AN ATP POOL WITH RAPID TURNOVER WITHIN THE CELL MEMBRANE

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SUMMARY: Seven-day-old cultures of rat leg muscle cells were double labelled by addition of [^{14}C]adenine in the culture medium (2 hrs 15 mins) and followed by addition of [^{32}P] phosphate (15 min). The specific activity (S.A.) of the isolated cyclic [^{14}C] adenine 3'-5'monophosphate (cAMP) was similar to that of the bulk ATP. The S.A. of [^{32}P] from cAMP was, however, higher than that of bulk ATP. The S.A. of [^{32}P] from cAMP could be further modified by prevention of normal muscle cell fusion. It is probable that the cAMP with high [^{32}P] S.A. was synthesized from a cell membrane pool of ATP with rapid turnover.

Radioactive adenine has been frequently used (1-4) to label cyclic adenosine 3'-5' monophosphate (cAMP) in a variety of cells and tissues. The experiments of Shimizu et al. (3), for example, show that cAMP from the guinea pig cerebral cortex is derived from a precursor pool of adenine nucleotide which is more rapidly labelled then the bulk cellular ATP. In the present report the S.A. of [^{14}C]-adenine and [^{32}P] in cAMP and bulk ATP were compared after double labelling of cultured muscle cells. Changes in the S.A. of [^{32}P] in cAMP could be demonstrated after modification of the plasma membrane by lysolecithin.

Methods: Rat leg muscle cells were plated in primary culture as described previously (5). The culture medium R, regularly used for these studies consisted of equal portions of F10 and medium 199 with 5% fetal calf serum and 10% horse serum. No antibiotics

were used. The advantage of using 7-day cultures was that 80-95% of the cells are of myogenic origin at this time -- the rest being classed as fibroblasts. The use of 15 mg lysolecithin per 100 ml of medium prevented natural fusion of cells (6). These cultures were labelled LL.

The assays for cAMP were conducted by using the method of Gilman (7) with protein kinase prepared from rat leg muscle. This preparation was tested for binding by phosphate, ATP and cGMP (cyclic 3'-5' guanosine monophosphate). The incubation conditions were: 200 λ of pH 4, 50 mM, acetate buffer, 5 µg protein kinase and 0.5 pmole [^{3}H] cAMP. After one hour at 0°C: (a) the kinase did not bind with [^{32}P] containing 3500 cpm, with or without combinations of unlabelled ATP (20 nmole) or cAMP (1.5 pmole). (b) Approximately half the expected radioactivity from 0.5 pmole of [^{3}H] cAMP was bound when 10 nmole ATP was used together with 1 pmole of unlabelled cAMP. (c) Approximately half the expected radioactivity of 0.5 pmole [^{3}H] cAMP was also bound under the same conditions but with 100 pmole cGMP together with 1 pmole cAMP. Under the assay conditions neither Pi, ATP or cGMP interferred significantly with binding of cAMP by the rat muscle protein kinase. The kinase activity of the preparation was 2.02 nmole per min per mg protein using unfractionated calf thymus histone (Worthington) as substrate.

ATP was assayed by the firefly luciferase method (8) using a Kettering Laboratory Photometer.

The cells in monolayer were sampled as follows: Medium was aspirated and cells washed rapidly in ice cold MEM (Minimum Eagle's Medium) followed by saline. This was followed by 10% TCA (trichloroacetic acid) aliquot. Eight to ten seconds elapsed between aspiration and the TCA step. The cells were scraped in the TCA

and stored at -70°. Later after centrifugation, the acid from the supernatant was removed by five washes with cold, water-saturated ether. An aliquot was removed and ATP separated by chromatography on PEI thin layer plates. The remainder was passed through charcoal, the loosely adsorbed nucleotides eluted with ammoniacal ethanol and assayed for cAMP and cAMP-associated radioactivity after removal of ethanol.

Radioactivity was determined with a Unilux II Model Nuclear Chicago Liquid Scintillation Spectrometer. Use was also made of a Packard Model 2420 Liquid Scintillation Spectrometer. The [^{14}C] isotope was counted with only 42% efficiency. This narrow window selection avoided [^{32}P] count spillover.

Culture-media, sera, chemicals and radioactive isotopes were obtained from the same sources as previously listed (5). The original S.A. of [^{3}H] cAMP was 25 Ci/mmol (Amersham-Searle) while that of [8-^{14}C] adenine was 52 mCi/mmol (Amersham-Searle). [^{32}P] was purchased from New England Nuclear.

Results and Discussion: The data shown in Table I and II were obtained from 7-day cultures. At this period, our cultures showed minimal basal values for cAMP concentrations. When the cultures of rat muscle cells were in the stage of proliferation (the first 5 days in culture) cAMP concentrations between 15 to 20 picomoles per mg protein were obtained. The cAMP decreased to minimal values between 1 to 3 picomoles per mg protein during fusion at 5 to 6 days of culture and then increased to 3 to 5 picomoles during further differentiation between 8 to 11 days. This latter small rise may reflect confluency of cultures with late growing fibroblasts (9). Rat muscle cells have shown positive cAMP responses to hormones after 8 to 11 days of differentiation in culture. Under our conditions of culture, 7-day muscle cells are somewhat

Table I - Specific Activities of Bulk ATP in 7-day Cultures of Muscle Cells.

