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Choline and Acetylcholine Metabolism in PC12 Secretory Cells†

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ABSTRACT: PC12, a clonal line of rat pheochromocytoma, synthesizes, stores, and secretes dopamine and acetylcholine. The cells take up choline by a saturable process and rapidly convert the accumulated choline to acetylcholine. This choline transport has a K_m of 12 μM, is Na^+ and energy independent, and is relatively insensitive to hemicholinium-3 ($IC_{50} \sim 50$ μM). Different ionic conditions can modulate the choline transport. Uptake was increased by pretreatment with 55 mM K^+ whereas it was decreased in the presence of 55 mM K^+. Choline uptake had similar characteristics in PC12 cells that had been induced to extend neurites by treatment with nerve growth factor. In undifferentiated PC12 cells, storage of newly synthesized acetylcholine was found in bound and free compartments as evidenced from subcellular fractionation. The free pool had a faster turnover rate. Most of the newly synthesized acetylcholine was rapidly degraded in the absence of a cholinesterase inhibitor while continuous incubation with labeled choline resulted in a slow incorporation of newly labeled acetylcholine into a bound pool. The accumulation of acetylcholine in the bound pool, but not acetylcholine synthesis, was inhibited by each of several agents that are known to interfere with the generation or maintenance of proton electrochemical gradients. The newly synthesized acetylcholine could be released from PC12 cells by incubation of the cells with 55 mM K^+. These properties indicate that PC12 cells are a good system for studying acetylcholine metabolism by secretory cells.

Studies on a variety of nervous tissues have revealed several important characteristics of the processes by which acetylcholine is stored and secreted by neurons. However, little has been learned about the molecular mechanisms involved in these processes. Progress has been impeded by technical problems inherent to the experimental systems (e.g., vertebrate neuromuscular junction, mammalian brain, mammalian autonomic ganglion, electric organ of rays) commonly used for biochemical studies of acetylcholine metabolism.

To overcome these technical problems, we have begun to study acetylcholine storage and secretion with another system that allows us to exploit the experimental advantages of cell culture. This system is PC12, a clonal line of rat pheochromocytoma (Greene & Tischler, 1976). PC12 cells synthesize acetylcholine and catecholamines (primarily dopamine), store each in separate granules, and secrete each by a Ca^{2+}-dependent process (Greene & Tischler, 1976; Greene & Rein, 1977a,b; Schubert & Klier, 1977; Rebois et al., 1980).

In nerve terminals, acetylcholine is synthesized to a great extent from choline that is transported into the terminals by a carrier-mediated process (Kuhar & Murrin, 1978; Jope, 1979), and a sizeable fraction of the newly synthesized acetylcholine is loaded into synaptic vesicles (Zimmermann & Denston, 1977). Upon depolarization of the nerve terminals, the acetylcholine is released from the terminals by a Ca^{2+}-dependent process; in several systems, there is preferential release of newly synthesized acetylcholine (Collier, 1969; Potter, 1970; Richter & Marchbanks, 1971; Dunant et al., 1972; Molenaar et al., 1973).

Here, we show that choline and acetylcholine metabolism in PC12 is similar to but not identical with that previously characterized in cholinergic neurons. Our results indicate that PC12 cells will have much utility for studying certain aspects of acetylcholine metabolism by secretory cells.

Materials and Methods

Chemicals. Bromopyruvate, eserine sulfate, iodoacetic acid, neostigmine bromide, oligomycin, ouabain, sodium fluoride, and valinomycin were obtained from Sigma Chemical Co. Sodium azide was from Matheson Coleman and Bell; hemicholinium-3 and *N,N'*-dicyclohexylcarbodiimide (DCCD)[1]

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[1] Abbreviations used: BETA, (2-benzoylethyl)trimethylammonium; DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; S-13, 2',5-dichloro-*N-tert*-butyl-4-nitrosalicylanilide.

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were from Eastman. A23187 was from Calbiochem-Behring, and paraoxon was from ICN. [^{3}H]Choline chloride (84 Ci/mmol) was obtained from New England Nuclear. The stable isotopic variants of acetylcholine and choline, which were prepared by the method of Jenden et al. (1973), and BETA were gifts of Dr. Donald Jenden. The following were also obtained as gifts: FCCP from Du Pont, nigericin from Hoffmann-La Roche, and S-13 from Monsanto Chemical Co. Nerve growth factor was purified from mouse submaxillary glands as described (Mobley et al., 1976).

Culture and Incubation of PC12 Cells in Defined Buffers. PC12 cells were cultured on plastic Petri dishes (100 × 20 mm) or flasks (25 cm^2) as described by Rebois et al. (1980). The growth medium was changed 24 h prior to each experiment. The cells were incubated at 37 °C while still attached to 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 $MgSO_4$, 1.2 mM KH_2PO_4, and 25 mM Hepes, pH 7.4. Where indicated, the KCl concentration was raised to 55 mM to provide a "high-K^+" buffer for depolarization of the cells. In these cases, the NaCl concentration was correspondingly lowered to maintain isoosmolarity. The cell density was such that each dish or flask contained, respectively, approximately 1.2 or 0.4 mg of protein. In cases where the cells were incubated with a drug, the drug treatment was usually found to cause no morphological changes detectable by phase-contrast microscopy. The exceptions are listed under Results.

