Release of Dopamine and ATP from PC12 Cells Treated with Dexamethasone, Reserpine and Bafilomycin A1

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ABSTRACT. The amounts and time courses of dopamine and ATP released from perfused PC12 cells were examined using a simultaneous on-line recording system. High KCl (60 mM) caused dopamine and ATP release with similar time courses. The relative amount of dopamine to ATP in the effluent was 9.5. In PC12 cells cultured with dexamethasone, reserpine or bafilomycin A1 for 2 days, these drugs did not affect increases of intracellular Ca\(^{2+}\) in response to high KCl. Dexamethasone doubled the amount of dopamine release induced by high KCl without changing the amount of ATP release. High KCl failed to cause dopamine release in reserpine-treated cells but evoked ATP release. Bafilomycin A1 decreased both high KCl-induced dopamine and ATP release. The ratio of released ATP to total adenine nucleotides and adenosine in response to high KCl was not changed by treatment with the drugs. These results suggest that dopamine and ATP are simultaneously released from secretory vesicles of PC12 cells, in which they are stored via different pathways. Similar to dopamine uptake into secretory vesicles, the H\(^+\)-gradient across the vesicular membrane developed by vacuolar ATPase may play an important role in the vesicular uptake of ATP.

KEY WORDS: ATP, dopamine, on-line, PC12 cell, V-ATPase.

It is well known that catecholamine (CA) is stored together with adenosine 5’-triphosphate (ATP) in secretory vesicles of the adrenal medulla [32] and pheochromocytoma (PC12) cells [31]. In adrenal chromaffin cells, it has been reported that CA is present in secretory vesicles but not in the cytosol [24] and that 75% of ATP in the cells is in secretory vesicles [7]. ATP stored in secretory vesicles has been shown to be released on exocytosis [5]. ATP has been reported to evoke CA release from adrenal chromaffin cells [1, 15] and PC12 cells [13] and to inhibit Ca\(^{2+}\) currents in adrenal chromaffin cells [3, 23].

Continuous and on-line assay methods for CA released from cultured adrenal chromaffin cells [11, 17] and perfused adrenal glands [4, 18] have been developed using an electrochemical detector. We previously reported a simultaneous on-line assay method for CA and ATP released from cultured porcine adrenal chromaffin cells using an electrochemical detector and luciferin-luciferase, respectively [14]. Using this system, we found that CA and ATP appeared in the effluent with similar time courses in response to various secretagogues including high KCl. In the sympathetic nerves of the guinea-pig vas deferens, however, it has been reported that the ratios of CA and ATP appearing in the effluent greatly change during stimulation, suggesting the presence of heterogeneous secretory vesicles with respect to the concentrations of CA and/or ATP [28] and post-release degradation of ATP by soluble ectonucleotidases [29]. Furthermore, the vesicles isolated from PC12 cells have been reported to contain CA and ATP at various concentration ratios (CA/ATP) ranging from 13 to 29 [31].

It is well known that ATP and CA are taken up into secretory vesicles from cytosol. A proton gradient across the vesicular membrane developed by vacuolar-type H\(^+\)-ATPase (V-ATPase) is reported to play an important role in the uptake of CA through the vesicular monoamine transporter (VMAT) [21]. It has been reported that ATP uptake is also inhibited by bafilomycin A1, a selective V-ATPase inhibitor, in bovine chromaffin granule ghosts [2]. In addition, reserpine which is a specific inhibitor of VMAT [10] decreases not only CA content but also ATP content in the vesicles of bovine chromaffin cells [6]. Therefore, using on-line assay method for CA and ATP, it is possible to examine the effects of various agents affecting the synthesis and storage of CA on the amount of ATP released from PC12 cells.

The aim of the present experiments was to study the time courses of dopamine and ATP release in response to high KCl in PC12 cells cultured for 2 days with dexamethasone, a synthetic glucocorticoid which up-regulate CA biosynthesis enzymes [16, 27], reserpine and bafilomycin A1 using a simultaneous on-line assay of dopamine and ATP and then discussed the mechanisms for uptake and storage of CA and ATP.

