Biochemical Pharmacology, Vol. 37, No. 12, pp. 2482–2485, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

Glutathione transferase isoenzymes in cultured rat hepatocytes

(*Received* 25 *March* 1987; *accepted* 22 *December* 1987)

Glutathione (GSH) transferase isoenzymes (EC 2.5.1.18) play an important role in catalysing the conjugation of a wide range of hydrophobic electrophiles with glutathione [1]. They are dimers of subunits with molecular weights in the region 23,000 to 30,000.

At least 12 subunits have been identified, and their distribution has been found to be tissue characteristic and to vary with their stage of development and exposure to hormones, inducers, etc. Adult rat liver is the tissue most abundant in the enzymes possessing GSH transferases 1-1, 1-2, 2-2, 3-3, 3-4, 4-4, 4-6 and 5-5 [2]. GSH transferase 7-7 [3], which has been identified in rat kidney, testis, lung and skeletal muscle [4], has so far been found in liver only during hepatocarcinogenesis and is regarded as a marker for hepatocellular preneoplasia and hepatoma [4, 5].

In a recent study [6], we have shown that GSH transferase activity levels in cultured hepatocytes vary depending on the culture conditions, i.e. the composition of the medium and whether cells are in conventional culture or co-culture. The results suggest that variations in the profile of GSH transferase isoenzymes might occur. In order to detect such variations, we have analysed GSH transferase subunit composition using a combination of GSH agarose affinity chromatography and reversed-phase HPLC according to Ostlund Farrants *et al.* [7].

Materials and methods

Cell isolation and culture. Rat hepatocytes were obtained from adult male Sprague–Dawley animals weighing 200–220 g [8]. They were seeded at a density of 15×10^6 cells per 175 cm^2 covered by 25 ml standard medium containing 10% foetal calf serum (FCS). This standard medium consisted of 75% minimal essential medium and 25% Medium 199, containing 200 μl/ml bovine serum albumin and 10 μg/ml bovine insulin.

Four hours after seeding, the medium was renewed. Three media were used, namely standard medium plus FCS (S + FCS), standard medium minus FCS (S + FCS) and standard medium minus FCS and made 25 mM with respect to nicotinamide (S + FCS + N) [6]. All media were 7×10^{-5} M with respect to hydrocortisone hemisuccinate.

Cell harvesting. After washing with chilled HEPES buffer (pH 7.6), cell monolayers were collected from 10 ml of HEPES by scraping with a rubber policeman. Cells from 10 petri dishes, i.e. approximately 150×10^6 cells, were pooled. This experiment was done in duplicate for each point.

Preparation of a combined GSH transferase fraction. Samples were prepared for analysis using a shortened version of the method of Vander Jagt *et al.* [9]. The pellets of cells were suspended in 3 ml phosphate/KCl buffer (33 mM phosphate adjusted to pH 7.0 containing 0.15 M KCl) which was 1 mM with respect to ethylenediamine tetraacetate and 0.25 mM with respect to phenylmethylsulphonylfluoride. The homogenised suspension was loaded on a GSH affinity column (1.6 × 1.2 cm) packed with epoxy-activated Sepharose 6B that had been reacted with glutathione. After washing the column with 3.5 ml of phosphate/KCl buffer, the GSH transferase fraction was eluted with 5 ml of a solution containing 5 mM GSH, 50 mM Tris and adjusted to pH 9.1 with NaOH at room temperature. The first millilitre of eluate was discarded and the next 3.2 ml was collected for HPLC analysis. The temperature was maintained at 4° throughout.

Analysis of subunits by reversed-phase HPLC. The separation and quantification of GSH transferase subunits was carried out by HPLC on a 10 × 0.8 cm Waters μ Bondapak C-18 reversed-phase column in a Z-module using a Waters system (Milford, U.S.A.) according to Ostlund Farrants *et al.* [7]. The solvents were water (A) and 0.1% trifluoracetic acid in acetonitrile (B). The samples were injected at 35% B. During a rung, a linear gradient was used from 35% to 55% B over 60 min with a flow rate of 15 ml·min^{-1}. Detection was carried out at 214 nm. Glutathione transferase isoenzyme 5-5 is not retained by the GSH affinity column [10] and therefore was not detected by the method used here.

The glutathione transferase subunits separated by HPLC were identified initially by comparison of their retention times with those of purified glutathione transferases. When necessary their identity was confirmed by a sodium dodecyl sulphate polyacrylamide gel electrophoresis. Analyses were quantitated using the values for the recovery of respective subunits from HPLC and their molar extinction coefficients at 214 nm as given by Ostlund Farrants *et al.* [7].