Uptake of [^{3}H]Choline. The cells were washed twice with 2 mL per flask of the buffer and then preincubated with buffer for 5 min at 37 °C. The buffer was removed and replaced with 4 mL of fresh buffer containing 1 μCi of [^{3}H]choline and various concentrations of unlabeled choline. After an additional incubation for the indicated times, the cells were washed 4 times with 5 mL of ice-cold incubation buffer containing 10 mM choline. The cells were dissolved in 0.1 N NaOH and samples added to 10 mL of 3a70B liquid scintillation cocktail (Research Products International) for counting of the radioactivity by liquid scintillation spectrometry. Uptake blanks, which were determined by incubation of the cells with [^{3}H]-choline at 4 °C, were usually about 4% of the total taken up at 37 °C. In all cases, the amount of radioactivity associated with the cells at 4 °C was subtracted from the radioactivity associated with the cells at 37 °C to give a value for accumulated choline. Where indicated, acetyl[^{3}H]choline was separated from [^{3}H]choline by thin-layer chromatography as described (Rebois et al., 1980).

Uptake and Metabolism of [$^2H_4$]Choline. The cells were washed twice with 5 mL per dish of cell incubation buffer and preincubated in 10 mL of the buffer for 10 min. The buffer was removed and replaced with 10 mL of fresh buffer containing various concentrations of [$^2H_4$]choline and further incubated for the indicated times. In some cases, 40 μM eserine was also present in the incubation buffer. After the incubation, the cells were washed twice with 10 mL per dish of ice-cold buffer and scraped from the dish with a rubber policeman into 2.4 mL of ice-cold 0.32 M sucrose and 10 mM Hepes, pH 7.3, and a sample of the cell suspension was added to 3 mL of 1 N formic acid/acetone (15/85) for determination of total cell acetylcholine. The remaining cell suspension was homogenized, and a granule fraction was obtained for the determination of the level of acetylcholine in granules. Acetyl[$^2H_4$]choline and acetylcholine were measured by combined gas chromatography–mass spectrometry as described by Freeman et al. (1975). Acetyl[$^2H_9$]choline and [$^2H_9$]choline were used as internal standards.

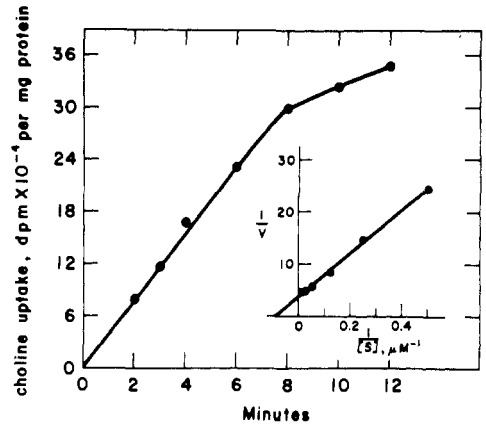

FIGURE 1: Time course of the accumulation of choline by PC12 cells. The cells were incubated with 2 μM [^{3}H]choline for the indicated times as described under Materials and Methods. The values are expressed as disintegrations per minute per milligram of cell protein. Each data point represents the mean for duplicate incubations. Inset: Double-reciprocal plot of the uptake of choline into PC12 cells. The amount of [^{3}H]choline per incubated flask was constant while the concentration of unlabeled choline was varied as indicated. The incubation was for 4 min. Velocity (V) is expressed as picomoles of choline accumulated per minute per milligram of cell protein. The data points represent the means for four incubations.

Isolation of Storage Granules. A cell homogenate was obtained by 15 up and down passes of a tight-fitting pestle in a Dounce homogenizer. The homogenate was centrifuged at 1000g for 10 min at 4 °C. The supernatant suspension was centrifuged at 20000g for 30 min. Almost all of the acetylcholine in the resulting pellet (P_2 fraction) was found to band in a sucrose-density gradient at regions determined by Schubert & Klier (1977) to be enriched in granules and transmitter. Therefore, we have assumed that the bound acetylcholine in the P_2 fraction is in storage granules, and the P_2 fraction was used without further purification for the determination of acetylcholine content of isolated granules. The amount of protein in the P_2 fraction was approximately 20% of that in whole cell homogenates.

Protein. Protein was determined by the method of Lowry et al. (1951).

Results

Choline Uptake. Figure 1 shows that when PC12 cells were incubated with [^{3}H]choline, the amount of radioactivity accumulated by the cells increased linearly with time for approximately 8 min. After a 10-min incubation with 0.5 μCi of [^{3}H]choline (concentration of added choline 0.01 μM), 30% of the accumulated radioactivity was associated with choline and 20% with acetylcholine. The K_m for choline transport was 12 μM, and the V_{max} was 270 pmol min^{-1} (mg of cell protein)$^{-1}$ as determined from a double-reciprocal plot (inset to Figure 1).