MATERIALS AND METHODS

Cell culture: Pheochromocytoma (PC12) cells were obtained from a cell bank (RIKEN, Tsukuba, Japan) and cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Grand Island, NY) with 10% horse serum (Gibco, Grand Island, NY), 10% fetal bovine serum (PAA, Linz, Austria), penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C in 5% CO\(_2\). The cells were treated with dexam-
ethasone (Wako, Osaka, Japan), reserpine (Ciba-Geigy, Osaka, Japan) or bafilomycin A1 (Sigma, St. Louis, MO) for 2 days and then used after these drugs were washed out. The culture medium was replaced every other day with or without drugs. Viability of cells treated with the drugs was examined with dye exclusion test for erythrosine B. The experiments for release of adenosine nucleotides and adenosine (AN) were performed with the cells cultured on polystyrene dishes (60 mm in diameter) to evaluate the breakdown of released ATP. After the cells were washed twice with physiological saline solution (PSS), high KCl (60 mM) solution was added. The solution was collected 3 min after incubation at 37°C and then subjected to HPLC. The composition of PSS was as follows (mM): NaCl, 135; KCl, 5; CaCl2, 2.5; MgCl2, 1.2; glucose, 10; HEPES, 5. The pH was adjusted to 7.4 with NaOH. In high KCl solution (60 mM), a corresponding amount of NaCl was replaced with KCl. To measure the cellular content of ATP and dopamine by HPLC, the cells cultured on 22 mm dishes were suspended with citrate-phosphate buffer and 0.4 N perchloric acid, respectively, and amounts of cellular content of AN and dopamine were expressed as pmol/µg DNA. For measurement of the intracellular Ca²⁺ concentration ([Ca²⁺]), the cells were cultured on cover slips (12 mm in diameter) placed in the dish. For on-line measurement of dopamine and ATP release, the cells were cultured on dishes (35 mm in diameter) and perfused continuously (See below). DNA content in each culture dish was determined with a spectrofluorometer (FP770, Jasco, Tokyo) according to the method described by Goppelt-Struebe and Golombek [8]. All experiments were done when PC12 cells allowed to grow to subconfluence (about 80% of bottom area of culture dish).

**On-line measurement of dopamine and ATP:** The experimental setup used for the on-line measurement of dopamine and ATP release was described elsewhere [14]. In brief, the culture dish was covered with a silicon rubber cap with an inlet and outlet (0.5 m in volume), and perfused with PSS using a peristaltic pump (MINIPULS 3, Gilson, France) at a flow rate of 2 ml/min at 37°C. Before starting experiments, cells were perfused over 30 min to remove cell debris and detached cells. The effluent from the chamber was separated into two equal parts. One was introduced into an electrochemical detector (840-EC, Jasco, Tokyo) equipped with a flow cell having a carbon plate electrode for continuous measurement of dopamine. The electrode potential was set at +0.45 V. The other part of the effluent was introduced into a reaction chamber (0.3 ml), which was placed in front of the photomultiplier of an ATP photometer (model 2000, JRB, LA) for measurement of ATP. Luciferin-luciferase mixture solution was also flowed into the chamber at a flow rate of 1 ml/min and mixed with the effluent by a mixer (Physicotoron, Niti-on, Funabashi, Japan) in the reaction chamber. The luciferin-luciferase mixture was prepared with synthetic D-luciferin (Sigma, St. Louis, MO) and luciferase, which was purified from fire fly lantern extracts (Sigma, St. Louis, MO) as described elsewhere [14].

Signals from the electrochemical detector and ATP photometer were recorded with a pen-writing recorder (Type 2210, LKB, Bromma, Sweden) and data recorder (Type 59ESJ, Sony, Japan) for later analysis using a personal computer in conjunction with an interface (MacLab, AD Instruments, Australia). A mixture of 0.1 µM of ATP and 1 µM of dopamine standard solution was applied in this system before experiments for calibration. The amounts of dopamine and ATP released into the effluent were expressed as pmol/µg DNA.

**[Ca²⁺]:** The intracellular Ca²⁺ concentration ([Ca²⁺]) in PC12 cells on cover slips was measured with a fluorescent Ca²⁺ indicator, fura-2 [9], by dual excitation microfluorometry using a spectrofluorometer (CAM-200, Jasco, Tokyo) as described previously [22]. To load fura-2 into PC12 cells, the cells were incubated with fura-2 acetoxymethyl ester (fura-2-AM, 5 µM; Wako, Osaka, Japan) for 0.5–1 hr at room temperature. Fluorescent signals were stored on the hard disk of the computer (Macintosh, Apple, Japan) through an A/D converter (MacLab 4e, AD Instruments, Australia) and on a data recorder (Neuro-corder DR-484, Neurodata Instrument, U.S.A.).

**Assay of dopamine, adenosine nucleotides and adenosine by HPLC:** Cell contents of dopamine were measured using an electrochemical detector (EC-100, EICOM, Kyoto, Japan) connected to the HPLC (801-SC, Jasco, Tokyo) system, after cells were treated with 0.4 N perchloric acid [26].

