Stimulation of secretion in permeabilized PC12 cells by adenosine 5'-[γ -thio]triphosphate: possible involvement of nucleoside diphosphate kinase

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The addition of Ca^{2+} , adenosine 5'-[γ -thio]triphosphate (ATP[S]) or guanosine 5'-[γ -thio]triphosphate (GTP[S]) to digitonin-permeabilized PC12 cells stimulates noradrenaline secretion. Both ATP[S] and GTP[S] stimulate release in the absence of Ca^{2+} . Whereas ADP and adenosine 5'-[$\beta\gamma$ -imido]triphosphate inhibited ATP[S]-stimulated release, they did not inhibit Ca^{2+} -stimulated release even in the absence of added ATP. This suggests that the kinase which uses ATP[S] to induce secretion may not play an essential role in Ca^{2+} -stimulated release. As GTP[S]-stimulated and ATP[S]-stimulated secretions were not additive, it seemed possible that stimulation by ATP[S] might in part result from the thiophosphorylation of GDP by nucleoside-diphosphate (NDP) kinase to form GTP[S]. The following results

are consistent with this possibility. (1) A low concentration of GDP increased ATP[S]-stimulated secretion, but not GTP[S]-stimulated or Ca^{2+} -stimulated secretion. (2) A variety of ribo- and deoxyribo-nucleoside di- and tri-phosphates inhibited ATP[S]-stimulated secretion, but not GTP[S]-stimulated or Ca^{2+} -stimulated secretion. Thus, like NDP kinase, the kinase which uses ATP[S] to cause noradrenaline release appears to have a very low specificity for ATP. (3) Incubation of permeabilized cells in a sucrose-containing buffer resulted in the preferential loss of ATP[S]-stimulated secretion and a decrease in the level of NDP kinase. The addition of rat liver NDP kinase to those depleted cells partially restored ATP[S]-stimulated secretion.

INTRODUCTION

In response to nicotine, ATP, or K^+ depolarization, rat pheochromocytoma PC12 cells secrete catecholamines in a Ca^{2+} -dependent manner [1–5]. Treatment of PC12 cells with a low concentration of digitonin permeabilizes the plasma membrane, but leaves the secretory vesicles intact [6,7]. Release of catecholamines by these permeabilized cells is Ca^{2+} -dependent [6–9] and appears to result from exocytosis [7].

The addition of adenosine 5'-[γ -thio]triphosphate (ATP[S]) to digitonin-permeabilized PC12 cells stimulates Ca^{2+} -independent noradrenaline (NA) secretion [9]. ATP inhibits this stimulation by ATP[S]. Although many kinases use ATP[S] to thiophosphorylate proteins, thiophosphorylated proteins are resistant to dethiophosphorylation by protein phosphatases. Adenosine 5'-[$\beta\gamma$ -imido]triphosphate (p[NH]ppA) is not a substrate for protein kinases, and it does not stimulate NA secretion [9]. These results suggest that thiophosphorylation can induce secretion in permeabilized PC12 cells. Since Ca^{2+} appeared to increase the rate of modification by ATP[S], we proposed that secretion in permeabilized PC12 cells might be regulated by a Ca^{2+} -stimulated protein phosphorylation [9]. The secretion evoked by ATP[S] in the absence of Ca^{2+} was thought to be due to the basal activity of this kinase. Experiments with protein phosphatase inhibitors and isolated protein phosphatases are consistent with protein phosphorylation playing an important role in regulating NA secretion [10].

Guanosine 5'-[γ -thio]triphosphate (GTP[S]) or guanosine 5'-[$\beta\gamma$ -imido]triphosphate (p[NH]ppG), but not GTP, also stimulates Ca^{2+} -independent NA secretion by digitonin-permeabilized PC12 cells [11]. Stimulation of NA secretion by GTP[S] or p[NH]ppG appears to be independent of any known second

messengers and may involve a low-molecular-mass GTP-binding protein [11,12]. The secretory activities of other types of cells are also affected by GTP[S] or p[NH]ppG [13].

