ATP CONTENT AND RELEASE IN CULTURED RECTAL GLAND CELLS FROM THE SHARK SQUALUS ACANTHIAS

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Recent studies from our laboratory have shown that the cystic fibrosis transmembrane conductance regulator (CFTR) is capable of conducting ATP as the charge carrier (Reisin et al., J. Biol. Chem., 269: 20584-20591, 1994). The physiological role of the ATP-conductive pathways may be associated with the autocoid delivery of ATP to the extracellular milieu, and the consequent stimulation of the outwardly rectifying chloride channel (ORCC, Schwiebert, et al. Cell 81: 1063-73, 1995). The CFTR-related protein, P-glycoprotein, responsible for the multidrug resistance phenotype in tumors, is also an ATP channel (Abraham et al., Proc. Natl. Acad. Sci. USA, 90: 312-316, 1993).

Little information is available on the ATP-permeable pathways of the shark. We recently determined that cultured shark rectal gland (SRG) cells express a cAMP-activated electrodiffusional pathway that is permeable to both ATP and Cl (Cantiello et al., Bull. MDIBL 33: 47-48, 1994). Although the ATP-conductive pathway of SRG cells was activated by cAMP, a behavior expected for a CFTR-like molecule, this pathway showed rectifying properties in symmetrical ATP, and a pharmacological profile (resistance to DPC, inhibition by nifedipine) more similar to that of the murine isoform of the P-glycoprotein. In this report we determined the intracellular ATP content and the characteristics of ATP release in SRG cells using the luciferin-luciferase assay.

Primary cultures of shark rectal gland cells were obtained from adult male Squalus acanthias, as previously described (Valentich, Bull. MDIBL 26: 91-94, 1986). Cells were seeded at a high density for primary cultures to spread onto coverslips. Extracellular and total cellular ATP were measured with a modified luciferin-luciferase assay as previously described (Abraham et al., op.cit.). Briefly, at the time of the experiment, the coverslips were placed in plastic cuvettes and held vertically in the cuvette with a microclip (Roboz Surgical Instruments, Inc., Rockville, MD). The assay solution contained 0.1 ml of the luciferin-luciferase assay mix (Sigma Chem. Co., St Louis, MO) and 0.5 ml of a Ca^{2+}-free solution containing 280 mM NaCl and 10 mM Hepes, pH 7.4. Whenever indicated, NaCl was replaced with Na-gluconate. Photon release was continuously measured in a luminometer (MonoLight 2010, Analytical Luminescence Lab., Ann Arbor, MI). The ATP release was determined by the photon release of the luciferin-luciferase assay for ~2 min (to reach a steady-state plateau) before membrane permeabilization to assess the total intracellular ATP. Permeabilization was accomplished by addition of alamethicin (10 μM) and sonication (30 sec, Ultrasonic sonicator, FS-14, Fisher Scientific). Photon release was again followed for another 2 min. To determine the amount of ATP released from cells, known concentrations of

ATP in solution were also measured to construct a calibration curve. The average ratio of extracellular to total ATP was obtained after the background signal level was subtracted and the ATP released was compared to total ATP content obtained in each case. Whenever indicated, cells were incubated overnight in the presence of cholera toxin (CTX, 6 μg/ml, Sigma).

Intracellular ATP content was measured after complete permeabilization with alamethicin as indicated above (Fig. 1). Intracellular ATP was 200% higher in Cl-free (gluconate) solution (35.2 ± 8.4, pmoles ATP/coverslip, n=13, vs. 107 ± 29.2, pmoles ATP/coverslip, n=8, $p<0.05$, for Cl and gluconate solutions, respectively). Thus, intracellular ATP was dependent on the presence of Cl in the extracellular milieu. Under these (physiological) conditions, however, no significant cAMP-dependent change in the ATP content was observed, at least according to the lack of effect of cholera toxin (Fig. 1), since the toxin-treated cells also had proportionally higher ATP in the Cl-free solution (289%, $p<0.005$).