Protein concentrations were determined using the Bio-Rad assay, using bovine serum albumin as a standard.

Results

Whole liver and freshly isolated hepatocytes. Identification of GSH transferases in rat tissue and isolated hepatocytes was based on retention times determined with purified rat GSH transferases [7]. GSH transferases were separated into subunits on the column and eluted in the order 3, 4, 7, 2 and 1 at retention times of 27, 31, 33, 35, and 48 min respectively. Subunit 1 was resolved into two major and two minor peaks, giving further evidence that this subunit is heterogeneous [11–13]. Subunit 7 was not detectable in either sample. Quantitative data obtained for both samples are shown in Table 1.

Cultured hepatocytes. Figure 1 compares a GSH transferase subunit separation from freshly isolated rat hepatocytes with the hepatocytes from the same preparation maintained in pure culture for 4 days in the three different media. Results calculated for all three different media are shown in Table 1. In each case there was a decrease in subunits 1 and 2 and the expression of subunit 7 which was not present in the original hepatocytes. The decrease in subunits 1 and 2 was less marked in the presence of nicotinamide.

Discussion

The reversed-phase HPLC technique recently described by Ostlund Farrants for the separation of the GSH transferase subunits of liver and kidney was applied here to analyse quantitively GSH transferase subunits in adult rat hepatocytes during culture.

Compared with the whole liver, freshly isolated hepatocytes exhibited negligible quantitative differences, except for subunit 3. However, after culture for four days, hepatocytes were characterised by a decrease of subunits 1 and 2, maintenance of the original levels of subunit 4, an