Cell extracts and the metabolites of ATP released from the cells were converted into ethenopurines [14, 20]. In brief, 10 µl of chloroacetalddehyde (40%, Wako, Osaka, Japan) was added to a mixture of 250 µl of samples and 90 µl of citrate-phosphate. The reaction mixture was incubated in a dry bath at 80°C for 40 min after addition of 9-β-D-arabinofuranosyladenine (Sigma, St. Louis, MO) as an internal standard. The separation of the ethenopurine derivatives was achieved by reverse-phase chromatography on an analytical Ultron N-phenyl HPLC column (0.46 × 15 cm, 5 µm particle size, Shinwa Chemical Industries, Tokyo) connected to a guard column (0.46 × 1 cm). Fluorescence of derivatives was measured using a spectrofluorometer (FP-540D, Jasco, Tokyo). The wavelengths for excitation and emission were set at 300 nm and 420 nm, respectively. The mobile phase contained 5 mM citrate buffer with 0.2% acetonitrile, the pH of which was adjusted to 4.5 with 2-diethylaminoethanol. The mobile phase was run at a flow rate of 1.0 ml/min at room temperature.

**Statistical analysis:** The data are presented as the mean ± S.E.M. (n = number of observations). Statistical significance was assessed using Student’s t-test in the case of the time to the peak of dopamine and ATP release and the Dunnett test in all other cases. P values of less than 0.05 were considered to be significant.

**RESULTS**

*On-line measurement of dopamine and ATP released from PC12 cells:* Figure 1A shows typical recordings of releases of dopamine and ATP from PC12 cells in response...
to high KCl (60 mM) and the relationship between the half decay time for dopamine and ATP release, which was defined as the time required until the response attenuated by half. High KCl caused transient increases in dopamine in the effluent. The time to the peak of dopamine and ATP releases induced by high KCl was 25.7 ± 1.4 and 26.0 ± 1.5 sec, respectively. There was no statistically significant difference in the time to peak between dopamine and ATP release. The relation of half decay time to dopamine and ATP release in response to high KCl shows almost unity (Fig. 1B), indicating that the time courses of dopamine and ATP release were almost identical. The molar ratio of total dopamine to total ATP evoked by high KCl was 9.5 ± 1.3 (n=15).

Effects of dexamethasone, reserpine and bafilomycin A1 on dopamine and ATP contents and increases in [Ca^{2+}], in response to high KCl: Dopamine and AN (ATP, ADP, AMP and adenosine) contents of PC12 cells (pmol/µg DNA) were examined after cells were treated for 2 days with dexamethasone (1 µM), reserpine (0.1 µM), bafilomycin A1 (0.1 µM) or without drugs (Table 1). The viability of the cells were more than 96% in each 2-days treatment. Under these conditions, dexamethasone increased dopamine content to about 280%, but had no effect on AN content in PC12 cells. Reserpine significantly decreased dopamine content but not AN content. Bafilomycin A1 decreased both dopamine and AN contents to the same extent.

Table 1. Cellular contents of dopamine and adenine nucleotide and adenosine

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Dopamine (pmol/µg DNA)</th>
<th>AN (pmol/µg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>103 ± 4.4</td>
<td>18.4 ± 2.9</td>
</tr>
<tr>
<td>Dexamethasone (1 µM)</td>
<td>282 ± 14*</td>
<td>15.3 ± 2.9</td>
</tr>
<tr>
<td>Reserpine (0.1 µM)</td>
<td>2.0 ± 0.6*</td>
<td>16.5 ± 2.3</td>
</tr>
<tr>
<td>Bafilomycin A1 (0.1µM)</td>
<td>46 ± 1.0*</td>
<td>10.7 ± 1.4*</td>
</tr>
</tbody>
</table>

Effects of drugs on cellular contents of dopamine and AN (ATP, ADP, AMP and adenosine) when PC12 cells were cultured with these drugs for 2 days (n = 7). *, Significant difference vs. control value (p<0.05).

We examined whether these treatments produced any changes in increases of the intracellular Ca^{2+} concentrations ([Ca^{2+}]), induced by high KCl. In control cells untreated with these drugs, high KCl (60 mM) caused a rapid rise in [Ca^{2+}], which attained a maximum within 10 sec and gradually decreased (Fig. 2A). As shown in Fig. 2B, there were no statistically significant differences of increases in [Ca^{2+}], induced by high KCl among these treatments.

