12. H.-C. J. Lai, N. R. Li, I. J. Weiss, C. C. Reddy and C.-P. D. Tu, *J. biol. Chem.* **259**, 5536 (1984).
13. B. Coles, D. J. Meyer, B. Ketterer, C. Stanton and R. C. Garner, *Carcinogenesis* **6**, 693 (1985).
14. B. Jernstrom, M. Martinez, D. J. Meyer and B. Ketterer, *Carcinogenesis* **6**, 85 (1985).
15. S. Pemble, J. B. Taylor, R. K. Craig and B. Ketterer, *Biochem. J.* **238**, 373 (1986).
16. C. Power, S. Sinha, C. Webber, M. M. Manson and G. E. Neal, *Carcinogenesis* **8**, 797 (1987).
17. A. Okuda, M. Sakai and M. Maramatsu, *J. biol. Chem.* **262**, 3858 (1987).

Biochemical Pharmacology, Vol. 37, No. 12, pp. 2485–2487, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

Effects of islet-activating protein on the catecholamine release, Ca^{2+} mobilization and inositol trisphosphate formation in cultured adrenal chromaffin cells

(*Received* 9 *October* 1987; *accepted* 13 *January* 1988)

Secretion of catecholamines from the adrenal chromaffin cells is considered to be a Ca^{2+}-dependent exocytosis [1]. An increase in the cellular Ca^{2+} uptake [2, 3] and subsequent rise in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$)* are correlated well with catecholamine release [4, 5].

It has been reported that in certain cell systems GTP binding protein may also participate in the secretory process [6, 7]. In permeabilized adrenal chromaffin cells, the reported effects of guanine nucleotide analogues on the catecholamine secretion are somewhat controversial [8, 9], and the role of GTP-binding protein in the intracellular mechanisms of secretory function still remains to be clarified.

Islet-activating protein (IAP), the active component of pertussis toxin, catalyses ADP-ribosylation of the GTP binding α subunit of G_i and prevents the effects of inhibitory hormones on adenylate cyclase [10]. It has been shown recently that a GTP-binding protein, which is different from G_i and is also ADP-ribosylated by IAP, participates in the control of cellular events such as the regulation of the linkage between receptors and phospholipase C [11, 12] or regulation of ionic channels [13, 14].

Our preliminary observation revealed that the pretreatment of the cells with IAP enhanced the CCh-induced catecholamine release without affecting Ca^{2+} mobilization [15]. In order to further elucidate the regulatory mechanisms of GTP-binding protein on catecholamine release, we examined the effects of IAP on catecholamine release, Ca^{2+} mobilization and inositol trisphosphate formation in cultured adrenal chromaffin cells.

Materials and methods

Primary culture of bovine adrenal chromaffin cells. According to the previous reports [16–18], chromaffin cells were isolated and cultured. The chromaffin cells were used for experiments 4–7 days after plating.

Catecholamine release. Catecholamine release was measured as described previously [17, 18].

Measurement of $[Ca^{2+}]_i$. The cultured chromaffin cells were collected by centrifugation (200 *g*, 6 min) and suspended in a Locke's solution supplemented with 0.1% bovine serum albumin. The cells were incubated at a density of 3–4 × 10^6 cells/ml with 2 μM fura-2 acetoxymethyl ester for 30 min at 37°. After the incubation, cells were washed and resuspended in the solution without fura-2 acetoxymethyl ester and incubated for another 30 min. Immediately before use, 0.5 ml cell suspension (2 × 10^6 cells/ml) was centrifuged (5000 *g*, 10 sec) and the cells were washed and resuspended in Locke's solution. The cell suspension was transferred to the thermostated quartz cuvette (37°) settled in a fluorescence spectrophotometer (CAF-100, Nihon Bunko, Tokyo, Japan). $[Ca^{2+}]_i$ was measured as described elsewhere [19, 20].

Measurement of [^{3}H]*inositol triphosphate accumulation.* [^{3}H]inositol trisphosphate accumulation of cultured adrenal chromaffin cells was measured as described previously [21, 22].

Chemicals. IAP was kindly provided by Kaken Seiyaku Co., Ltd., Tokyo, Japan. The following chemicals were obtained from the companies indicated. *myo*-[2-^{3}H]Inositol (17 Ci/mmol), from Amersham International Inc, Bucks, U.K.; Carbamylcholine chloride (CCh), from Sigma Chemical Company, (St Louis, MO); monensin, from Calbiochem (La Jolla, CA); fura-2 acetoxymethylester from Molecular Probes Inc. (Junction City, OR).