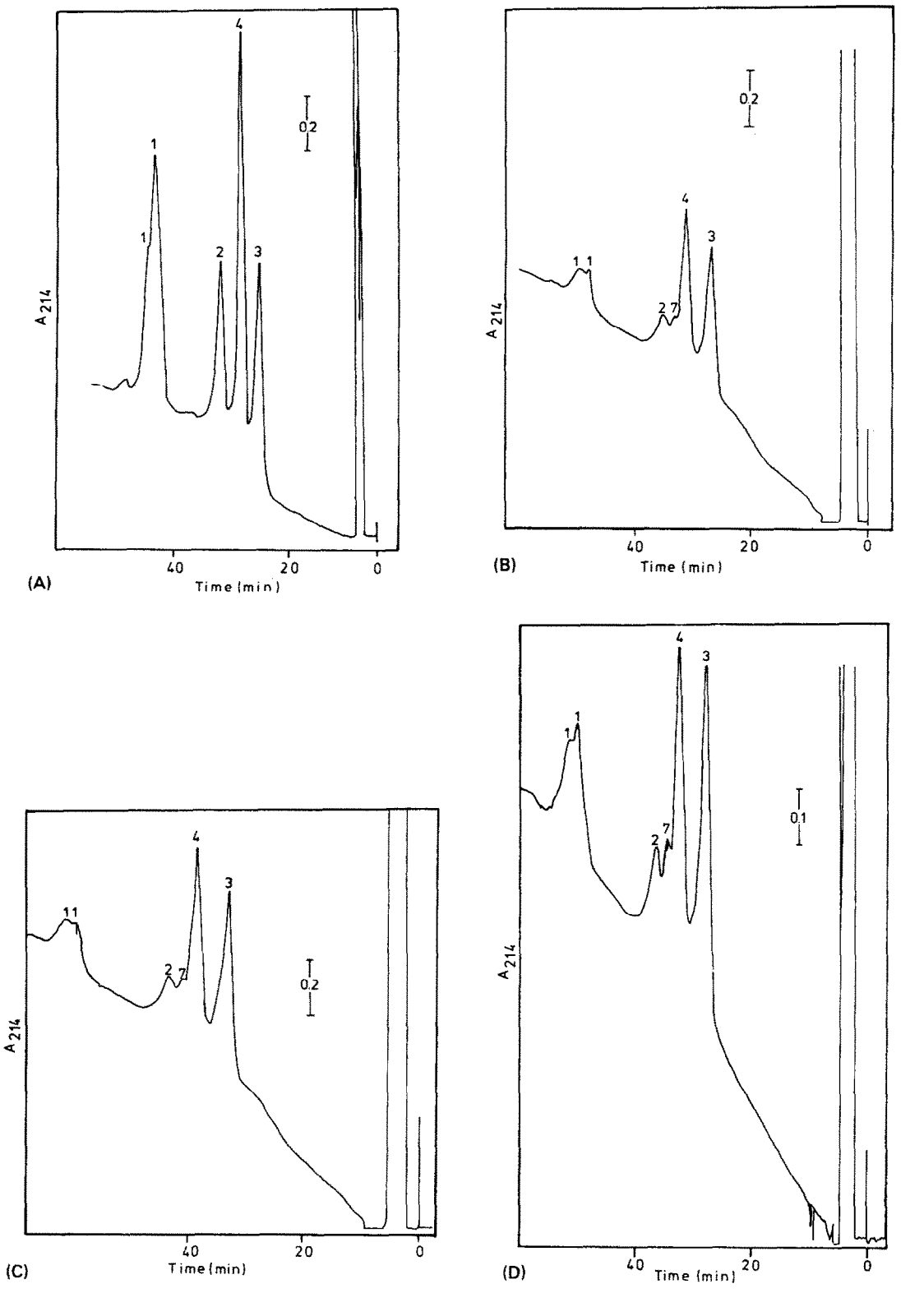

Fig. 1. Separation of rat GSH transferase subunits by reversed-phase HPLC. (A) GSH transferase subunit composition of freshly isolated hepatocytes. (B) GSH transferase subunit composition of 4 days pure cultured rat hepatocytes in medium S + FCS. (C) GSH transferase subunit composition of 4 days pure cultured rat hepatocytes in medium S-FCS. (D) GSH transferase subunit composition of 4 days pure cultured rat hepatocytes in medium S-FCS + N. The samples were applied to a μBondpack C-18 column and eluted with a gradient of 0.1% trifluoracetic acid in acetonitrile as described in Materials and Methods. Numbers refer to GSH transferase subunits.

Table 1. Quantitative analysis of GSH transferase subunits

Source of enzyme	(μg/mg)Cytosol. proteins 1	2	3	4	7
Whole liver	43.3	15.6	7.7	37.7	nil
Freshly isolated hepatocytes	39.9	14.8	13.5	38.8	nil
Hepatocytes after 4 days in culture					
+foetal calf serum	19.9	8.1	18.9	37.2	12.9
−foetal calf serum	22.2	2.7	19.5	41.6	5.1
−foetal calf serum +nicotamide	26.4	8.9	26.8	40.0	8.1

Values were obtained after separation of the GSH transferase subunits by HPLC, as described in Materials and Methods. Each point represents a pool of 10 petri dishes of 15×10^6 hepatocytes.

increase in subunit 3 and expression *de novo* of subunit 7. Variations in subunit composition occurred according to the composition of the nutrient medium. For example the addition of nicotinamide which tends to preserve normal morphology [6] tended to reduce the loss of subunits 1 and 2 but did not prevent the increase of subunit 3 nor the expression of subunit 7.

From the values for μg subunits per mg cytosol protein given in Table 1 and for specific activities for a number of substrates derived from the literature [3], it is possible to arrive at calculated values for the activity of each preparation with respect to the substrates used in previous work on homogenates of cells in culture. In such calculations the most striking changes in culture are a 2-fold increase in activity towards 2,4-dichloro-1-nitrobenzene and a decrease to one half in cumene hydroperoxide reducing activity. This is in accord with previous activity measurements [6] and is apparently due to the relative increase in subunit 3 and the relative decrease in subunits 1 and 2.

Because of these changes in GSH transferase isoenzyme distribution the ability of hepatocytes in culture to deal with substrates may differ from hepatocytes *in situ* in the liver and freshly isolated hepatocytes. For example since aflatoxin B_1 oxide is a substrate only for subunits 1 and 2 [13], cultured hepatocytes would be expected to detoxify this substrate very poorly. They should be very susceptible to its toxicity. On the other hand, since benzo(*a*)pyrene-7,8-diol-9, 10-oxide is a good substrate for both GSH transferases 4-4 [14] and 7-7 [3] and since levels of subunit 4 are maintained in cultured hepatocytes and subunit 7 is expressed *de novo*, cultured hepatocytes should be better able to deal with this toxin than normal hepatocytes. Also, since GSH transferase 7-7 is good for GSH transferase and for GSH peroxidase (at least for linoleic acid hydroperoxide) [3], its presence may compensate to some degree for the loss of subunits 1 and 2.