There was a sizeable efflux of choline from the PC12 cells while the cells were in the incubation (Figure 2). However, after a 4-min incubation of PC12 cells in 5 mL of the buffer, the concentration of extracellular choline reached only 0.13 μM. Thus, the efflux of choline was not sufficient to alter the K_m value determined in Figure 1.

Table I gives the effects of various conditions of incubation on the uptake of choline by PC12 cells. Choline uptake was temperature dependent, Na^+ independent, and only slightly inhibited by 0.1 mM ouabain. Hemicholinium-3, a very potent inhibitor of high-affinity choline uptake into mammalian brain synaptosomes, was not an effective inhibitor of choline uptake

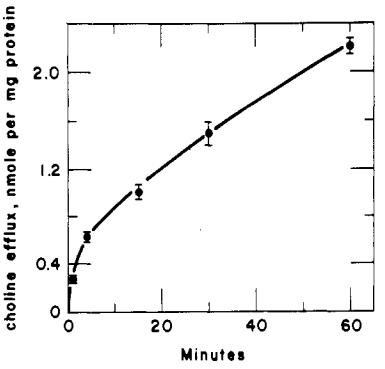

FIGURE 2: Time course of choline efflux from PC12 cells. The cells were washed and incubated in the choline-free incubation buffer described under Materials and Methods. At the indicated times, the buffer was removed, and the level of choline in the buffer was determined. The values are expressed as nanomoles of choline per milligram of cell protein. The data points represent the means for four incubations, and the error bars indicate the standard error (SE).

Table I: Effect of Various Agents on Choline Accumulation by PC12 Cells

incubation condition[a]	choline accumulation[b]
control	100 ± 6 (4)
0 °C	4 ± 1 (4)
omit Na^+	145 ± 14 (4)
ouabain, 0.1 mM	84 ± 3 (2)
hemicholinium-3, 5 μM	103 ± 2 (2)
hemicholinium-3, 50 μM	52 ± 2 (4)
iodoacetate, 1 mM	109 ± 3 (4)
oligomycin, 4 μg/mL	123 ± 1 (2)
azide, 3 mM	105 ± 12 (2)
iodoacetate, 1 mM, + azide, 3 mM	266 ± 30 (6)
iodoacetate, 1 mM, + azide, 3 mM, 4 °C	5 ± 2 (2)
iodoacetate, 1 mM, + oligomycin, 4 μg/mL	134 ± 8 (2)

[a] The cells were preincubated for 10 min in incubation buffer modified as indicated and subsequently incubated for 4 min with 5 μM [^{3}H]choline (0.25 μCi/mL). The cells were washed and harvested, and the accumulated radioactivity was counted by scintillation spectrometry. When sodium was omitted from the buffer, the sucrose concentration was correspondingly increased to maintain the osmolarity. [b] The results, which are expressed as the percent of choline accumulated by control cells, are the means ± SE for the number of incubations given in parentheses. Control cells accumulated 107 ± 6 pmol of choline min^{-1} (mg of protein)$^{-1}$.

by PC12 cells. The IC_{50} of hemicholinium-3 was approximately 50 μM.

Table I also shows that choline uptake into PC12 cells was not blocked by iodoacetate, oligomycin, or sodium azide, three inhibitors of energy metabolism. One of these inhibitors, oligomycin, caused a slight increase in choline uptake. Under the conditions employed for Table I, only iodoacetate, which inhibits glycolysis, decreased cellular pools of ATP (20% of control levels). Oligomycin and sodium azide, which inhibit oxidative phosphorylation, did not alter ATP stores in PC12 because the cells derive a major portion of their ATP from glycolysis rather than mostly from oxidative phosphorylation (E. Reynolds, unpublished experiments). An unexpected effect was obtained by treatment of the cells with iodoacetate and sodium azide simultaneously. Under this condition, choline uptake increased approximately 2.5-fold. This marked increase in choline uptake did not occur when the cells were treated with these agents at 4 °C or with a combination of iodoacetate and oligomycin.

Table II: Choline Accumulation by PC12 Cells as a Function of K^+ Concentration in the Incubation Buffer[a]

K^+ concn (mM)		
preincubation	incubation	choline accumulation[b]
6	6	118 ± 8
55	6	142 ± 3
55	55	92 ± 2

[a] The cells were preincubated for 10 min in incubation buffer and then for 5 min in buffer containing 5 μM [^{3}H]choline (0.25 μCi/mL). The K^+ concentrations of the buffers are listed. After the 5-min incubation, the cells were washed and harvested, and the radioactivity was counted. [b] The values [pmol min^{-1} (mg of protein)$^{-1}$] are means ± SE for four to six incubations. The differences between the first and second values and between the second and third values are significant at $P < 0.02$ and $P < 0.001$, respectively, as determined by Student's t test.