Results and discussion

In the present study, we examined the effects of IAP on catecholamine release, Ca^{2+} mobilization and inositol trisphosphate formation in cultured adrenal chromaffin cells to elucidate the role of GTP-binding proteins in the mechanism of stimulus-secretion coupling in these cells.

The maximal catecholamine release induced by 300 μM CCh was augmented by pretreating the cells with IAP in a concentration- and time-dependent manner. The maximum effect (about 150% of control) of IAP was observed by pretreating the cells with 250 ng/ml IAP for 20 hr. Pretreatment with the same concentration of IAP for 2 hr was not sufficient to exert its potentiating effect. The EC_{50} value of CCh for catecholamine release (50 μM) was not altered obviously by IAP pretreatment. Furthermore, 56 mM KCl (high K^+)-induced catecholamine release was also enhanced by pretreating the cells with IAP (Table 1). The basal catecholamine release also tended to be increased by IAP pretreatment. These results are essentially in agreement with the communication by Tanaka *et al.* [23]. IAP, however, failed to affect the monensin-induced catecholamine release (Table 1). According to the observation reported by Izumi *et al.* [24], catecholamine release induced by monensin occurs by a nonexocytotic mechanism.

Since the increase in $[Ca^{2+}]_i$ plays an essential role in catecholamine release from adrenal chromaffin cells [2, 3], we examined the effects of IAP pretreatment on $[Ca^{2+}]_i$ of these cells. As shown in Table 2, neither basal $[Ca^{2+}]_i$ nor CCh- and high K^+-induced rises in $[Ca^{2+}]_i$ were affected by IAP pretreatment (Table 2). Furthermore, both CCh- and high K^+-induced $^{45}Ca^{2+}$ uptake were not altered by IAP pretreatment (data not shown).

All of the above results suggest that IAP acts on the common process of the depolarization-mediated and the receptor-mediated secretory mechanisms. In addition, our present results suggest that IAP augments catecholamine release by acting on a secretory process distal to the rise in $[Ca^{2+}]_i$, such as an exocytotic process.

* Abbreviations used: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; IAP, islet-activating protein; CCh, carbamylcholine; high K^+, KCl (56 mM).

Table 1. Effects of IAP pretreatment on CCh-, KCl- and monensin-induced catecholamine release from adrenal chromaffin cells

Additions	Catecholamine release (% of total content) IAP (−)	IAP (+)
Experiment A		
None	2.4 ± 0.2	2.9 ± 0.2
CCh (300 μM)	12.3 ± 0.8	18.3 ± 0.4*
KCl (56 mM)	11.2 ± 0.6	14.8 ± 0.9*
Experiment B		
None	4.1 ± 0.3	4.8 ± 0.2
Monensin (0.3 μM)	24.7 ± 0.9	25.2 ± 1.6

The cells were pretreated with IAP (250 ng/ml) for 20 hr. In Experiment A and B, the cells were incubated with or without stimulant for 5 and 60 min, respectively. The released catecholamines were determined as described in Materials and Methods. The results shown are mean ± SEM of quadruplicate determinations. Typical data obtained from two different cell preparations are presented.

* $P < 0.05$ vs corresponding IAP (−).

Forsberg *et al.* reported that muscarinic receptor-mediated inositol trisphosphate formation may prime the cells for nicotinic receptor-mediated catecholamine release, and suggested that inositol trisphosphate formation may modulate catecholamine release in chromaffin cells [25]. Thus, we examined the effects of IAP pretreatment on CCh- and high K^+-induced inositol trisphosphate accumulation in these cells (Table 3). Although the CCh-induced inositol trisphosphate accumulation was partially inhibited by IAP pretreatment, the high K^+-induced inositol trisphosphate accumulation was not affected (Table 3). On the other hand, both CCh- and high K^+-induced catecholamine release were potentiated by IAP pretreatment (Table 1). Therefore, the potentiation of catecholamine release caused by IAP pretreatment was not due to the changes in inositol trisphosphate formation.

In conclusion, our present results suggest that GTP-binding protein may play a role in the catecholamine secretion at a step distal to Ca^{2+} mobilization, such as an exocytotic process.