This study is the first report of the presence of subunit 7 in cultured hepatocytes. Using a cDNA probe and Northern blots, mRNA for subunit 7 has also been found in lung, kidney, epididymis, testis and spleen [15]. It is barely detectable in normal liver [15] and freshly isolated hepatocytes, but can be detected in hepatocytes which have been observed in culture for 48 hr [16]. Both the protein and message, however, have been observed in liver undergoing chemical carcinogenesis.

Immunohistochemical techniques have shown it occurs in preneoplastic foci and hyperplastic nodules [5, 16]. It is also present in carcinogen-induced hepatomas [3, 5, 16] and its message has been demonstrated in minimal deviation Morris hepatomas [17]. It is regarded as the most reliable available marker of hepatocellular preneoplasia and neoplasia in the rat.

Thus, it is necessary merely to take the hepatocyte out if its normal tissue environment and place it in culture to express GSH transferase 7-7. It is noteworthy that nicotinamide which tends to preserve normal morphology and reduces the loss of subunit 1 does not modify subunit 7 expression.

In summary, the present study shows that after 4 days in culture, in either the presence or absence of foetal calf serum, hepatocytes undergo marked changes in GSH transferase subunit profile, including reduced levels of subunit 1 and 2, increased levels of subunit 3 and the expression of subunit 7, which is not associated with the normal hepatocytes, but is a tumor marker. Addition of nicotinamide tended to reduce the decrease of subunits 1 and 2, but did not prevent the expression of subunit 7.

Acknowledgements—DJM and BK thank the Cancer Research Campaign of which BK is a fellow, for their generous support.

We thank Drs V. Rogiers and A. Vercruysse for helpful discussions and Ms L. Christodoulides and Ms M. Van den Berghe for typing the manuscript.

YV thanks the National Fonds voor Wetenschappelijk Onderzoek (NFWO) for supporting his stay in Rennes.

* *Vrije Universiteit Brussel*
Department of Toxicology
1090 Brussel, Belgium
† *INSERM, U49*
Unité de Recherches Hépatologiques
35033 Rennes Cedex France
§ *Cancer Research Campaign Molecular Toxicology Research Group*
Middlesex Hospital Medical School
London W1P 6DB, U.K.

YVES VANDENBERGHE*†‡
DENISE GLAISE†
DAVID J. MEYER§
ANDRE GUILLOUZO†
BRIAN KETTERER§

‡ To whom correspondence should be addressed at Department of Toxicology, Vrije Universiteit Brussel, Laarbeeklaan, 103 1090 Brussel, Belgium.

REFERENCES

1. E. Boyland and L. F. Chasseaud, *Adv. Enzymol.* **32**, 173 (1969).
2. B. Ketterer, D. J. Meyer, B. Coles, J. B. Taylor and S. Pemble, in *Antimutagenesis and Anticarcinogenesis* (Eds. D. B. Shankel, P. E. Hartman, T. Kada and Hollaender), p. 103. Plenum, New York (1986).
3. D. J. Meyer, D. Beale, K. H. Tan, B. Coles and B. Ketterer, *FEBS Lett.* **184**, 139 (1985)
4. S. Pemble, J. B. Taylor and B. Ketterer, *Biochem. J.* **240**, 885 (1986).
5. A. Kitahara, K. Satoh, K. Nishimura, T. Ishikawa, K. Ruike, K. Sato, H. Tsuda and N. Ito, *Cancer Res.* **44**, 2698 (1984).
6. Y. Vandenberghe, A. Ratanasavanh, D. Glaise and A. Guillouzo, *In Vitro and Cellular Developmental Biology*. in press.
7. A.-K. Ostlund Farrants, D. J. Meyer, B. Coles, C. Southan, A. Aitken, P. J. Johnson and B. Ketterer, *Biochem. J.* **245**, 423 (1987).
8. C. Guguen, A. Guillouzo, M. Boisnard, A. Le Cam and M. Bourel, *Biol. Gastroenterol.* **8**, 223 (1975).
9. D. C. Vander Jagt, L. A. Hunsaker, K. B. Garcin and R. E. Royer, *J. biol. Chem.* **260**, 11603 (1985).
10. D. J. Meyer, L. G. Christodoulides, H. K. Tan and B. Ketterer, *FEBS Lett.* **173**, 327 (1984).
11. C. B. Pickett, C. A. Telakowski-Hopkins, G. J.-F. Ding, L. Argenbright and A. Y. H. Lu, *J. biol. Chem.* **259**, 5 182 (1984).

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).