Sympathetic neurons extend neurites in a culture medium containing cyanide and dinitrophenol but not iodoacetate

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A culture medium circulated through the rat heart and supplemented with insulin, transferrin and nerve growth factor leads to a massive proliferation of neurite outgrowth from neurons of peripheral sympathetic ganglia of the chick embryo. Addition of 1 mM cyanide or 50 μM dinitrophenol to such medium for 2 days had no adverse effect on the neurite outgrowth and ATP content of these neurons. However, 0.5 μM iodoacetate lowered ATP content 65% without affecting the number of surviving neurons up to 2 days. Only when ATP content was reduced to 80% by 2.5 μM iodoacetate was the number of surviving neurons significantly reduced (30%). It is concluded that the glycolytic pathway is the major route of ATP synthesis in embryonic sympathetic neurons maintained in culture, and only a small fraction of ATP is utilized for the survival and neurite extension.

Neuronal culture *Neuronal metabolism* *ATP* *Cyanide* *Glycolysis* *Nerve growth factor*

1. INTRODUCTION

Neuronal cultures are used extensively for a variety of investigational purposes. A small population of neurons derived from sympathetic ganglia of the chick embryo survives in culture in the presence of nerve growth factor [1–3] and is mostly noradrenergic in character [2]. In a previous investigation these noradrenergic neurons were used to study a relationship between ATP content and [^{3}H]norepinephrine uptake [4]. It was found that inhibitors of oxidative metabolism were ineffective in lowering ATP content but those of glycolysis rapidly and almost completely depleted ATP stores of cultured noradrenergic neurons. Furthermore, in the same preparation it was shown that 10% of the total ATP stores were sufficient for the physiological function of sympathetic neurons in culture (i.e uptake and storage of sympathetic transmitter [4]).

This article is dedicated to the memory of the late Professor S.M. Kirkepar, our colleague and the uncle of the author (A.R.W.)

Most recently, we have shown that essentially all the plated neurons of the peripheral sympathetic ganglia of chick embryo survive in a culture medium, previously circulated through the rat heart, and supplemented with insulin, transferrin and nerve growth factor [5]. Under these conditions, neurons put out a massive network of very fine neurites and the process begins minutes after plating the neurons in such a medium. Therefore, the chronic effects of most well-studied inhibitors, such as cyanide, dinitrophenol and iodoacetate, were examined on the neurite outgrowth of neurites and ATP content of neurons plated in the heart perfusate supplemented with other growth factors. It will be demonstrated that only iodoacetate caused a dose-dependent decrease in ATP content of neurons over a 2-day period, and these neurons survived and extended neurite outgrowth even when ATP levels were reduced over 70%.

2. MATERIALS AND METHODS

Sympathetic neurons derived from lumbo-sacral

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paravertebral ganglia of 11- to 13-day-old chick embryos were cultured in serum-free culture medium as described before [1–3]. Briefly, sympathetic ganglia were digested with trypsin (0.1%) for 30 min, washed with excess phosphate-buffered saline, and then dissociated by trituration in F14 medium. The suspension of ganglionic cells (final volume 5 ml) was poured in a 100 mm plastic dish (Falcon) to which 500 μl of heat-inactivated horse serum was added. The dish was kept in a Co_2 incubator for 2–3 h. During this preplating step most of the non-neuronal cells attached to the plastic, whereas neuronal cells remained in suspension [1,2,6]. Suspended cells were then gently removed and counted. Approx. 2000 or 8000 cells were plated on polyornithine-coated dishes containing 1.5 ml F14 culture medium first circulated through the rat heart (see below) and then supplemented with 1 μg/ml each of insulin and transferrin and 20 ng/ml 7s nerve growth factor. All media contained 100 μ/ml penicillin and 100 μg/ml streptomycin.

The number of surviving neurons was determined by scanning along a strip with an area of 1/24th of the total surface area of the dish, using a Nikon Diphot phase-contrast microscope, magnification $\times 100$. Neurons were defined as being those cells having retractile and well-defined compact cell bodies, with neurites of at least 5 cell diameters in length [2].

Rat hearts were perfused with F14 medium at a rate of 5 ml/min through the aorta (Langendorff technique) at 37°C for 30 min. The perfusate was sterilized by filtration (0.2 μ filter).

After counting the cells, the same dish was used to measure ATP content of neurons in culture. To each culture dish, 400 μl ice-cold 50% ethyl alcohol was added, and the dishes were frozen over a bath of dry ice and ethanol. 15–20 μl of defrosted solution was assayed for ATP [7]. Briefly, the 10 μl sample was thoroughly mixed with 500 μl glycine buffer (pH 7.5) to which 15 μl of luciferase-luciferin solution was added, vortex-mixed, and immediately (less than 30 s) counted in Beckman liquid scintillation counter (model LS7000) for the chemoluminescence. Standard solutions of ATP (0.1–10 ng) were analyzed along with each batch of unknowns for conversion of cpm of unknown samples to ng/dish after correction for volume changes. Standard solutions and the unknown samples contained equal amounts of ethyl alcohol. Means ± SE of number of observations are given in the paper. Student's *t*-test was used for comparison of mean values.

