Real-time measurements of acetylcholine-induced release of ATP from bovine medullary chromaffin cells

Eduardo Rojas, Harvey B Pollard and Eli Heldman

Laboratory of Cell Biology and Genetics, NIADDK, National Institutes of Health, Bethesda, MD 20050, USA

Received 29 April 1985

The luminescent oxidation of luciferin has been used to monitor acetylcholine-induced ATP release from cultured bovine chromaffin cells Acetylcholine (1–100 μM) evoked ATP release of up to 30% of the total cellular ATP This secretion required external free calcium and could also be elicited by K^+-induced membrane depolarization The size of the cytosolic ATP compartment was estimated as 5% of the ATP in the cell by solubilising the cell membrane using digitonin (20 μM) or by application to the cells of brief pulses (2 μs) of high electric field (2000 V/cm) Blockers of the voltage-gated Ca^{2+} channel effectively blocked K^+-induced ATP release, while the acetylcholine antagonists d-tubocurarine and β-bungarotoxin inhibited the acetylcholine-induced release of ATP These data support the concept that ATP is released together with the catecholamines by exocytosis of chromaffin granule contents

ATP secretion *Chromaffin cell* *Acetylcholine channel* *Exocytosis* *Catecholamine*

1 INTRODUCTION

In many types of secretory cells the secretory granules and vesicles contain ATP in addition to the specific transmitters or hormones. Examples include chromaffin cells [1], pancreatic B-cells [2], nerve cells [3–5], platelets [6,7] and mast cells [8] It should therefore be possible, at least in principle, to obtain quantitative, real-time measurements of the kinetics of secretion of vesicle contents by monitoring ATP release [9] using the luciferase-catalysed luminescent oxidation of luciferin.

Using a new preparation of highly purified luciferase, we have measured the kinetics of Ca^{2+}-dependent acetylcholine-induced ATP release from these cells. The size of the cytosolic ATP compartment was measured by permeabilizing the cells with digitonin [11,12] or high electric field discharges [13]. In addition, some of the properties of stimulus-secretion coupling through the cholinergic receptors were determined

2. MATERIALS AND METHODS

2 1 *Preparation of chromaffin cells*

Chromaffin cells were prepared from bovine adrenal medulla by collagenase digestion as detailed in [14]. The cells were placed in Eagle's essential medium at a concentration of 50×10^6 cells per flask (30 cm^3 medium), and allowed to recover from the isolation procedure for a 3 day period in a CO_2 incubator at 37°C. After this incubation the cells were collected by centrifugation and resuspended in a modified Krebs solution (135 mM NaCl, 10 mM Hepes-NaOH, pH 7.2, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM D-glucose). The cell preparation was kept at room temperature prior to initiation of experiments at a concentration of 10^7 cells/cm^3 Krebs solution.

2.2. *Luciferin/luciferase preparation*

The enzyme preparation (Analytical Luminescence Laboratory, San Diego, CA) consisted of a mixture of highly purified luciferase, purified

Published by Elsevier Science Publishers B V (Biomedical Division)
00145793/85/$3 30

bovine serum albumin and luciferin. The contents of a sealed vial were dissolved in 5 cm^3 Krebs solution The reaction mixture (130 μl Krebs solution, 15–25 μl cells, 50 μl luciferin-luciferase mixture) was placed in a plastic tube specifically supplied for use in the luminescence instrument (Turner Instruments, CA), which we modified to extend the frequency response up to 100 Hz Cell density in the reaction mixture was kept below 2.5×10^6 cells/cm^3 to maintain ecto-ATPases at low activity. Under resting conditions the light output of the reaction mixture containing cells was constant at about 0.25% of the maximum signal recorded in each experiment. Various secretagogues were added in small volumes (10 μl) to the cell mixture with a microliter pipetter (Gilson) through a modified pipette holder. Emitted light levels were constant and linearly related to ATP levels up to 1400 pmol in the reaction medium Light levels, although reduced by the dilution of the ATP during the additions of small volumes, were unaffected by Triton X-100 (up to 1%) and digitonin (up to 200 μM) Furthermore, the reaction was inhibited by K^+ (25% at 100 mM K^+). The assays with the cells were performed either at 20 or 32°C with similar results. The digitonin (Calbiochem-Behring) solution used was passed through a cationic exchanger column (Dowex-300)