Activation of K^+ -channel currents in atrial myocytes by ATP[S] [14], stimulation of superoxide formation in membranes of HL-60 leukaemic cells by ATP[S] [15] and activation of adenylate cyclase in human platelet membranes by ATP[S] [16] are thought to result from the thiophosphorylation of GDP by nucleoside diphosphate kinase (NDP kinase) to give GTP[S]. Since 1 mM GTP did not inhibit secretion induced by 1 mM ATP[S], but did cause about a 50% inhibition of secretion induced by 1 mM GTP[S], we originally thought that the stimulation of NA secretion in permeabilized PC12 cells by ATP[S] did not result from the formation of GTP[S] [9].

We have performed a number of experiments to test our hypothesis that the stimulation of NA secretion of ATP[S] in the absence of Ca^{2+} results from the basal activity of a Ca^{2+} -stimulated kinase. The results of these experiments do not support our original conclusions, but rather suggest that at least part of the effect of ATP[S] on Ca^{2+} -independent secretion results from the formation of GTP[S] by NDP kinase. The time course of ATP[S]-stimulated secretion and the relatively poor inhibition of ATP[S]-stimulated secretion by GTP suggest that the formation of GTP[S] from ATP[S] by NDP kinase may occur in close proximity to the GTP-binding protein responsible for stimulating NA secretion.

MATERIALS AND METHODS

PC12 cells were cultured as described previously [9]. For secretion experiments, the cells were plated on polylysine-coated 48-well

Abbreviations used: ATP[S], adenosine 5'-[γ -thio]triphosphate; p[NH]ppA, adenosine 5'-[$\beta\gamma$ -imido]triphosphate; p[NH]ppG, guanosine 5'-[$\beta\gamma$ -imido]triphosphate; GTP[S], guanosine 5'-[γ -thio]triphosphate; NDP kinase, nucleoside-diphosphate kinase; NA, noradrenaline.

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multiwell plates at about 3×10^5 cells/cm² and used 2 or 3 days after plating.

The protocol used for measuring NA release is similar to that described previously [9]. [³H]NA-labelled PC12 cells were permeabilized at room temperature with 7.5 μ M digitonin in 125 μ l of KG-buffer (139 mM potassium glutamate/2 mM MgCl₂/5 mM EGTA/20 mM Pipes, pH 6.6). After 6 min, the permeabilization buffer was removed, and [³H]NA secretion was measured in KG-buffer or in KG-buffer containing the nucleotides indicated. When nucleotides were added to the KG-buffer, an equal concentration of MgCl₂ was also added. After 15 min, the release media were removed and placed on ice until centrifugation at 12000 *g* for 4 min. The supernatant from this centrifugation was removed and counted for radioactivity. The cells attached to the dish were solubilized with 125 μ l of 5 mM Pipes and 1% Triton X-100, combined with the pellet from the above centrifugation and counted for radioactivity. [³H]NA release is expressed as percentage calculated by multiplying the amount of [³H]NA in the supernatant by 100 and dividing by the total amount of [³H]NA recovered. Three wells were used for all determinations, and release values are reported as means $\pm$ S.D. In some experiments the permeabilized cells were incubated in KG-buffer for 10 or 30 min before addition of release media. The level of secretion is expressed relative to the amount of NA present after these preincubations. Free Ca²⁺ concentrations were calculated as described by Fabiato and Fabiato [17]. All experiments were repeated on at least three separate occasions with similar results.

Rat liver NDP kinase was purified as described by Kimura and Shimada [18]. Antibody against rat liver NDP kinase was raised in rabbits and affinity-purified [18]. To determine relative amounts of NDP kinase, the digitonin-permeabilized PC12 cells were lysed with 0.2 M NaCl/5 mM EDTA/5 mM EGTA/25 mM Tris (pH 7.5)/0.5% Triton X-100. The extracted proteins from 1.5×10^6 cells were separated by SDS/PAGE, transferred to poly(vinylidene difluoride) membranes, and analysed by immunoblotting using affinity-purified anti-(NDP kinase) antibodies. Immunoreactive bands were made visible by using goat anti-rabbit antibodies coupled to alkaline phosphatase and a chemiluminescent substrate (Bio-Rad Laboratories) in accordance with the manufacturer's instructions.

