor cells is phosphorylation of tyrosine residues (Collett & Erikson, 1978; Hunter & Sefton, 1980). We have not found phosphotyrosine in proteins from PC12 cells (Figure 6), but this modification may occur in PC12 at a level below that detectable by our procedure. Furthermore, another type of protein modification might be involved.

Acetylcholine release from nerve growth factor treated PC12 cells may be more dependent on ATP than is release from undifferentiated PC12. Certain conditions of treatment with deoxyglucose and oligomycin resulted in a slight (13–27%) inhibition of K^+-evoked release of acetylcholine from the differentiated cells. Therefore, the protein modification that we have postulated to account for the ATP independence of release in undifferentiated PC12 cells may be present to a lesser extent at release sites in differentiated cells.

Acknowledgments

We thank Karen Jones for her assistance in the analysis of phospho amino acids from PC12.

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