Table 2. Effects of IAP pretreatment on $[Ca^{2+}]_i$ of adrenal chromaffin cells

Additions	$[Ca^{2+}]_i$ (nM) IAP (−)	IAP (+)
None	172 ± 25	168 ± 38
CCh (300 μM)	690 ± 47	704 ± 65
KCl (56 mM)	740 ± 38	720 ± 30

The cells were pretreated with IAP (250 ng/ml) for 20 hr. $[Ca^{2+}]_i$ was measured as described in Materials and Methods. Maximum rises in $[Ca^{2+}]_i$ induced by CCh or KCl were presented. The results shown are mean ± SEM of triplicate determinations. Typical data obtained from two different cell preparations are presented.

Acknowledgement—This work was supported in part by a Grant-in-Aid for Scientific Research, 1986, from the Ministry of Education, Culture and Science, Japan, and Research Aid of Inoue Foundation For Science, 1986. We thank Miss Hiroko Une for her excellent technical assistance.

Table 3. Effects of IAP pretreatment on inositol trisphosphate accumulation of adrenal chromaffin cells

Additions	Inositol trisphosphate accumulation (dpm) IAP (−)	IAP (+)
None	2650 ± 160	2310 ± 150
CCh (300 μM)	6470 ± 220	5230 ± 40*
KCl (56 mM)	4250 ± 70	3990 ± 200

The cells were pretreated with IAP (250 ng/ml) for 20 hr. Thereafter the cells were stimulated with 300 μM CCh or 56 mM KCl for 2 min and the accumulated [^{3}H]inositol trisphosphate was determined as described in Materials and Methods. The results shown are mean ± SEM of quadruplicate determinations. Typical data obtained from two different cell preparations are presented.

* $P < 0.05$ vs corresponding IAP (−).

Department of Pharmacology
School of Medicine
Keio University
35 Shinanomachi
Shinjuku-ku
Tokyo 160, Japan

NOBUYUKI SASAKAWA
SATOSHI YAMAMOTO
TOSHIO NAKAKI
RYUICHI KATO

REFERENCES

1. A. D. Smith and H. Winkler, in *Catecholamines* (Eds. H. Blaschko and E. Musholl), p. 538. Springer-Verlag, Berlin (1972).
2. W. W. Douglas, in *Handbook of Physiology, Section 7: Endocrinology, VI: Adrenal Gland* (Eds. H. Blaschko, J. Sayers and A. D. Smith), p. 367. American Physiological Society, Washington, DC (1975).
3. O. H. Viveros, in *Handbook of Physiology, Section 7: Endocrinology, VI: Adrenal Gland* (Eds. H. Blaschko, J. Sayers and A. D. Smith), p. 389. American Physiological Society, Washington, DC (1975).
4. N. Sasakawa, K. Ishii, S. Yamamoto and R. Kato, *Jap. J. Pharmac.* **40**, 14P (1986).
5. L. S. Kao and A. S. Schneider, *J. biol. Chem.* **261**, 4881 (1986).
6. M. M. Barrowman, S. Cockcroft and B. D. Gomperts, *Nature, Lond.* **319**, 504 (1986).
7. B. D. Gomperts, M. M. Barrowman and S. Cockcroft, *Fedn Proc. Fedn Am. Socs exp. Biol.* **45**, 2156 (1986).
8. D. E. Knight and P. F. Baker, *FEBS Lett.* **189**, 345 (1985).
9. M. A. Bittner, R. W. Holtz and R. R. Neubig, *J. biol. Chem.* **261**, 10182 (1986).
10. T. Katada and M. Ui, *Proc. natn. Acad. Sci. U.S.A.* **79**, 3129 (1982).
11. S. Cockcroft and B. D. Gomperts, *Nature, Lond.* **314**, 534 (1985).
12. S. J. Brandt, R. W. Dougherty, E. G. Lapetina and J. E. Niedel, *Proc. natn. Acad. Sci. U.S.A.* **82**, 3277 (1985).
13. P. J. Pfaffinger, J. M. Martin, D. D. Hunter, N. M. Nathanson and B. Hille, *Nature, Lond.* **317**, 536, (1985).
14. G. G. Holz, S. G. Rane and K. Dunlap, *Nature, Lond.* **319**, 670 (1986).
15. S. Yamamoto, N. Sasakawa, T. Nakaki and R. Kato, *Jap. J. Pharmac.* **43**, 165P (1987).
16. K. Kumakura, A. Guidotti and E. Costa, *Molec. Pharmac.* **16**, 865 (1979).
17. N. Sasakawa, K. Kumakura, S. Yamamoto and R. Kato, *Life Sci.* **33**, 2017 (1983).
18. N. Sasakawa, S. Yamamoto and R. Kato, *Biochem. Pharmac.* **33**, 2733 (1984).
19. G. Grynkiewicz, M. Poenie and R. Y. Tsien, *J. biol. Chem.* **260**, 3440 (1985).
20. D. A. Williams, K. E. Fogarty, R. Y. Tsien and F. S. Fay, *Nature, Lond.* **318**, 558, (1985).
21. N. Sasakawa, T. Nakaki, S. Yamamoto and R. Kato, *FEBS Lett.* **223**, 413 (1987).
22. T. Nakaki, N. Sasakawa, S. Yamamoto and R. Kato, *Biochem. J.* **251**, 397 (1988).
23. T. Tanaka, H. Yokohama, M. Negishi, H. Hayashi, S. Ito and O. Hayaishi, *Biochem. biophys. Res. Commun.* **144**, 907 (1987).
24. F. Izumi, A. Wada, N. Yanagihara, H. Kobayasi and Y. Toyohira, *Biochem. Pharmac.* **35**, 2937 (1986).
25. E. J. Forsberg, E. Rojas and H. B. Pollard, *J. biol. Chem.* **261**, 4915 (1986).