RESULTS

Comparison of Ca²⁺-stimulated with ATP[S]-stimulated NA release

The amount of NA released from digitonin-permeabilized PC12 cells in the absence of Ca²⁺ is much greater with ATP[S] than with ATP or no added nucleotide (Table 1). In the presence of saturating Ca²⁺ (10 μ M), comparable levels of NA secretion are obtained with ATP and ATP[S]. We have proposed that this stimulation of NA secretion by ATP[S] in the absence of Ca²⁺ might result from the basal activity of a Ca²⁺-stimulated kinase and that Ca²⁺-stimulated secretion results from the stimulation of this kinase by Ca²⁺ [9].

If Ca²⁺-stimulated secretion requires the action of a Ca²⁺-stimulated kinase, it should be ATP-dependent. However, when secretion was measured immediately after permeabilization, there was a high level of Ca²⁺-stimulated NA release in the absence of added ATP (Table 1). Even after the permeabilized cells were incubated for 30 min in KG-buffer, there was still a significant amount of Ca²⁺-stimulated NA secretion in the absence of added ATP (Table 1).

One explanation for the high level of Ca²⁺-stimulated release in the absence of added ATP is that the cells contain sufficient residual ATP for the kinase to phosphorylate its target protein. To examine this possibility, we compared the effects of ADP and

p[NH]ppA on Ca²⁺-stimulated NA release in the absence of added ATP and on NA release in the presence of 1 mM ATP[S] and no Ca²⁺. ATP[S]-stimulated NA secretion was inhibited by about 60% by 2 mM MgADP, by 85% by 10 mM MgADP and by 70% by 10 mM Mg-p[NH]ppA. These inhibitions are consistent with these nucleotides competing with ATP[S] for the kinase. In contrast, 10 mM MgADP or 10 mM Mg-p[NH]ppA caused no inhibition of Ca²⁺-stimulated release even in the absence of added ATP.

If the same kinase is responsible for both ATP[S]-stimulated and Ca²⁺-stimulated NA secretion, why were both reactions not inhibited by ADP and p[NH]ppA? This difference does not appear to result from the kinase having a much higher affinity for ATP than for ATP[S], as 1 mM ATP gave only about a 50% decrease in the secretion caused by 1 mM ATP[S] [9].

Comparison of ATP[S]-stimulated with GTP[S]-stimulated NA secretion

GTP[S] and p[NH]ppG also stimulate Ca²⁺-independent NA secretion by digitonin-permeabilized PC12 cells. Immediately after permeabilization, GTP[S]-stimulated NA secretion was slightly dependent on added ATP (Table 2). The amount of NA release in the presence of 1 mM GTP[S] plus 1 mM ATP[S] was only slightly greater than that obtained in the presence of 1 mM

Table 1 Ca²⁺-stimulated and ATP[S]-stimulated NA secretion

Cells prelabelled with [³H]NA were permeabilized with digitonin. After 6 min, the permeabilization buffer was removed, and either [³H]NA secretion was measured immediately in KG-buffers in the presence of the nucleotides indicated, or the permeabilized cells were first incubated for 30 min in KG-buffer without added nucleotides, and then [³H]NA secretion was measured in KG-buffers in the presence of the nucleotides indicated. Ca²⁺-stimulated release is [³H]NA released in 10 μ M Ca²⁺ minus that released in the absence of Ca²⁺.

Nucleotide added to release buffers	NA released (%)		
	No Ca ²⁺	10 μ M Ca ²⁺	Ca ²⁺ -stimulated
[³ H]NA release measured immediately after permeabilization			
None	4.7 $\pm$ 0.3	18.2 $\pm$ 0.8	13.5
2 mM MgATP	4.9 $\pm$ 0.5	25.8 $\pm$ 0.3	20.9
1 mM MgATP[S]	13.7 $\pm$ 0.3	24.7 $\pm$ 0.7	
[³ H] NA release measured after 30 min incubation in the absence of ATP			
None	5.9 $\pm$ 0.3	15.2 $\pm$ 0.5	9.3
2 mM MgATP	5.5 $\pm$ 0.2	20.6 $\pm$ 1.0	15.1
1 mM MgATP[S]	11.3 $\pm$ 0.2	16.4 $\pm$ 0.1	

Table 2 Effects of ATP and ATP[S] on GTP[S]-stimulated NA release

Cells prelabelled with [³H]NA were permeabilized with digitonin. After 6 min, the permeabilization buffer was removed, and [³H]NA secretion measured in KG-buffer in the presence of the nucleotides indicated.