We repeated these experiments using [$^2H_4$]choline to label PC12 stores of choline and acetylcholine; under such conditions, choline and acetylcholine levels were measured by the gas chromatography-mass spectrometry technique of Freeman et al. (1975). This procedure allows the simultaneous determination of endogenous choline (unlabeled choline), newly accumulated choline ([$^2H_4$]choline), newly synthesized acetylcholine (acetyl[$^2H_4$]choline), and unlabeled acetylcholine, which at least in part reflects older acetylcholine stores. When the cells were treated with iodoacetate plus sodium azide for 10 min and then incubated for an additional 10 min with 10 μM [$^2H_4$]choline, the results were as follows: compared to the situation with control cells, the cellular level of choline and [$^2H_4$]choline was increased 12-fold and 18-fold, respectively. Acetylcholine and acetyl[$^2H_4$]choline levels were the same as those in control cells. These results are consistent with those obtained with [^{3}H]choline. We have yet to determine the mechanism of this effect of iodoacetate plus azide on choline levels; however, it appears that membrane properties in general were not altered because the uptake of deoxy[^{3}H]glucose by the cells was not increased by such treatment (data not presented).

Simon et al. (1976) showed that choline uptake into nerve terminals of mammalian brain was increased when the terminals had been depolarized prior to the period of choline uptake. A similar phenomenon occurs in PC12 cells as is shown in Table II. Cells that were exposed for 10 min to a depolarizing level of K^+ (55 mM) and then returned to the normal low-K^+ buffer prior to the addition of [^{3}H]choline took up significantly more label than did control cells that were never exposed to the high-K^+ buffer. However, the presence of 55 mM K^+ during the incubation with labeled choline inhibited the uptake of choline.

We have characterized choline uptake in PC12 cells that had been induced to extend neurites by a 14-day treatment with nerve growth factor as described by Greene & Tischler (1976). Under these conditions, there was no change in the K_m or V_{max} of choline transport. The uptake of choline was Na^+ independent and insensitive to hemicholinium-3.

Acetylcholine Synthesis. For all of the subsequent experiments, [$^2H_4$]choline was used to label acetylcholine stores. As shown in Figure 3, the specific activity of the acetylcholine found in the cells after a 30-min incubation with [$^2H_4$]choline varied with the concentration of [$^2H_4$]choline in the incubation medium. The specific activity is the mole fraction acetyl-[$^2H_4$]choline/(acetyl[$^2H_4$]choline + [$^2H_0$]acetylcholine). Approximately 30% of the cells' acetylcholine was labeled as acetyl[$^2H_4$]choline after a 30-min incubation with 10 μM [$^2H_4$]choline; when the concentration of extracellular [$^2H_4$]-

Table III: Effects of Ionophores and DCCD on the Cellular and Granule Fraction Levels of Acetylcholine[a]

	levels[b]			
	total AcCh		granular AcCh	
treatment	AcCh	[$^2H_4$]AcCh	AcCh	[$^2H_4$]AcCh
control	100 ± 10 (12)	100 ± 8 (12)	100 ± 7 (14)	100 ± 12 (14)
DCCD, 1 μM	95 ± 8 (6)	95 ± 9 (6)	92 ± 9 (6)	47 ± 4 (6)
FCCP, 0.5 μM	91 ± 8 (4)	74 ± 5 (4)	83 ± 6 (6)	30 ± 7 (6)
S-13, 0.1 μM	87 ± 7 (4)	73 ± 3 (4)	87 ± 10 (8)	43 ± 12 (8)
nigericin, 0.01 μg/mL	81 ± 2 (4)	81 ± 5 (4)	74 ± 3 (2)	35 ± 4 (2)
valinomycin, 3 μg/mL	ND	ND	83 ± 6 (2)	100 ± 2 (2)
A23187, 1 μg/mL	75 ± 4 (4)	56 ± 9 (4)	100 ± 2 (4)	64 ± 10 (4)

[a] The cells were washed and incubated as described under Materials and Methods for 30 min with 10 mL of buffer containing the indicated drugs per plate. The cells were harvested, an aliquot was removed for determination of cell levels of acetylcholine, and a granule fraction was prepared from the cell homogenate. [b] The results, which are expressed as the percentage of control values, are means ± SE for the number of incubations given in parentheses. Control cells contained, per mg of protein, 1.1 ± 0.13 nmol of acetylcholine and 0.32 ± 0.03 nmol of acetyl[$^2H_4$]choline; control granules contained, per mg of protein, 0.96 ± 0.08 nmol of acetylcholine and 0.09 ± 0.012 nmol of acetyl[$^2H_4$]choline. Abbreviations used: AcCh, acetylcholine; ND, not determined.