Biochemical Pharmacology, Vol. 37, No. 12, pp. 2487–2489, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

N-Alkyl colchiceineamides: their inhibition of GTP or taxol-induced assembly of tubulin

(*Received* 24 *July* 1987; *accepted* 8 *December* 1987)

Colchicine is a potent drug that interferes with microtubule assembly both *in vitro* and *in vivo*, by forming a complex with the dimeric subunit of the microtubule, tubulin [1]. The complex formed incorporates into the microtubule and inhibits the assembly process [2, 3].

Many colchicine derivatives have been extracted from plants or synthesized over the years [4]. We have synthesized a group of 10-*N*-alkyl derivatives of colchicine (see Table 1) in order to examine their anti-microtubule activities. The 10-methoxy position of colchicine is known to be liable to mild hydrolysis [4]. In contrast, the 10-amino derivatives are resistant to hydrolysis due to the high pK_a values of the amine. The activities of these compounds as tubulin assembly inhibitors were studied using GTP and taxol as promoters of tubulin assembly, and in cells. GTP is an essential component in the tubulin assembly mechanism and will promote assembly of uninhibited protein under proper experimental conditions [5]. Taxol, a novel diterpenoid, promotes the assembly of microtubules under conditions that normally do not support microtubule polymerization, by interacting directly with microtubule polymers and stabilizing them against depolymerizing agents such as Ca^{2+} and cold temperature [6]. Using both agents, we have studied the structure–activity relationships of colchiceineamides.

Materials and methods

Preparation of colchicine analogs. To 1-ml solutions of colchicine (200 mg, 0.5 mmol) in dry acetonitrile, several amines (20 mmol) were added: methyl-, ethyl-, propyl-, isopropyl-, butyl-, isobutyl-, *tert*-butyl-, 3-dimethyl-amino-1-propyl-amine, 1,6-hexadiamine and dimethylhydrazine (Table 1 summarizes the analogs prepared). The reactions were carried out at room temperature (except for the reaction of 1,6-hexadiamine which was carried out in a hot water bath). The formation of the desired products was monitored by TLC. Retention times (R_f) for the 10-amino derivatives that eluted on Alumina (5:95 ethanol–CH_2Cl_2) were: 55, 55, 53, 54, 57, 45, 50, 56, 35 and 62% respectively. Silica TLC plates (1 mm) were used for preparative separation. The reaction mixtures were eluted by ethanol–CH_2Cl_2 (2.5:97.5), and the appropriate compounds were extracted from the silica by hot chloroform and recrystallized from an ethylacetate–hexane mixture. The melting points of the products were as follows: 150–155, 160–165, 110, 150, 110–115, 220, 155–160, 205, 160, and 110–120° respectively. The structures of the resulting compounds were confirmed by NMR measurements (Varian 400 MHz spectrometer), mass spectrometry, elemental analysis and, in one case (compound IX), by single crystal X-ray