3. RESULTS

3.1 *Acetylcholine-induced ATP release. calcium dependence*

In chromaffin cells, the secretion of catecholamines is evoked by stimulation of a nicotinic cholinergic receptor and depends on calcium in the medium [15–17]. As shown in fig.1, this is also the case for stimulation of ATP release For the experiment illustrated in fig 1A, 10 μM acetylcholine was applied to cells in the luciferin-luciferase medium and prompt secretion of ATP was observed. The time course of secretion showed that the ATP secretory activity was complete in less than 1 min, consistent with data for secretion of catecholamines [18]. In a modified Krebs medium with no added calcium (1 μM), the response of the cells to acetylcholine was abolished (fig.1B).

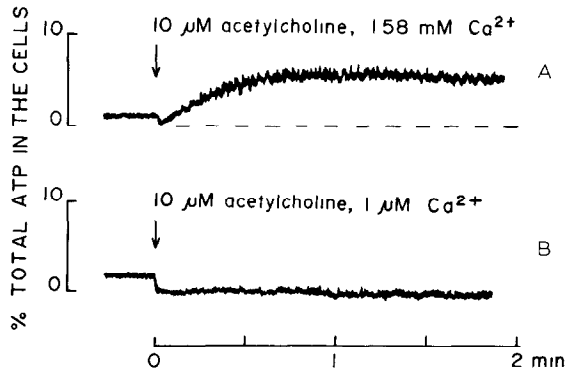

Fig 1 Calcium dependence of acetylcholine-induced ATP release Chart records of the photomultiplier current output when the reaction mixture contained 250000 cells These represent the time integral of the ATP release Vertical calibration 10% of maximum signal obtained by addition of Triton X-100 (1% final concentration) at the end of the experiment Arrows indicate the addition of acetylcholine (10 μM) (A) Free calcium concentration (measured with a calcium electrode) was 1 58 mM (B) Free calcium concentration was 1 μM The rapid decrease of the light signal to below the basal level upon addition of 10 μl Krebs solution with acetylcholine (200 μM) was caused by the dilution of the ATP present in the medium

3.2. *Nicotinic acetylcholine receptor desensitization*

Acetylcholine receptors in chromaffin cells can be desensitized by prolonged exposure to high concentrations of agonists. Operationally, this means that further addition of agonist does not elicit further catecholamine release. This property of the acetylcholine receptor is also evident in the case of the ATP release. As shown in fig 2A and B, submaximal doses of both acetylcholine (10 μM) or nicotine (32 μM) can evoke substantial ATP release Application of a further identical dose 2 min later (20 μM for record A) resulted in no further ATP release. This refractoriness of ATP secretion was not due to the depletion of ATP since 20 μM acetylcholine could have elicited more secretion than that observed by the divided 10 μM doses. Furthermore, a sudden elevation of $[K^+]_o$ (keeping the sum $[Na^+]_o + [K^+]_o$ in the modified Krebs solution constant) after the stimulation with either acetylcholine or nicotine did elicit a substantial additional ATP release response (not shown)

Additional experiments showed that the nicotinic receptor was specifically involved in the coupling between acetylcholine stimulation and ATP release Fig.2C illustrates the substantial inhibition of the acetylcholine-evoked ATP release induced by a brief pretreatment of the cells with 25 μM d-tubocurarine. We also found that β-bungarotoxin and hexamethonium, specific inhibitors of nicotinic acetylcholine receptors, but not muscarinic acetylcholine receptors, blocked acetylcholine-induced ATP release. As expected, muscarine was ineffective (not shown).

3.3. *Origin of the secreted ATP*

In chromaffin cells, ATP is distributed both in the secretory granules and in the cytosol [1,19]. It was therefore necessary to establish the origin of the secreted ATP. This was of particular importance since it is known that chromaffin cells can also release cytosolic components such as ascorbate in a calcium sensitive manner in response to acetylcholine [20].