Penicillin, streptomycin, bovine crystalline insulin, human transferrin, and luciferase-luciferin were from Sigma (St. Louis, MO); nerve growth factor from Collaborative Research (Lexington, MA); sodium cyanide from BDH Chemicals (Poole, England); 2,4-dinitrophenol, iodoacetic acid and sodium salt were from Aldrich (Milwaukee, WI).

3. RESULTS

The relationship between the number of surviving neurons and their ATP content under control conditions and in the presence of various metabolic inhibitors is shown in fig.1. Continued presence of cyanide (100, 500 and 1000 μM) for 2 days had no adverse effect on the number of neurons surviving in culture. The same figure also shows that none of the concentrations of cyanide significantly reduced ATP content of the surviving neurons. Almost identical results were obtained

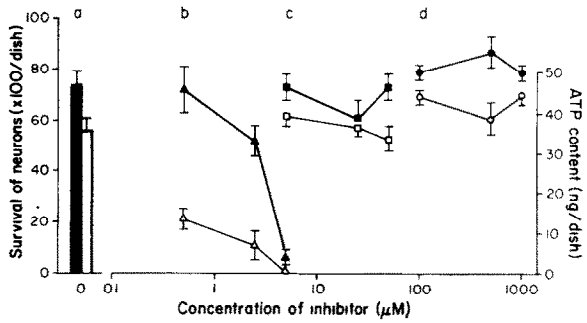

Fig.1. Effects of metabolic inhibitors on the survival and ATP content of neurons maintained in culture for 2 days. The number of surviving neurons (solid column) and their ATP content (open column) after 2 days in culture in the absence of metabolic inhibitors is shown in (a). Increasing concentrations of iodoacetate (b), 2,4-dinitrophenol (c) and cyanide (d) were added, as shown, to separate 35 mm dishes along with other supporting factors at the start of culture. The number of surviving neurons (solid symbols) and their ATP content (open symbols) were measured 2 days after beginning of culture. After counting the number of surviving neurons, the same dish was used to analyze the ATP content. Each column and a symbol represents a mean of 4–12 experiments. Vertical lines show SE of mean.

when 2,4-dinitrophenol was added (5–50 μM) for 2 days. In contrast to these findings, addition of iodoacetate (0.5 μM) produced a modest decline in the number of surviving neurons, but the reduction was statistically not significant ($P > 0.5$). However, the same concentrations of iodoacetate produced a substantial decrease in ATP content (65%) within 2 days. A further increase in the concentration of iodoacetate (2.5 μM) caused a significant reduction (about 30%) in the number of surviving neurons and was accompanied with a sharp fall (about 80%) in ATP content. In 5 μM iodoacetate, all the neurons died within 2 days and ATP was undetectable.

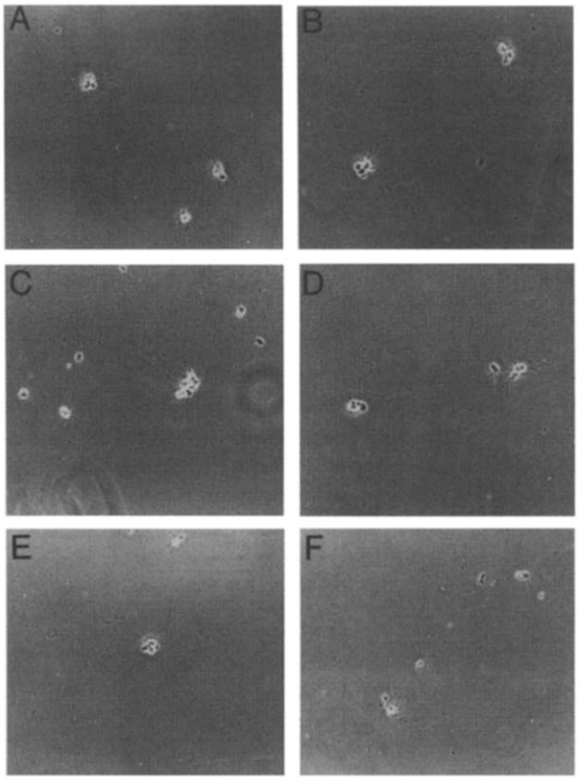

Fig.2. Phase-contrast photomicrographs of neurons in the absence and presence of metabolic inhibitors. The experimental protocol of culturing neurons and exposure to various metabolic inhibitors right from the start of the culture was identical to that described in fig.1, except that 2000 cells were plated in each dish. (A) Control (no inhibitor), (B) 1 mM cyanide, (C) 5 μM dinitrophenol, (D) 50 μM dinitrophenol, (E) 2.5 μM iodoacetate and (F) 5 μM iodoacetate. Magnification 133×.