Nucleotides added to release buffers	NA released (%)	
	No Ca ²⁺	Increase over basal
None	3.5 $\pm$ 0.9	
1 mM MgGTP[S]	14.0 $\pm$ 0.5	10.5
1 mM MgGTP[S] and 2 mM MgATP	17.8 $\pm$ 0.9	14.3
1 mM MgGTP[S] and 1 mM MgATP[S]	15.9 $\pm$ 0.2	12.4
1 mM MgATP[S]	11.1 $\pm$ 0.2	7.6

Figure 1 Time courses of NA secretion

Cells prelabelled with [3 H]NA were permeabilized with digitonin, and the amount of [3 H]NA released was determined after various lengths of time in KG-buffer containing 1 mM MgATP[S] (○), 1 mM GTP[S] plus 2 mM MgATP (●), 100 μ M GTP[S] plus 2 mM MgATP (■), or 2 mM MgATP (-----).

Figure 2 Effect of GTP on ATP[S]-stimulated and GTP[S]-stimulated NA secretion

Cells prelabelled with [3 H]NA were permeabilized with digitonin, and then [3 H]NA secretion was measured in KG-buffer containing 1 mM MgATP[S] (○), 1 mM MgGTP[S] plus 2 mM MgATP (●), or 100 μ M MgGTP[S] plus 2 mM MgATP (■). To these solutions were added the indicated concentration of MgGTP.

GTP[S] by itself, and much less than the sum of GTP[S]-stimulated and ATP[S]-stimulated NA releases (Table 2). Since the effects of ATP[S] and GTP[S] were not additive, a more detailed comparison was made between the stimulation of NA release by ATP[S] and by GTP[S]. In the rest of the experiments described here, GTP[S]-stimulated secretion was measured in the presence of 2 mM ATP to inhibit the possible use of GTP[S] by ATP-dependent protein kinases [7].

The time courses of NA release in the presence of 1 mM ATP[S] and in the presence of either 100 μ M or 1 mM GTP[S] are shown in Figure 1. There was no obvious delay between the time of addition of either 1 mM ATP[S] or 1 mM GTP[S] and the stimulation of NA, but there was about a 3 min delay between the time of addition of 100 μ M GTP[S] and stimulation of NA release.

GTP inhibited both GTP[S]-stimulated and ATP[S]-stimulated NA secretion (Figure 2). Although 1 mM ATP[S] evoked less NA secretion than did 100 μ M GTP[S], 1 mM GTP gave nearly

Table 3 Effects of different nucleotides on ATP[S]-stimulated and GTP[S]-stimulated NA secretion

[3 H]NA secretion was measured in the absence of Ca^{2+} and in the presence of the nucleotides indicated. ATP[S]-stimulated secretions are expressed relative to the amount of [3 H]NA released in the presence of 1 mM MgATP[S] and no added nucleotide. GTP[S]-stimulated secretions are expressed relative to the amount of [3 H]NA released in the presence of 1 mM MgGTP[S] and 2 mM MgATP. The results shown are means $\pm$ S.D. of determinations from three wells. All the relative values were rounded to the nearest 5%.