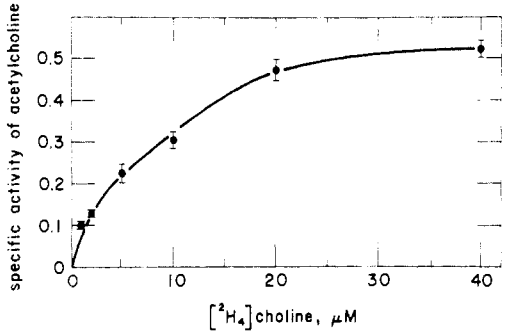

FIGURE 3: Specific activity of acetyl[$^2H_4$]choline in PC12 cells after incubation with [$^2H_4$]choline. The cells were incubated with varying concentrations of carrier-free [$^2H_4$]choline for 30 min, and then the levels of acetyl[$^2H_4$]choline and acetylcholine in the cells were determined. The specific activity is the mole fraction of acetyl[$^2H_4$]-choline/total acetylcholine. The data points represent the means for four incubations, and the error bars indicate the SE.

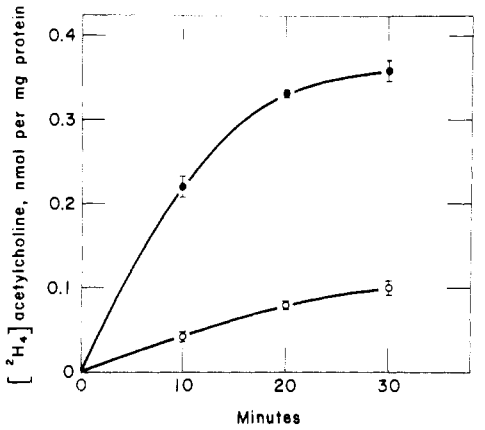

FIGURE 4: Time course of acetylcholine synthesis and accumulation by granules. The cells were incubated for varying times with 10 μM [$^2H_4$]choline as described under Materials and Methods. After the incubation, acetyl[$^2H_4$]choline was measured in samples of whole cells (●) and a granule fraction (○) obtained from a cell homogenate. The results are expressed as nanomoles of acetyl[$^2H_4$]choline per milligram of cell protein or granule fraction protein, respectively. The data points represent the means for six incubations, and the error bars indicate the SE.

choline was 20–40 μM, approximately half of the cells' acetylcholine was labeled.

Time Course of Loading of Acetylcholine into Storage Granules. Previous studies have shown that approximately 35% of the acetylcholine in PC12 cells is present in a bound form that is resistant to cholinesterase and sediments in the mitochondrial (P_2) fraction of cell homogenates (Rebois et al., 1980). Almost all of the remaining acetylcholine in cell homogenates is found in the supernatant after high-speed centrifugation. The bound acetylcholine is presumably in storage granules because most of the acetylcholine in the P_2 fraction was found to band in a sucrose-density gradient at the same region determined by Schubert & Klier (1977) to be enriched in granules. Acetylcholine-containing granules have a buoyant density different from that of the catecholamine-containing granules present in PC12 cells.

Figure 4 describes the time course of labeling of total cell acetylcholine and acetylcholine in the granule fraction of cell homogenates when the cells were incubated with [$^2H_4$]choline. Acetyl[$^2H_4$]choline appeared soon after the cells were exposed to [$^2H_4$]choline, and the level of total cell acetyl[$^2H_4$]choline reached a plateau within 30 min. The loading of acetyl-[$^2H_4$]choline into granules occurred more slowly; the level of granule acetyl[^2H]choline increased linearly with time for up to 120 min (data not shown). The levels of total cell acetyl[$^2H_4$]choline and granule acetyl[$^2H_4$]choline were each increased approximately 1.5–2-fold at each time point when the incubation buffer also contained the cholinesterase inhibitor eserine at a concentration of 40 μM (data not shown).

Inhibition of the Loading of Acetylcholine into Storage Granules. The bioenergetic mechanism by which acetylcholine is transported into storage vesicles is not understood. Toll & Howard (1980) proposed that an ATPase and a proton electrochemical gradient are involved. They found that the accumulation of newly synthesized acetyl[^3H]choline in storage granules in intact PC12 cells was inhibited by treatment of the cells with agents that are known to interfere with the generation or maintenance of proton electrochemical gradients. We have extended these studies using combined gas chromatography-mass spectrometry to characterize more completely the effect of such agents on the synthesis and storage of acetylcholine in PC12 cells.

PC12 cells were incubated for 30 min in buffer containing 10 μM [$^2H_4$]choline and one of the compounds listed in Table III. DCCD is an inhibitor of several membrane-bound (Ca^{2+}/Mg^{2+})-ATPases, including the (Ca^{2+}/Mg^{2+})-ATPase of cholinergic synaptic vesicles from *Torpedo* (Beechey et al., 1966; Toll et al., 1977; Pick & Racker, 1979; Rothlein & Parsons, 1979; Michaelson et al., 1980). The other compounds are ionophores (Hanstein, 1976; Pressman, 1976). S-13 and FCCP are proton ionophores, and valinomycin is a K^+ ionophore. Nigericin causes a H^+/K^+ exchange while A23187 causes a H^+/Ca^{2+} exchange. All of these compounds can inhibit mitochondrial synthesis of ATP. However, under the