Sample	picomole/mg protein ($x10^3$)	[8-^{14}C] Adenine cpm/pmole	[^{32}P] Phosphate cpm/pmole	$^{32}P/^{14}C$
R	19.9 ± 0.9	4.7 ± 0.7	0.03 ± 0.004	0.008
LL	23.8 ± 1.8	5.2 ± 0.6	0.04 ± 0.002	0.008

18 hours after introduction of 4 ml of fresh non-radioactive medium, [8-^{14}C] adenine was added to each culture plate (50 mm diam). The final concentration of [^{14}C] was 0.13 µCi/ml medium. [^{32}P] was introduced into the medium 2-1/4 hrs. after [^{14}C] adenine. The plates were sampled 15 mins after the addition of [^{32}P]. When S.A. determinations were made the [^{32}P] was calculated at 1.87×10^6 cpm/ml medium. The concentration of phosphate measured in the medium collected from the culture plates after incubation was 1.28 micromole/ml (see text). In 7-day cultures, the intracellular inorganic phosphate is 60 nanomoles/mg protein. Eight plates per group were sampled with 1.5 to 2.1 mg protein per plate. Averages from the eight plates ± standard deviation are indicated for R - regular or the LL - regular + lysolecithin medium respectively. In this experiment, 0.15 mg monopalmitoyl phosphoryl choline/ml medium was added. The cell extracts were chromatographed for separation of labelled adenine nucleotides on PEI (polyethylene imine) cellulose sheets (Baker). The chromatography was carried out with water, 0.2 N LiCl and 1.0 N LiCl in successive steps. The radioactive spots were localized with co-chroma tography of standards and after radioautograph with Kodak Blue-Brand X-ray film.

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refractory vis-a-vis the adenyl cyclase response to hormones like insulin and glucagon (10).

As noted previously, the use of lysolecithin in the culture medium (LL) prevented fusion of muscle cells. Return of cultures to regular medium (R) at any time brought about massive cell fusion by 6 to 8 hours. The major effect of lysolecithin on muscle cells

Table II - Specific Activities of Cyclic Adenine 3'5' Monophosphate in 7-Day Cultures of Rat Leg Muscle Cells.

Sample	picomole/mg protein	[8-^{14}C] Adenine[(a)] cpm/picomole	[^{32}P] Phosphate cpm/picomole	^{32}P/^{14}C
R	1.9 ± 0.07	4.0 ± 0.8	0.39 ± 0.05	0.1
LL	2.3 ± 0.06	4.6 ± 0.7	0.91 ± 0.06	0.2

(a) cAMP was isolated after two dimensional chromatography of charcoal absorbed eluates of experiment of Table I. cAMP was located after co-chromatography with standards and location by radioautography as with ATP. First direction chromatography performed in sequential system of water; 0.2 and 1.0M LiCl. Second direction chromatography performed in sequential system of pH 3.5 formate 0.5 M and 2.0 M respectively. cAMP was determined by method of Gilman (7) using 3 to 5 µgm of protein and competing with [^{3}H] cAMP (S.A. 25 Ci/mmole) Rechromatography of the cAMP with LiCl gave S.A. of ^{14}C similar to that shown above. Isotope decay and limited samples size prevented recheck of ^{32}P radioactivity at the same time.

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was therefore, at the level cell membranes. This has been verified chemically by using various isolated membrane fractions from cells labelled with [2-^{3}H] glycerol. (Experiments to be published.) Data of replicate plates treated with LL are also shown in Tables I and II.

The tables show that both types of cultures had equilibrated to the [8-^{14}C] adenine label after a total time of 2-1/2 hours because the S.A. of ATP and cAMP were similar. A contaminant chromatographing close to cAMP has been noted in the PEI system used. Isolation of cAMP by at least two dimensional PEI chromatography is necessary to obtain constant S.A.

The S.A. of [^{32}P] in cAMP should be a third of that for ATP if equilibration was achieved during this 15 min interval. The

cAMP S.A. for [^{32}P] is, however, exceptionally high when compared with ATP. The value of Pi in the medium after incubation was measured at 1.28 micromole/ml. The S.A. of [^{32}P] in the medium was calculated to be 1.45 cpm/pmole and may account for the high S.A. of [^{32}P] in the cAMP. The cAMP [^{32}P] S.A. was further increased in LL treated cells. Therefore, neither the adenyl cyclase nor the phosphodiesterase of the cell membrane (11-14) may be as drastically affected by the LL treatment as the putative cell membrane ATP-pool system.

The ^{14}C S.A. for ATP as well as cAMP were similar between cells grown in regular medium (R) and those in LL. The difference in ^{32}P/^{14}C ratios between bulk ATP and cAMP can be most simply interpreted as being due to a special ATP pool in the plasma membrane or due to an unknown contaminant containing phosphate. After two dimensional chromatography, cGMP, and inorganic pyrophosphate the most likely contaminants, are well separated from cAMP. The presence of another contaminant in the cAMP sampled from the muscle cells seems less probable since the S.A. of adenine in cAMP and ATP are similar.

It is proposed that an ATP precursor pool for cAMP can be present within the cell membrane and change in response to changes of the membrane structure. The fluid hydrophobic nature of cell membrane (15) can of itself act as a barrier to small quantities of ATP which may be stable in this hydrophobic environment in a manner analogous to some model systems (16).

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