Other nucleotides added to release	Relative levels of NA secretion	
	ATP[S]-stimulated	GTP[S]-stimulated
None	100	—
2 mM MgATP	30 $\pm$ 5	100
5 mM MgATP	10 $\pm$ 5	90 $\pm$ 5
2 mM MgGTP	40 $\pm$ 5	50 $\pm$ 5
5 mM MgGTP	15 $\pm$ 5	15 $\pm$ 5
5 mM MgITP	50 $\pm$ 10	110 $\pm$ 5
5 mM MgUTP	20 $\pm$ 5	95 $\pm$ 5
5 mM MgCTP	50 $\pm$ 5	95 $\pm$ 10
2 mM MgADP	60 $\pm$ 5	—
5 mM MgADP	25 $\pm$ 5	85 $\pm$ 10
2 mM MgGDP	40 $\pm$ 5	45 $\pm$ 5
5 mM MgGDP	10 $\pm$ 5	20 $\pm$ 5
5 mM MgIDP	70 $\pm$ 10	110 $\pm$ 10
5 mM MgUDP	45 $\pm$ 5	90 $\pm$ 5
5 mM MgCDP	90 $\pm$ 5	125 $\pm$ 5
2 mM MgAMP	25 $\pm$ 5	95 $\pm$ 5
5 mM MgAMP	10 $\pm$ 5	95 $\pm$ 5
5 mM MgGMP	35 $\pm$ 5	70 $\pm$ 5
2 mM Mg dATP	45 $\pm$ 5	100 $\pm$ 5
2 mM Mg dADP	60 $\pm$ 10	100 $\pm$ 10
2 mM Mg dGTP	10 $\pm$ 5	20 $\pm$ 5
2 mM Mg dGDP	10 $\pm$ 5	15 $\pm$ 5
5 mM Mg dUTP	75 $\pm$ 5	110 $\pm$ 5

complete inhibition of secretion evoked by 100 μ M GTP[S] and only about a 30% inhibition of that evoked by 1 mM ATP[S]. Low concentrations of GTP caused slightly more inhibition of secretion induced by 1 mM GTP[S] than of that induced by 1 mM ATP[S] (Figure 2).

Effects of different nucleotides on ATP[S]-stimulated, GTP[S]-stimulated and Ca^{2+} -stimulated NA secretion

The GTP-binding protein which is responsible for GTP[S] stimulation of NA release in permeabilized PC12 cells is very specific for GTP, as 100-fold excesses of ATP, ITP, UTP and CTP gave little or no inhibition of GTP[S]-stimulated secretion [12]. In contrast, 5-fold excesses of these nucleoside triphosphates gave at least 50% inhibitions of secretion evoked by addition of ATP[S] (Table 3). ATP[S]-stimulated NA release was also inhibited by deoxynucleoside triphosphates, nucleoside diphosphates and deoxynucleoside diphosphates and by GMP and AMP. Only guanosine-containing nucleotides and deoxynucleotides inhibited GTP[S]-stimulated release. dGTP and dGDP were the most potent inhibitors of both ATP[S]-stimulated and GTP[S]-stimulated releases. Ca^{2+} -stimulated release in 1 mM ATP or in the absence of added ATP was not inhibited by any of the nucleotides or deoxynucleotides listed in Table 3.

The inhibition of ATP[S]-stimulated NA release by a variety of nucleotides and deoxynucleotides (Table 3) suggests that the kinase which uses ATP[S] to cause NA release has a very low specificity for ATP. One such kinase is NDP kinase. NDP kinase

Table 4 Effect of addition of NDP kinase on ATP[S]-stimulated secretion

Cells prelabelled with [3 H]NA were permeabilized with digitonin and then incubated for 30 min in KG-buffer or in sucrose buffer (320 mM sucrose, 5 mM EGTA and 20 mM Pipes, pH 6.6). After this incubation, either [3 H]NA secretion was measured in KG-buffer or the cells were incubated for an additional 15 min in KG-buffer containing either 0.4 mg/ml BSA or 0.4 mg/ml NDP kinase (NDPK). After this incubation, [3 H]NA secretion was measured in KG-buffer. ATP[S]-stimulated release is [3 H]NA released in the presence of 1 mM MgATP[S] and no added Ca^{2+} minus that released in the presence of 2 mM MgATP and no added Ca^{2+} . GTP[S]-stimulated release is [3 H]NA released in the presence of 200 μM MgGTP[S], 2 mM MgATP and no added Ca^{2+} minus that released in the presence of 2 mM MgATP and no added Ca^{2+} . Ca^{2+} -stimulated release is [3 H]NA released in the presence of 10 μM Ca^{2+} and 2 mM MgATP minus that released in the presence of 2 mM MgATP and no added Ca^{2+} .