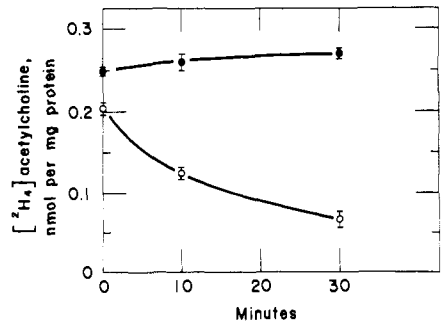

FIGURE 5: Effect of paraoxon on the time course of the disappearance of newly synthesized acetyl[$^2H_4$]choline from PC12 cells. The cells were incubated for 10 min with 10 μM [$^2H_4$]choline, washed, and further incubated for the indicated times with 10 μM choline. All incubations were in the presence (●) or absence (○) of 10 μM paraoxon. After the incubations, the cellular level of acetyl[$^2H_4$]choline was determined. The results are expressed as nanomoles of acetyl-[$^2H_4$]choline per milligram of cell protein. The data points represent the means for four incubations, and the error bars indicate the SE.

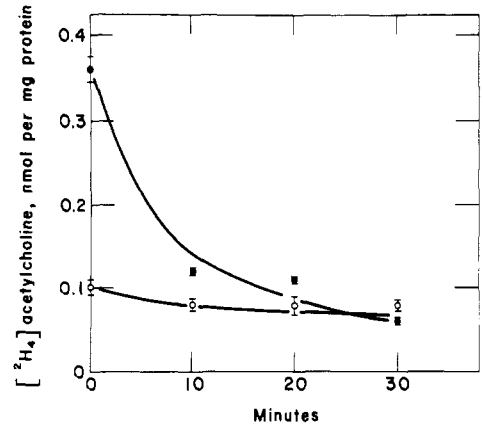

FIGURE 6: Time course of disappearance of newly synthesized acetyl[$^2H_4$]choline from PC12 cells and from PC12 granules. The cells were incubated for 30 min with 10 μM [$^2H_4$]choline, washed, and further incubated for the indicated times with 10 μM choline. After the incubations, the level of acetyl[$^2H_4$]choline was determined in an extract of whole cells (●) and in the granule fraction (○) of a cell homogenate. The results are expressed as nanomoles of acetyl-[$^2H_4$]choline per milligram of cell protein or granule fraction protein, respectively. The data points represent the means for four incubations, and the error bars indicate the SE.

conditions used, these agents do not deplete the PC12 cells store of ATP (Toll & Howard, 1980).

As shown in Table III, only the K^+ ionophore valinomycin failed to affect cell or granular levels of acetylcholine. DCCD, S-13, FCCP, and nigericin each markedly reduced the level of newly synthesized acetyl[$^2H_4$]choline in the storage granules while having much less effect on granule levels of acetylcholine and on total cell levels of acetylcholine and acetyl[$^2H_4$]choline. The results demonstrate that these agents block the loading of newly synthesized acetylcholine into granules but neither inhibit acetylcholine synthesis nor cause granule lysis. The results are also consistent with the hypothesis that an ion gradient drives the transport of acetylcholine into the granules.

A23187 was used in an attempt to determine whether a Ca^{2+} gradient might be the driving force for the acetylcholine transport. However, A23187 inhibited acetylcholine synthesis, resulting in a decrease in both granule and total cell levels of acetyl[$^2H_4$]choline. Thus, we could not determine whether A23187 had any direct effect on acetylcholine transport into granules.

Disappearance of Acetylcholine. Figure 5 describes the time course of the disappearance of acetyl[$^2H_4$]choline from PC12 cells that had been incubated with [$^2H_4$]choline for 10 min, washed, and further incubated in buffer that contained unlabeled choline instead of [$^2H_4$]choline. The cellular levels of acetyl[$^2H_4$]choline decreased rapidly after [$^2H_4$]choline was washed from the cells, and by 30 min, it was less than 35% of the value determined at the beginning of the postwashout incubation period. The level of total acetylcholine did not change much during this time. The decrease in cellular acetyl[$^2H_4$]choline was likely due primarily to hydrolysis by cellular cholinesterase because when the cholinesterase inhibitor paraoxon was present at a concentration of 10 μM, the cellular level of acetyl[$^2H_4$]choline did not decline during the postwashout incubation period.

In contrast to the rapid decrease in total cell acetyl[$^2H_4$]-choline during the postwashout incubation period, there was little change in the level of acetyl[$^2H_4$]choline in granules during this time (Figure 6). The incubation conditions for the experiments of Figure 6 were similar to those for Figure 5 except the initial incubation with [$^2H_4$]choline was for 30 min to increase the amount of acetyl[$^2H_4$]choline in the granules. The results of Figures 4 and 6 thus show that in the absence of a cholinesterase inhibitor, acetyl[$^2H_4$]choline in the granules turns over much more slowly than does total cell acetyl[$^2H_4$]choline.