On-line measurement of dopamine and ATP released...
from PC12 cells treated with dexamethasone, reserpine and bafilomycin A1: Figure 3A shows typical dopamine and ATP releases evoked by high KCl (60 mM) for 30 sec after 2-days treatment without (control) or with dexamethasone, reserpine or bafilomycin A1. In control cells, high KCl caused the release of dopamine and ATP, the amounts of which were 21.6 ± 1.8 and 2.9 ± 0.4 pmol/µg DNA (n=15), respectively. In dexamethasone-treated cells, the amount of dopamine released from PC12 cells in response to high KCl was greatly increased about 2.5-fold of the control response (n=6), but the amount of released ATP unchanged. In reserpine-treated cells, however, the amount of dopamine release induced by high KCl was significantly decreased to 3.3% of the control level without any effects on the amount of ATP release. On the other hand, in bafilomycin A1-treated cells, both dopamine and ATP release in response to high KCl were significantly decreased to 49% and 25% of control responses, respectively. The time to peak of dopamine release in cells treated with dexamethasone, bafilomycin A1 and without drugs was 26.5 ± 1.7, 25.6 ± 1.0 and 27.4 ± 1.0 sec, respectively. There were no statistical differences among them. The time to peak of ATP release in control cells and cells treated with dexamethasone, reserpine and bafilomycin A1 was 26.0 ± 0.9, 25.4 ± 2.3, 27.3 ± 1.9 and 25.6 ± 1.0 sec, respectively. There were also no statistical differences among them (n=15: control, n=6: with drug).

Breakdown of released ATP: The released ATP might decompose to ADP, AMP or adenosine, because ectonucleotidases are present on the plasma membrane [19] and the release of soluble nucleotidases by stimulation is also evident in PC12 cells and sympathetic neurons [29]. Therefore, we measured the amounts of ATP, ADP, AMP and adenosine appearing in the supernatant in response to high KCl from PC12 cells treated with dexamethasone, reserpine or bafilomycin A1 (Table 2). ATP, ADP, AMP and adenosine (AN) were not detectable in the supernatant without stimulation. The relative amounts of ATP, ADP, AMP and adenosine to AN (adenine nucleotides plus adenosine) appearing in the supernatant after high KCl stimulation were not significantly different among these treatments.

DISCUSSION

The present results clearly indicated that high KCl caused dopamine and ATP release with equal time course. The present study is the first report to show the simultaneous measurement of dopamine and ATP secretion from PC12 cells. The relative amount of dopamine to ATP (molar ratio: dopamine/ATP) in the effluent was 9.5, the value of which was similar to that of catecholamine (CA) to ATP observed in porcine adrenal chromaffin cells [14]. In addition, acetylcholine and BaCl2 also produced dopamine and ATP secretion with the same time courses and the similar molar ratio (data not shown). It has been proposed that there are different stages in chromaffin vesicles, such as immature and releasable vesicles in adrenal chromaffin cells [30]. These results suggest that releasable vesicles in PC12 cells contain dopamine and ATP at a constant molar ratio like adrenal chromaffin cells. On the other hand, the vesicle fraction biochemically isolated from PC12 cells is reported to contain dopamine and ATP at various concentration ratios ranging from 13 to 29 [31]. This might be due to the presence of immature vesicles with different molar ratios of dopamine to ATP.

The enzymatic degradation of released ATP has been reported to occur with ectonucleotidases present in the plasma membrane [19] and with soluble nucleotidases [29]. In the present experiment, however, the ratio of released ATP to AN (adenine nucleotides and adenosine) in response to high KCl was almost constant in PC12 cells treated with dexamethasone, reserpine or bafilomycin A1. These results suggest that the enzymatic degradation of released ATP is constant under these conditions. Moreover, these treatment
did not modify the rise in \([Ca^{2+}]_i\) in response to high KCl.

Dexamethasone is known to up-regulate all CA biosynthetic enzymes such as tyrosine hydroxylase, aromatic L-amino acid decarboxylase and dopamine \(\beta\)-hydroxylase [16] and to increase the amount of CA in vesicles of PC12 cells [27], whereas reserpine is reported to specifically bind to the vesicular monoamine transporter (VMAT) [25] and result in the loss of CA in the vesicles. This was true in the present experiment. However, ATP release induced by high KCl was not affected by these treatments. This split of dopamine and ATP release may be due to differences in the amounts of dopamine and ATP in releasable vesicles. Although Caughey and Kirshner [6] reported that CA depletion by reserpine leads to the loss of vesicular ATP in bovine chromaffin cells, this was not the case in PC12 cells treated with reserpine for 2 days. It has been reported that CA stored in the secretory vesicles makes a complex with ATP at a constant molar ratio (CA:ATP=4:1) [12]. Our results indicate that this is not a only mechanism for CA storage in secretory vesicles of PC12 cells. Taken together, these results suggest that the vesicular storage of dopamine and ATP are independently regulated in PC12 cells.