Cells first preincubated for 30 min in:	Conditions used for second preincubation	NA released (%)		
		ATP[S]-stimulated	GTP[S]-stimulated	Ca^{2+} -stimulated
KG-buffer	No second preincubation	6.3 $\pm$ 0.6	8.4 $\pm$ 0.8	13.1 $\pm$ 1.0
	15 min in KG-buffer + BSA	7.1 $\pm$ 0.8	8.2 $\pm$ 0.8	11.0 $\pm$ 1.0
	15 min in KG-buffer + NDPK	7.0 $\pm$ 1.1	6.3 $\pm$ 0.9	12.3 $\pm$ 0.4
Sucrose buffer	No second preincubation	1.7 $\pm$ 0.4	8.1 $\pm$ 0.7	10.9 $\pm$ 0.3
	15 min in KG-buffer + BSA	2.4 $\pm$ 0.4	6.2 $\pm$ 0.6	7.8 $\pm$ 0.3
	15 min in KG-buffer + NDPK	4.4 $\pm$ 0.9	5.7 $\pm$ 0.5	7.1 $\pm$ 0.8

Figure 3 Detection of NDP kinase (NDPK) by immunoblotting

Samples were resolved by SDS/PAGE and analysed by immunoblotting with affinity-purified anti-NDP kinase antibody. A, proteins from PC12 cells lysed immediately after digitonin permeabilization; B, proteins from digitonin-permeabilized cells that had been incubated for 30 min in KG-buffer; C, proteins from digitonin-permeabilized cells that had been incubated for 30 min in sucrose buffer. The bottom panel shows densitometer scans of lanes B and C.

catalyses the phosphorylation of nucleoside 5'-diphosphates to triphosphates by a Ping Pong mechanism:

N_1 and N_2 can be any ribo- or deoxyribo-nucleotide with either purine or pyrimidine bases. AMP and GMP inhibit both dephosphorylated and phosphorylated NDP kinase [19]. NDP kinase can use ATP[S] to convert GDP into GTP[S] [14–16].

Effects of low concentrations of GDP on ATP[S]-stimulated secretion

If stimulation of NA secretion by ATP[S] results from the thiophosphorylation of GDP by NDP kinase to give GTP[S], ATP[S]-stimulated NA secretion should be dependent on the presence of GDP. To examine this possibility, permeabilized PC12 cells were incubated for 10 min in the absence of Ca^{2+} and ATP to lower endogenous nucleotide concentrations, and then NA secretion was measured in the absence and presence of 100 μM GDP or ADP. Addition of 100 μM GDP to the release media caused almost a 50% increase in ATP[S]-stimulated NA secretion, from 7.4 $\pm$ 0.2% NA released in the absence of added GDP to 11.0 $\pm$ 1.3% NA released in the presence of 100 μM GDP. The addition of 100 μM GDP had no effect on Ca^{2+} -stimulated NA secretion, and it had no effect on, or caused a slight inhibition of, GTP[S]-stimulated release. Addition of 100 μM ADP had no effect on ATP[S]-stimulated NA release. As expected from the results shown in Table 3, the addition of higher concentrations of GDP or ADP inhibited ATP[S]-stimulated release. Inhibition by higher GDP concentrations could result from its competing both with ATP[S] for the dephosphorylated NDP kinase [19] and with GTP[S] for the GTP-binding protein.

Preferential loss of ATP[S]-stimulated secretion and NDP kinase

Permeabilized PC12 cells were incubated for 30 min in KG-buffer or in a sucrose buffer (320 mM sucrose, 5 mM EGTA and 20 mM Pipes, pH 6.6). These preincubation buffers were removed, and NA secretions were measured in KG-buffer (Table 4). This preincubation in sucrose buffer resulted in the preferential loss of ATP[S]-stimulated secretion. ATP[S]-stimulated release from cells preincubated in sucrose buffer was about 30% of that from cells preincubated in KG-buffer. Ca^{2+} -stimulated and GTP[S]-stimulated releases from cells preincubated in sucrose buffer were 80–95% of those from cells preincubated in KG-buffer (Table 4).