Exposure of the cells to 55 mM K^+ induces the release of acetylcholine from the cells in a Ca^{2+}-dependent fashion (Greene & Rein, 1977a; Schubert & Klier, 1977; Rebois et al., 1980). We have determined that approximately 9% of the acetyl[$^2H_4$]choline formed after a 30-min incubation with [$^2H_4$]choline is released by the cells upon a subsequent exposure of the cells to 55 mM K^+ for 5 min. Under these conditions, the specific activities (mole fractions) of the total cell acetylcholine, granule acetylcholine, and released acetylcholine were 0.24, 0.1, and 0.14, respectively. These specific activities indicate that newly synthesized acetylcholine was not preferentially released as it appears to be under other conditions in some other cholinergic systems.

Depletion of Acetylcholine in Granules. For certain studies of acetylcholine metabolism in PC12, it would be useful to have cells in which the granular stores of acetylcholine were depleted. We attempted to produce this condition by treating the cells for prolonged periods with agents that either inhibit acetylcholine synthesis or induce secretion of acetylcholine.

One of the agents was bromopyruvate, which at a concentration of 100 μM inhibits acetylcholine synthesis in synaptosomes from rat brain by inhibiting pyruvate dehydrogenase and thereby decreasing acetyl-CoA availability (Jope et al., 1978). Unfortunately, 50 μM bromopyruvate in growth medium proved to be cytotoxic after only a 20-min incubation. A 3-h incubation with 10 μM bromopyruvate effected only an 18% reduction in cell levels of acetylcholine.

BETA, a keto analogue of acetylcholine, is a potent, reversible, and stable inhibitor of human placental choline acetyltransferase with an IC_{50} of 3.1 μM (Rowell et al., 1978). A 10-min treatment of cells in incubation buffer containing 20 μM BETA and 10 μM [$^2H_4$]choline resulted in a marked decrease (33% of control) in the synthesis of acetyl[$^2H_4$]-choline. The level of acetylcholine was only slightly affected (83% of control) by this treatment, and there was no change in the amount of [$^2H_4$]choline accumulated.

When the cells were exposed for 1–16 h to growth medium containing 20 μM BETA, the total cell and granule levels of acetylcholine decreased only slightly (a maximum decrease of 24% and 15%, respectively, for total cell and granule acetylcholine). To exclude the possibility that BETA was ineffective in growth medium because it was inactivated during

the longer incubation, we changed the medium every 15 min for 1 h with fresh medium containing 20 μM BETA. After this treatment, the medium was removed, and the cells were washed twice and incubated for 10 min in buffer containing 10 μM [$^2H_4$]choline but without BETA. Under this condition, there was no inhibition of the synthesis of acetyl[$^2H_4$]choline nor a decrease in cell acetylcholine measured at the end of the 1-h incubation in BETA-containing growth medium. Other causes for the ineffectiveness of BETA in growth medium are currently under investigation.

We also examined whether treatment of PC12 cells with 55 mM K^+, which induces acetylcholine secretion, would deplete the cells' granular stores of acetylcholine. Cells that were incubated for 1 h in buffer containing 55 mM K^+ had only 47% of the acetylcholine found in control cells that were incubated for 1 h in buffer containing 6 mM K^+. The granule fraction of cells exposed to 55 mM K^+ had 40% of the acetylcholine content of granules from control cells. Further studies on cells containing depleted granular stores of acetylcholine are now in progress.

Discussion

Several properties of acetylcholine synthesis and storage in PC12 appear similar to those exhibited by cholinergic neurons. PC12 cells take up choline from the extracellular medium. In these cells, as in cholinergic neurons from mammalian brain, the newly accumulated choline serves as an important precursor pool for acetylcholine synthesis (Jope, 1979). When PC12 cells were incubated for 30 min in buffer containing 10 μM [$^2H_4$]choline, approximately 30% of the cells' acetylcholine was labeled with $^2H_4$ (Figure 3). After a similar incubation with 40 μM [$^2H_4$]choline, 50% of the acetylcholine was acetyl[$^2H_4$]choline. Furthermore, there is rapid acetylation of a significant fraction of the choline that is transported into PC12 cells. Approximately 20% of the total choline accumulated by PC12 cells during a 10-min incubation period was converted to acetylcholine; this percentage is comparable to that obtained during a similar incubation with synaptosomes from some regions (brain stem, hypothalamus) of rat brain although synaptosomes from other regions of rat brain acetylate a considerably higher percentage of accumulated choline (Jope, 1979). In contrast, murine neuroblastoma cells in culture were found to convert only 0.1–0.5% of the accumulated choline to acetylcholine, almost all of the remainder being phosphorylated to form phosphorylcholine (Lanks et al., 1974).

PC12 cells, like neurons, store some of their acetylcholine in vesicles or granules. Approximately 35% of the acetylcholine in homogenates of PC12 cells is contained in granules (Rebois et al., 1980). The percentage of acetylcholine located in granules in intact cells could differ from this value because the homogenization procedure may cause granule damage with a resultant efflux of some of the granule acetylcholine. However, the turnover studies shown in Figures 4 and 6 indicate that the percentage of acetylcholine located in granules in intact PC12 cells is not substantially greater than 35%. Most of the acetylcholine in the cells turns over rather rapidly while the acetylcholine found in granules after homogenization turns over much more slowly. If the percentage of acetylcholine in granules in intact cells were substantially greater than the value (35%) found in homogenates, we would expect the experiments described in Figures 4 and 6 to show less of a discrepancy between the turnover rates for total cell acetylcholine and acetylcholine in granules.