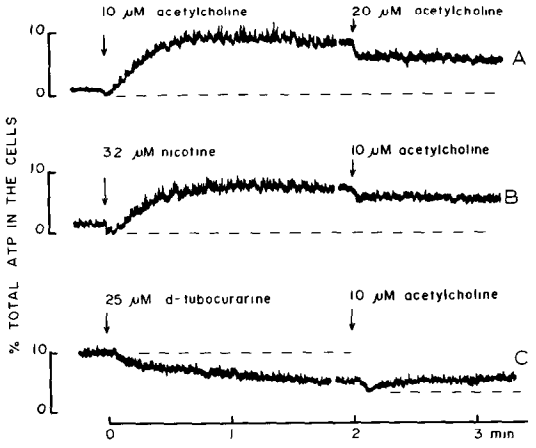

Fig 2 Desensitization of the nicotinic acetylcholine receptor for ATP release. Records represent the time integral of the ATP released from 250000 chromaffin cells (A) Responses to consecutive applications of acetylcholine The arrow on the left indicates the first addition (concentration in the reaction mixture 10 μM). Arrow on the right shows the effects of a second addition (20 μM) (B) Response to an initial stimulation with nicotine (32 μM) and a further stimulation with acetylcholine (10 μM) (C) Response of chromaffin cells pretreated with d-tubocurarine (25 μM) to acetylcholine

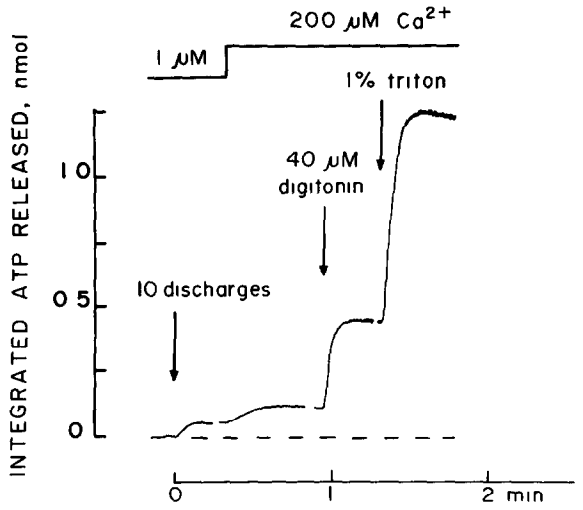

Fig 3 ATP release from permeabilized chromaffin cells Records of the time integral of the ATP release from 150000 cells in Cl^--free, isethionate Krebs solution. Left arrow. application of 10 high-voltage discharges (frequency 0.25 s^{-1}). The concentration of free calcium was augmented from 1 to 200 μM as indicated above the records. Middle arrow· application of digitonin Right arrow· application of 1% Triton

To determine the size of the cytosolic ATP compartment, we permeabilized the cells by brief exposures (2 μs) to an electrical field (2000 V/cm) and by application of digitonin (20–40 μM) in a medium with isethionate in place of chloride. Fig.3 shows that when chromaffin cells were subjected to 10 high-voltage discharges in a medium containing 1 μM Ca^{2+}, 4–5% of the cellular ATP was rapidly released into the medium (fig.3, left arrow) Increasing Ca^{2+} to 200 μM induced a further release of ATP, while application of 40 μM digitonin in the presence of 200 μM Ca^{2+} (fig 3, middle arrow) gave rise to a further ATP liberation of 30–35% of the total ATP in the cells. Finally, treatment of the reaction mixture with 1% Triton released the remainder of the ATP in the cells (fig.3, right arrow). The ATP level reached with the Triton treatment gave the total ATP as 1200 pmol (fig 3). Since the reaction mixture contained ~150000 cells, the measured ATP content per chromaffin cell was 8 fmol. Under these experimental conditions, namely isethionate in place of chloride and low $[Ca^{2+}]_o$ (0.1–1 μM) in the external medium, we calculated that 4–5% of the

ATP in the cells is liberated with both permeabilization methods (average of 9 experiments). Increasing $[Ca^{2+}]_o$ to 200 μM induces a further liberation of ATP in the range from 9 to 10% of the total ATP in the cells.