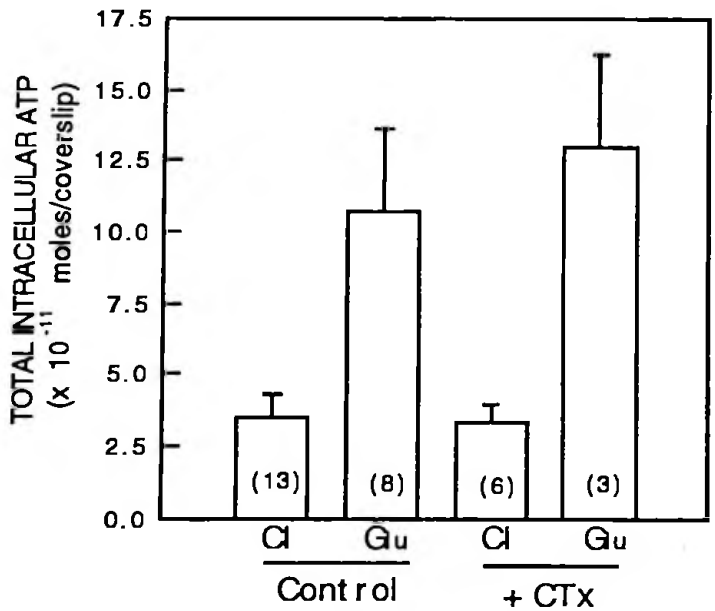

Fig. 1. Effect of cholera toxin (6 μg/ml) on ATP content of cultured SRG cells. ATP content was measured in 280 mM NaCl (Cl) or a Cl-free solution (Glu) where NaCl was replaced with Na-gluconate. Values were obtained after complete permeabilization of cells with alamethicin. Values are the mean ± SEM for experiments indicated in the bars.

Whenever the rate of ATP release (Prat et al., Am. J. Physiol., 270: In press, 1996), was determined as the extracellular to total ATP ratio, it was observed that no significant differences were found (Fig. 2) between Cl-containing and Cl-free solutions for control and CTX-treated cells. However, the ATP ratio was 63.5% lower in CTX-treated cells in the Cl-free solution. Although this difference did not reach statistical significance, the data are suggestive of a possible cAMP- (CTX)-dependent inhibition of ATP release in the absence of extracellular Cl (Fig. 2). Because the rate of ATP release did not change in the presence of external gluconate (Fig. 1), the data suggested that the higher ATP contents in the Cl-free solution may be only partially explained by changes in the rate of ATP release. This is further supported by the data of Fig. 3, where the rate of ATP release and intracellular ATP were inversely related, albeit independent of treatment with CTX. The data indicate that intracellular ATP is inversely proportional to the rate

of ATP release. The correlation (Natural log-Linear) is consistent, however, with a constitutive mechanism for the release of cellular ATP, and which is not activated by cAMP.

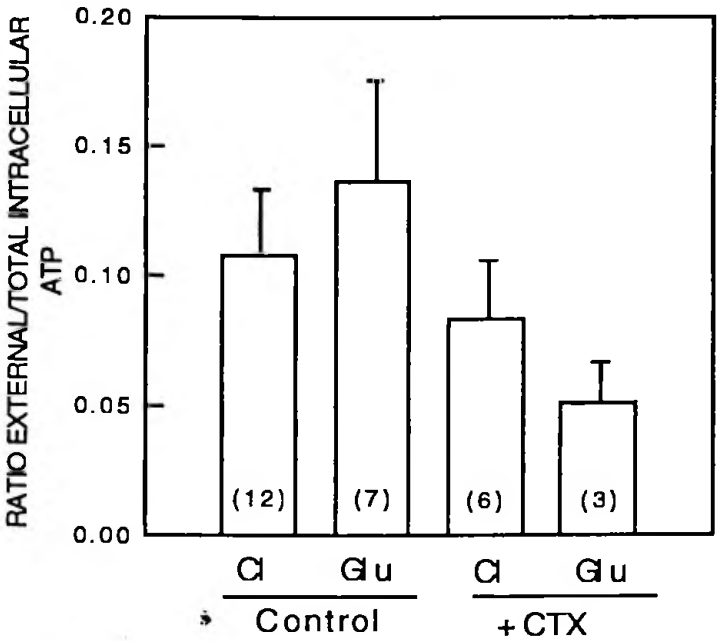

Fig. 2. Effect of cholera toxin (6 μg/ml) on ATP released by of cultured SRG cells. Data are expressed as the ratio between external vs. total ATP. ATP content was measured in 280 mM NaCl (Cl) or a chloride-free solution (Glu) where NaCl was replaced with Na-gluconate. Values of total intracellular ATP were obtained after complete permeabilization of cells with alamethicin. Values are the mean ± SEM for experiments indicated in the bars.