Although the metabolism of acetylcholine in PC12 is comparable in several respects to that in cholinergic neurons, some striking differences are also apparent. For example, the uptake of choline by PC12 cells differs from choline transport into cholinergic neurons; the choline uptake system of PC12 cells has a lower affinity for choline, is independent of energy stores and extracellular Na^+ concentration, and is relatively insensitive to hemicholinium-3 ($IC_{50} \sim 50$ μM). In these respects, choline uptake by PC12 cells is more similar to that by murine neuroblastoma cells in culture (Lanks et al., 1974; Breakefield, 1976).

The temperature dependence and saturability of choline uptake into PC12 cells indicate that the uptake of choline is carrier mediated. The observation that the uptake is independent of energy stores suggests a facilitated diffusion mechanism.

Another apparent distinction between the PC12 cells and a variety of cholinergic neurons is that, under the conditions employed, depolarized PC12 cells did not release newly synthesized acetylcholine preferentially over older stores of acetylcholine. Perhaps different results would be obtained under other conditions of incubation or with PC12 cells that had been induced to extend neurities by treatment with nerve growth factor. One explanation for the preferential release of newly synthesized acetylcholine by other systems comes from the finding that the cholinergic synaptic vesicles of *Torpedo* electric tissue are metabolically heterogeneous (Zimmermann & Denston, 1977). The releasable acetylcholine may be stored in a subpopulation of vesicles that are loaded preferentially with newly synthesized acetylcholine. It is not known whether the cholinergic vesicles in PC12 cells are metabolically heterogeneous in a like manner.

The transport of catecholamines into granules or synaptic vesicles of adrenal medulla, PC12 cells, or neurons is driven by a proton electrochemical gradient, which is established by the activity of a proton-translocating ATPase associated with the granules or vesicles (Bashford et al., 1976; Toll & Howard, 1978; Johnson & Scarpa, 1979; Rebois et al., 1980). The results presented in Table III suggest that a similar mechanism of transport may function in the transport of acetylcholine into storage granules of PC12 cells. Accumulation of newly synthesized acetylcholine in PC12 granules was blocked by DCCD, which is known to inhibit the (Ca^{2+}/Mg^{2+})-ATPase associated with cholinergic synaptic vesicles from *Torpedo* (Rothlein & Parsons, 1979; Michaelson et al., 1980), or by ionophores that are known to dissipate transmembrane proton gradients. These compounds do not exert their effects on acetylcholine transport in PC12 cells by interfering with mitochondrial ATP synthesis and thereby depleting cellular stores of ATP (Toll & Howard, 1980).

Recently, two groups have described the ATP-stimulated uptake of Ca^{2+} by cholinergic synaptic vesicles from *Torpedo* (Israel et al., 1980; Michaelson et al., 1980). Their findings suggest the possibility that the ATPase of cholinergic vesicles or granules is a Ca^{2+}-translocating ATPase, and acetylcholine transport into the vesicles or granules is driven by a transmembrane gradient of Ca^{2+} rather than a proton gradient. Our results do not exclude this mechanism. Thus, DCCD could inhibit acetylcholine transport into PC12 granules by inhibiting a Ca^{2+}-translocating ATPase on PC12 cholinergic granules as it does the Ca^{2+}-translocating ATPase of sarcoplasmic reticulum (Pick & Racker, 1979); the ionophores could act by releasing Ca^{2+} from PC12 mitochondria (Saris & Åkerman, 1980), resulting in a diminished gradient of Ca^{2+} across the granule membrane.

Alternatively, Ca^{2+} transport into *Torpedo* cholinergic vesicles may not be directly coupled to the vesicle (Ca^{2+}/Mg^{2+})-ATPase activity. If the vesicle ATPase is indeed a

proton-translocating ATPase, in addition to driving acetylcholine transport, a transmembrane proton electrochemical gradient could drive Ca^{2+} transport into the vesicles as it does for mitochondria (Saris & Åkerman, 1980). This latter mechanism of Ca^{2+} transport into cholinergic vesicles appears more likely in view of the findings that (1) proton ionophores inhibit the ATP-stimulated uptake of Ca^{2+} into isolated *Torpedo* vesicles and (2) the K_m for Ca^{2+} of the vesicle (Ca^{2+}/Mg^{2+})-ATPase differs widely from that of Ca^{2+} uptake by the vesicles (Michaelson et al., 1980).

Our studies of acetylcholine storage in PC12 granules have been limited by our inability to demonstrate acetylcholine transport in vitro into cell-free preparations of the granules. Recently, acetylcholine has been found to be transported into isolated synaptic vesicles from *Torpedo* (Giompres & Luqmani, 1980; Parsons & Koenisberger, 1980). This system may prove useful for examining the bioenergetic mechanisms of acetylcholine loading into storage vesicles.

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