Our basis for making these assignments of ATP localization is based on the following calculations. Taking the volume of the granule as 4.6×10^{-15} cm^3, the ATP concentration in the granule as 130 μmol/cm^3, and 10^4 granules per cell [19], the granular ATP content per cell is estimated as 6 fmol. Taking the cytosolic cell volume as 50% of the cell volume, i.e , $2\,5 \times 10^{-10}$ cm^3 and the cytosolic concentration of ATP as 1.5 μmol/cm^3, the ATP in this compartment is estimated as 0.4 fmol per chromaffin cell, or less than 7% of the total ATP that can be extracted from the cell. By comparison, the ATP released after permeabilization of the cells in the experiment illustrated in fig.3 amounted to 4.8% of the total ATP We believe these values to be very close

One other interesting observation from the data in fig.3 was that digitonin permeabilization seemed

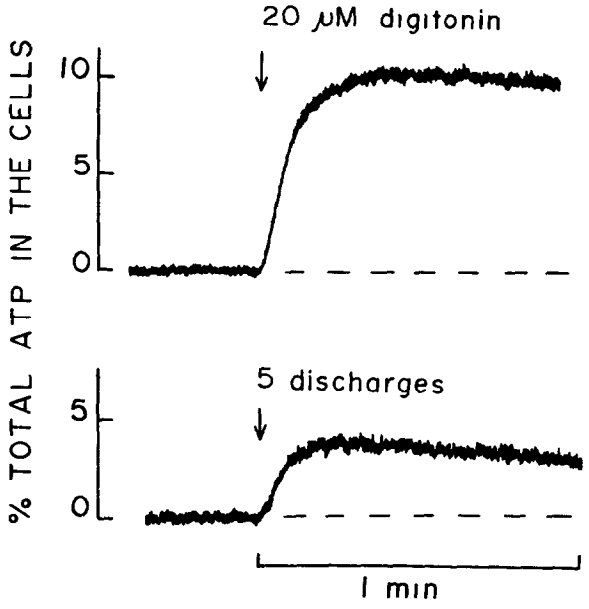

Fig 4 Comparison between ATP release induced by 20 μM digitonin (upper) and by 5 high-voltage discharges (lower) Experiments with 250000 cells in Cl^--free, isethionate medium in presence of 1 μM Ca^{2+} High-voltage discharges were applied at a frequency of 0 2 s^{-1}

to provoke further ATP release when added after high-voltage permeabilization Although both techniques have been used on chromaffin cells, no side-by-side comparison of the two methods has been reported. Therefore we exposed two aliquots of the same cell preparation to either 20 μM digitonin, a level found optimal by ourselves and others [11,12], or 5 high-voltage discharges in a Cl^--free, isethionate medium in the presence of 1 μM Ca^{2+}. A careful comparison of ATP release evoked by different numbers of discharges revealed that with our apparatus no further release could be elicited by more than 5 discharges. As shown in fig.4, we measure ATP release from chromaffin cells induced by either 20 μM digitonin (upper trace) or by 5 high electric field discharges (lower trace). Clearly, digitonin evoked twice as much release as did an optimal number of high-voltage discharges. When the $[Ca^{2+}]_o$ was adjusted to 0.1 μM, both methods released similar amounts of ATP from the cytosol (not shown). On the other hand, release of catecholamines from permeabilized cells is known to depend also on the presence of additional ATP in the medium [11,13] yet, due to the nature of our assay, we could not increase the $[ATP]_o$ to reach the levels reportedly required (5 mM) for optimal exocytotic catecholamine release without complete oxidation of the luciferin in the reaction mixture. Therefore, the measured stimulation of the ATP release from permeabilized cells by a sudden increase in $[Ca^{2+}]_o$ from 1 to 200 μM as in fig.3, or the quantitative differences between digitonin and high-voltage permeabilization in fig.4, may only represent fractions of the release possible if the optimal $[ATP]_o$ could be included in the reaction mixtures. Very little more can be said at present because the action of ATP during secretion remains unknown.

4. DISCUSSION

The main conclusion from our study is that acetylcholine-induced, Ca^{2+}-dependent ATP secretion from chromaffin cells [9] can be quantitatively measured using a highly purified luciferase preparation to assay released ATP and that pharmacological modulations of ATP release seem to parallel catecholamine secretion. One particularly attractive feature of this assay of ATP is that it can allow us to monitor exocytosis with a time resolu-

tion in the millisecond range. Indeed, the emitted light signals may be suitable for measurements of quantal events, a process in which we are presently engaged.