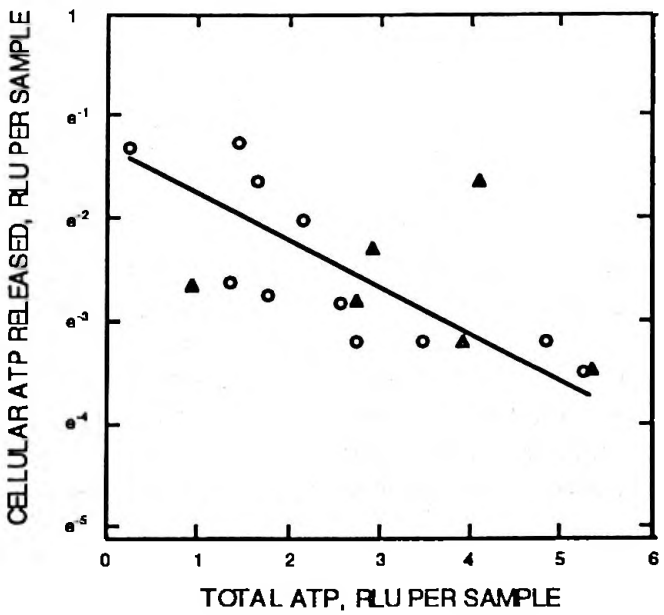

Fig. 3. Correlation between cellular ATP released and total ATP. Data are expressed in arbitrary light units (RLU). Values were obtained for control (circles) and cholera toxin-treated SRG cells (triangles) in chloride-free solution. Ordinate axis is a natural log of RLU, thus indicating the exponential correlation between released ATP and total nucleotide content.

The data in the present study indicate that the maintenance of intracellular ATP content of cultured SRG cells is largely dependent on replacing the extracellular anion, which is most consistent with the presence of a constitutive (not cAMP-stimulated) ATP pathway which elicits the spontaneous release of cellular ATP. This is in contrast to our previous patch-clamp observations indicating that cAMP-stimulation activates a pathway that is permeable to both Cl and ATP. However, the cells were largely insensitive to CTX. Although this may be due to a possible resistance of shark cells to cholera toxin, SRG cells were also insensitive to cAMP analogs, isobutyl-methyl-xanthine, and forskolin (data not shown), thus indicating that the batches of cells used in this study failed to express functional "CFTR-like" ATP pathways. However, a basal ATP pathway was also observed in our previous studies, thus indicating that putative ATP transporters other than CFTR may also be present. In this regard, the data on the decrease of the rate of release of ATP by CTX on the Cl-free-bathed cells may be suggestive of a transport mechanism that is likely to be down-regulated by cAMP-stimulation. Although this hypothesis will require further studies to be confirmed, recent evidence indicates that stimulation of the cAMP pathway down-regulates the expression of P-glycoprotein (Scala et al., J. Clin. Invest., 96: 1026-34, 1995).

An interesting aspect of the data in this report relates to the fact that in extracellular Cl, SRG cells had much lower intracellular ATP than in Cl-free solution. This may be explained by the competition between cellular ATP and Cl for transport through the same transport mechanism. CFTR, for example, is able to move either one of the anions. However, previous studies on P-glycoprotein indicate that this channel only moves ATP and is impermeable to Cl. The higher ATP content in external gluconate, therefore, may be associated with a higher electrochemical Cl gradient which then disallows ATP from coming out, and/or the possibility that in the absence of external Cl, a major contributor to ATP movement, the resting potential of the cell may have changed. Considering the depolarizing effect of external Cl replacement, the possibility exists for a change in the electrochemical driving force for cellular ATP movement. This is supported by recent studies where it was calculated that the resting potential of the cell is perhaps the most relevant acute regulator of ATP release (Prat et al., Am. J. Physiol., 270: In press, 1996). This is also consistent with the fact that the rate of ATP release was not modified, thus suggesting that it is not the transport mechanism itself, but the driving force associated with the ATP movement which is modified. The data argue in favor of a potential electrodiffusional ATP pathway, at least in the context of its sensitivity to cell depolarization. This constitutive pathway for ATP release may play a relevant role in the regulation of the epithelial cell response associated with adenosine nucleotide derivatives in SRG cells.

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