Fig.2 shows the photomicrographs of neurons surviving for 2 days in different types of culture media.

The general appearance of neurons surviving under control conditions (A) and those surviving in the presence of 1 mM cyanide (B), 5 μM dinitrophenol (C) and 50 μM dinitrophenol (D) was almost the same. 2.5 μM iodoacetate caused a modest decrease in the number of surviving neurons (E). However, higher concentrations of iodoacetate (5 μM) killed all of the plated neurons (F).

In 4 other experiments, cultures were initiated in F14 medium supplemented with insulin, transferrin and nerve growth factor. Under these conditions, about 40% of the plated neurons survived in culture [2–3]. The effects of metabolic inhibitors on ATP content and the survival of this subpopulation of neurons was identical to that reported above for the entire population of the neurons (not shown).

It is well recognized that survival and extension of neurites is greatly influenced by the interaction between the neuronal cell surface and the matrix on which neurons are plated [8]. Therefore, one can argue that iodoacetate could influence the matrix-neuron interaction in such a way that the plated cells fail to attach to the substrate and therefore died in the presence of this metabolic inhibitor, whereas cyanide and dinitrophenol do not interfere with the attachment of neurons to the substrate. To check such a possibility, the following series of experiments were carried out. Neurons were cultured in the heart perfusate supplemented with insulin, transferrin and nerve growth factor. After 2 days, the medium was changed over to either the same (control) or that containing 1 mM cyanide, 50 μM dinitrophenol, or 5 μM iodoacetate. The number of surviving neurons remained virtually unchanged in cyanide, dinitrophenol and the control media for 2 days, but practically all the neurons died in iodoacetate containing culture medium within the same time period (not shown).

4. DISCUSSION

It was astonishing to find that neurons derived from sympathetic ganglia of the 10- to 12-day-old chick embryos survived and extended neurite outgrowth even in the continued presence of 1 mM

cyanide. Cyanide also did not significantly lower ATP levels of cultured neurons within 2 days. These observations lead us to believe that neurons in culture do not depend on oxidative metabolism of glucose or other substrates. Additional support comes from experiments with a classical uncoupler of oxidative metabolism 2,4-dinitrophenol. This agent also had no significant effect on neuronal ATP stores, nor was there any loss of neurons in culture over a 2-day period. It appears that embryonic neurons of sympathetic ganglia continue to survive and grow in the absence of oxidative metabolism. In an earlier study we found that short-term exposure to high concentrations of various types of inhibitors of oxidative metabolism also had no adverse effect on ATP content of cultured neurons [4]. The same effects of metabolic inhibitors were obtained if cells were plated at low (2000 or 8000 cell/dish) (present study) or high (30 000 cell/dish) density [4].

From our previous study [4] and this study it is clear that the breakdown of glucose via glycolysis was sufficient and necessary for the synthesis of ATP. Whether glucose does not penetrate mitochondria or some of the enzymes of the Krebs cycle are not fully functional is not known. We also do not know why the adult neuronal cells mainly depend on oxygen whereas embryonic neurons continue to function in its absence. It may be of considerable importance to determine the factors that lead to a change from glycolytic to oxidative metabolism during development.

It is well known that glucose is utilized by neuronal tissues via glycolysis and then the products of glycolytic metabolism are fed into the citric acid cycle [9]. If energy production is blocked via oxidative metabolism, then the glycolysis of glucose should still continue to synthesize ATP, although to a much lesser extent. This study shows that ATP levels of neurons were reduced in a dose-dependent manner by the inhibitor of glycolysis. In fact, iodoacetate was the only metabolic inhibitor that was able to reduce ATP content and kill all the plated neurons. The results provide evidence that the main source of ATP synthesis is from the glycolytic pathway.

The dose-response curve of iodoacetate revealed an interesting relationship between ATP content and neuronal survival. When ATP stores were reduced 70%, the survival was still nearly 100%, and over 80% reduction in ATP content led to about a 30% decline in the survival of neurons. These data indicate that only a small fraction of total ATP content is utilized for the survival of neurons in culture.

The results are of special interest because the effects of various metabolic inhibitors were observed on the entire population of neurons of peripheral sympathetic ganglia in a culture medium that induces an extensive neurite outgrowth essentially from the very start of the culture [5]. One would expect that under these culture conditions the demand for metabolic energy might be accelerated and the oxidative metabolic pathway may be called upon to meet the excess needs. However, in normal culture medium or that circulated through the heart, the effects of metabolic inhibitors on neuronal ATP content were identical. These observations reinforce the conclusion regarding the origin of ATP from the glycolytic pathway.

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