An immunoblot with affinity-purified anti-NDP kinase antibody showed that cells that had been incubated for 30 min in sucrose buffer contained less NDP kinase than did cells incubated in KG-buffer (Figure 3). Anti-(NDP kinase) antibodies were detected by a chemiluminescent assay, and the relative amounts

of NDP kinase determined by densitometry (Figure 3). Cells incubated in sucrose buffer contained $40 \pm 2\%$ less NDP kinase than did cells incubated in KG-buffer. A similar decrease was observed in three separate experiments. Cells incubated in KG-buffer for 30 min had 10–20% less NDP kinase than they did immediately after permeabilization.

Thus cells incubated in sucrose buffer had a lower level of NDP kinase and gave less NA secretion when stimulated with ATP[S] than did cells incubated in KG-buffer. Although this is consistent with NDP kinase playing a role in ATP[S]-stimulated release, it is possible that the decrease in ATP[S]-stimulated release is related not to the loss of NDP kinase, but to the loss of some other protein(s).

Rat liver NDP kinase in KG-buffer was added to cells that had been preincubated for 30 min in sucrose buffer or in KG-buffer (Table 4). After 15 min, these second preincubation buffers were removed, and NA secretions measured. The addition of NDP kinase caused an increase in ATP[S]-stimulated NA secretion from cells that had been preincubated in sucrose buffer, but not from cells that had been preincubated in KG-buffer. These second preincubations resulted in no changes or decreases in Ca^{2+} -stimulated and GTP[S]-stimulated NA secretions. The increase in ATP[S]-stimulated secretion that results from the addition of NDP kinase to sucrose-incubated cells shows that NDP kinase is capable of using ATP[S] to induce secretion. This increase in ATP[S]-stimulated release may be related to the normal mechanism by which ATP[S] induces secretion, as the addition of NDP kinase to cells incubated in KG-buffer did not cause a similar increase in ATP[S]-stimulated NA release.

DISCUSSION

We previously proposed that the stimulation of NA secretion by ATP[S] in the absence of Ca^{2+} might result from the basal activity of a Ca^{2+} -stimulated kinase and that Ca^{2+} -stimulated secretion results from the stimulation of this kinase by Ca^{2+} [9]. However, Ca^{2+} -stimulated release in the absence of added ATP was not inhibited by either 10 mM ADP or 10 mM p[NH]ppA. In contrast, these two nucleotides almost completely inhibited ATP[S]-stimulated secretion. This difference suggests either that the kinase responsible for ATP[S]-stimulated release is different from that responsible for Ca^{2+} -stimulated release or that Ca^{2+} -stimulation does not require the action of a Ca^{2+} -stimulated kinase.

Although ATP increases Ca^{2+} -stimulated release in digitonin-permeabilized PC12 cells, it also increases both GTP[S]-stimulated secretion [11] and secretion from cells preincubated with ATP[S] [9]. This suggests that ATP is used at a step in the secretory response other than or in addition to that regulated by Ca^{2+} . Results with semi-intact PC12 cells suggest that ATP is used at a step preceding that at which Ca^{2+} acts to trigger secretion [20].

The following results are consistent with ATP[S] stimulation of NA secretion in digitonin-permeabilized PC12 cells resulting, at least in part, from the formation of GTP[S] by NDP kinase: (1) GTP[S]-stimulated and ATP[S]-stimulated secretions were not additive; (2) low concentrations of GDP increased ATP[S]-stimulated secretion, but not GTP[S]-stimulated or Ca^{2+} -stimulated secretions; (3) a large variety of ribo- and deoxyribonucleotides inhibited ATP[S]-stimulated secretion, but not GTP[S]-stimulated or Ca^{2+} -stimulated secretion; (4) permeabilized PC12 cells converted a small amount of ATP[^{35}S] into GTP[^{35}S], and nucleotides which inhibited ATP[S]-stimulated secretion decreased the amount of GTP[^{35}S] formed (results not

shown); and (5) incubation of permeabilized cells in a sucrose-containing buffer resulted in the preferential loss of ATP[S]-stimulated secretion and a decrease in the level of NDP kinase. Addition of rat liver NDP kinase to these depleted cells partially restored ATP[S]-stimulated secretion. These results also point out a number of differences between ATP[S]-stimulated and Ca^{2+} -stimulated release that suggest the kinase which uses ATP[S] to induce secretion may not play an essential role in Ca^{2+} -stimulated release.