The value of the digitonin and high electric field permeabilization experiments is profound, and our main conclusions are dependent upon them. ATP is distributed both in secretory granules and cytosol, as mentioned above, and thus the origin of secreted ATP would continue to be hypothetical without recourse to the direct measurement of the cytosolic ATP using the permeabilization techniques. Digitonin, for reasons which are not well understood, appears to solubilize the plasma membrane of the chromaffin cell, leaving the organelles such as chromaffin granules, mitochondria and endoplasmic reticulum intact [11,12]. The permeabilized cell loses large molecules such as lactate dehydrogenase (LDH), but not DBH or catecholamines which are found in the granules.

By contrast, the high electric field permeabilization step seems based on quite well understood physical principles [13,21]. Thus, the application of high electric field pulses, a few microseconds in duration, makes holes in the plasma membrane estimated to be 4–5 Å in diameter. These holes do not allow LDH to escape [13] and thus externally applied luciferase cannot enter the cells. Therefore, the detection of ATP in the case of high-voltage-permeabilized cells takes place outside the cell boundary, unlike the situation with digitonin-permeabilized cells. Therefore, the fact that both techniques generate similar data with regard to the exact amounts of ATP in the cytosol and in the granules is encouraging with respect to the dependability of the data and therefore the conclusions.

However, the consequences of this new advance may be even more far-reaching. The luciferase system, as presently designed, can now be viewed as a quantitative and possibly universal detector of secretion for cells in which ATP is co-stored and co-secreted with hormones or transmitters of particular interest.

ACKNOWLEDGEMENTS

The authors are pleased to thank Dr R. Ornberg for comments and Diane Seaton for preparation of the chromaffin cells.

REFERENCES

[1] Winkler, H. and Westhead, E. (1980) Neuroscience 5, 1803–1823
[2] Sussman, K E and Leitner, J W (1977) Endocrinology 101, 694–701
[3] Dowdall, M J., Boyne, A F and Whittaker, V P (1974) Biochem J 140, 1–12
[4] Wagner, J.A., Carlson, S S and Kelly, R B (1978) Biochemistry 17, 1199–1206.
[5] Pollard, H B and Pappas, G (1979) Biochem. Biophys Res. Commun. 88, 1315–1321
[6] Code, C.F (1952) Physiol Rev 32, 47–65
[7] Born, G V R (1958) Biochem J. 68, 695–704
[8] Uvnas, B (1974) Life Sci 14, 2355–2366
[8] Weil-Malherbe, H. and Bone, A D (1958) Biochem J 70, 14–22
[9] Ito, S , Nakazato, Y and Ohga, A (1980) Adv Biosci 36, 87–94
[10] Leitner, J.W., Sussman, K E , Vatter, A.E and Schneider, F H (1975) Endocrinology 95, 662–677
[11] Dunn, L.A and Holz, R.W (1983) J Biol. Chem 258, 4989–4993
[12] Wilson, S P and Kirshner, N (1983) J Biol Chem 258, 4994–5000
[13] Knight, D.E. and Baker, P.F (1982) J Membrane Biol. 68, 107–140
[14] Greenberg, A. and Zinder, O. (1982) Cell Tissue Res. 226, 655–656
[15] Douglas, W W (1968) Br. J. Pharmacol. 34, 451–474.
[16] Douglas, W W and Rubin, R P (1961) J Physiol. 159, 40–57.
[17] Kidokoro, Y and Ritchie, A.K. (1980) J Physiol 307, 199–216.
[18] Oka, M., Isosaki, M and Watanabe, J. (1980) Adv Biosci 36, 29–36
[19] Ungar, A and Phillips, J H (1983) Physiol Rev 63, 787–843
[20] Levine, M , Asher, A , Pollard, H. and Zinder, O (1983) J Biol. Chem 258, 13111–13115.
[21] Zimmermann, U , Pilwat, G and Riemann, F. (1974) Biophys J 14, 881–899