The transport of monoamines into secretory vesicles through VMAT is driven by the \(H^+\) gradient generated by vacuolar-\(H^+\)-ATPase [25]. In our experiment, ATP and dopamine release induced by high KCl were greatly decreased by bafilomycin A1, which is a specific V-ATPase inhibitor. It has been reported that ATP transport into chro-
moffin granule ghosts is reduced by bafilomycin A1 [2]. Therefore, it seems likely that the inhibition of vesicular V-
ATPase by bafilomycin A1 results in the loss of both vesic-
ular substances of PC12 cells. Similar to dopamine uptake
into secretory vesicles, the H+-gradient across the vesicular
membrane developed by vacuolar ATPase may play an
important role in the vesicular uptake of ATP.

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REFERENCES

1. Asano, T., Otsuguro, K., Ohta, T., Sugawara, T., Ito, S. and
Nakazato, Y. 1995. Characteristics of ATP-induced catechol-
amine secretion from adrenal chromaffin cells of the guinea-pig.
transport into chromaffin granule ghosts. J. Biol. Chem. 271:
17132–17138.
90.
toring of catecholamine release from perfused cat adrenals. J.
5. Burnstock, G. 1981. Neurotransmitters and trophic factors in
the autonomic nervous system. J. Physiol. (Lond.) 313: 1–35.
tetrazenine on catecholamine and ATP storage in cultured
bovine adrenal medullary chromaffin cells. J. Neurochem. 49:
563–573.
and storage of newly synthesized adenine nucleotides in bovine
determination of the DNA content of the cells cultured in tissue
generation of Ca2+ indicators with greatly improved fluores-
10. Henry, J.P., Sagné, C., Botton, D., Isambert, M.F. and Gasnier,
B. 1998. Molecular pharmacology of the vesicular monoamine
11. Hillarp, N.A. and Thieme, G. 1959. Nucleotides in the cate-
cholamine granules of the adrenal medulla. Acta Physiol.
Scand. 45: 328–333.
Extracellular adenosine 5’-triphosphate-evoked norepinephrine
secretion not relating to voltage-gated Ca channels in pheo-
1999. On-line measurement of adenosine triphosphate and cat-
echolamine released from adrenal chromaffin cells. Comp. Bio-
from extracellular and intracellular sources are different
as shown by simultaneous measurements of cytosolic Ca2+ and
tion of catecholamine biosynthetic enzymes by dexamethasone
in PC12 cells. J. Neurochem. 60: 946–951.
toring of the secretory function of cultured adrenal chromaffin
of total catecholamine secretion from the adrenal gland in
Methods 24: 39–43.
19. Mohri, K., Takeuchi, K., Shinozuka, K., Bujr, R.A. and West-
fall, D.P. 1993. Simultaneous detection of nerve-induced ade-
nine nucleotides and nucleosides released from rabbit pulmo-
membrane proton-adenosinetriphosphatases. Physiol. Rev. 79:
361–385.
currents induced by muscarinic receptor activation in guinea
22. Otsuguro, K., Ohta, T., Ito, S. and Nakazato, Y. 1996. Modula-
tion of calcium current by ATP in guinea-pig adrenal chromaf-
chromaffin granules: a model for hormone and neurotransmit-
The role of Na+ in muscarinic receptor-mediated catechola-
mine secretion in the absence of extracellular Ca2+ in cat per-
26. Tischler, A.S., Perlman, R.L., Morse, G.M. and Shepard, B.E.
1983. Glucocorticoids increase catecholamine synthesis and
storage in PC12 pheochromocytoma cell cultures. J. Neuro-
27. Todorov, L.D., Mihaylova-Todorova, S., Craviso, G.L., Bju-
release of the cotransmitters ATP and noradrenaline from symp-
thetic nerves of the guinea-pig vas deferens. J. Physiol. 496:
731–748.
28. Todorov, L.D., Mihaylova-Todorova, S., Westfall, T.D., Sned-
ronal release of soluble nucleotidases and their role in
29. Uvnas, B. 1991. The molecular mechanism of nondegranula-
tive release of biogenic amines. J. Physiol. Pharmacol. 42:
211–219.
30. Wagner, J.A. 1985. Structure of catecholamine secretory ves-
31. Winkler, H. and Westhead, E. 1980. The molecular organiza-
tion of adrenal chromaffin granules. Neuroscience 5: 1803–
1823.