The effects of ATP[S] and GTP[S] on NA secretion by permeabilized PC12 cells were not additive (Table 2), which is consistent with these nucleotides acting through a common mechanism to stimulate secretion. Since ATP enhances GTP[S]-stimulated release, the small increase in secretion observed when both ATP[S] and GTP[S] were added compared with when only GTP[S] was added could result from the cell using ATP[S] for another ATP-dependent process which increases the secretory activity.

Most protein kinases are specific for ATP, but some will also use GTP [21]. In contrast, the kinase which uses ATP[S] to induce secretion in permeabilized PC12 cells appears to have a very low nucleotide specificity (Table 3). This low specificity is similar to that of NDP kinase [19]. The order of inhibition of ATP[S]-stimulated secretion by nucleoside diphosphates was $\text{GDP} > \text{ADP} > \text{UDP} > \text{CDP}$, which parallels their apparent relative affinities for rat liver NDP kinase [18]. The inhibition of ATP[S]-stimulated release by the various nucleoside diphosphates could result either from their competing with GDP for thiophosphorylated NDP kinase or from their inhibiting ATP[S] binding to dephosphorylated NDP kinase [19]. UDP has been used in several other systems to inhibit either the phosphorylation or thiophosphorylation of endogenous GDP by NDP kinase [15,22–24]. GDP and dGDP could also inhibit ATP[S]-stimulated release by competing with any GTP[S] produced for binding to the GTP-binding protein.

Inhibition of ATP[S]-stimulated release by these different nucleotides does not appear to result from some general inhibition of secretory activity, as they do not inhibit Ca^{2+} -stimulated release either in the absence of added ATP or in the presence of 1 mM ATP. Also, except for those containing guanosine as their base, they had little or no effect on GTP[S]-stimulated release.

We originally believed that the effect of ATP[S] on NA secretion did not result from the formation of GTP[S]. This conclusion was based primarily on the observation that 1 mM GTP caused a 50% decrease in NA secretion evoked by 1 mM GTP[S], but no inhibition of NA secretion evoked by 1 mM ATP[S] [9]. These experiments were performed on cells that had been preincubated for 30 min in the absence of added nucleotides. When the comparison was made immediately after permeabilization, GTP was found to inhibit both GTP[S]-stimulated and ATP[S]-stimulated NA secretion (Figure 2). However, even under these latter conditions, GTP was not a very potent inhibitor of ATP[S]-stimulated NA release.

The amount of NA released by 1 mM ATP[S] was less than that released by 100 μM GTP[S]. If secretion evoked by ATP[S] results from the formation of GTP[S], the amount of GTP[S] produced from 1 mM ATP[S] caused less stimulation of NA release than that caused by the direct addition of 100 μM GTP[S]. However, 1 mM GTP gave nearly complete inhibition of secretion evoked by exogenously added 100 μM GTP[S] and only about a one-third inhibition of that evoked by 1 mM ATP[S]. One possible explanation as to why GTP appears to give more inhibition of secretion induced by exogenously added GTP[S] than that formed from ATP[S] is that the conversion of ATP[S] into GTP[S] by NDP kinase occurs in the vicinity of the

GTP-binding protein responsible for stimulating NA secretion. NDP kinases have been reported to be associated with GTP-binding proteins [25–28], and it has been suggested either that NDP kinase can phosphorylate GDP while it remains bound to a GTP-binding protein or that the GTP produced by NDP kinase is somehow channelled to the GTP-binding protein [18,25–27,29]. Either of these types of interactions could explain why exogenously added GTP is not as effective an inhibitor of GTP[S] produced by the action of NDP kinase as it is of exogenously added GTP[S].

Activation of K⁺-channel currents in atrial myocytes by ATP[S] [14] and stimulation of superoxide formation in membranes of HL-60 leukaemic cells by ATP[S] [15] occur after time lags which are much longer than those obtained with GTP[S]. These long lag times are thought to reflect the time it takes for NDP kinase to form enough GTP[S] to activate the GTP-binding protein. In contrast, there was no long delay between the time of addition of ATP[S] and stimulation of NA secretion by digitonin-permeabilized PC12 cells. This is consistent with GTP[S] being formed in the vicinity of the GTP-binding protein responsible for stimulating NA secretion.

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