Biochimica et Biophysica Acta, 1112 (1992) 14–18

BBAMEM 75797

Effects of alcohols on fluorescence anisotropies of diphenylhexatriene and its derivatives in bovine blood platelets: relationships of the depth-dependent change in membrane fluidity by alcohols with their effects on platelet aggregation and adenylate cyclase activity

Shuji Kitagawa and Hirotaka Hirata

Niigata College of Pharmacy, Niigata (Japan)

(Received 20 May 1992)

Key words: Alcohol; Diphenylhexatriene; Blood platelet; Membrane fluidity; Adenylate cyclase; (Bovine blood)

The effects of three short-chain alkyl alcohols and benzyl alcohol on the membrane fluidity of bovine blood platelets were investigated by studies on the fluorescence anisotropies of diphenylhexatriene (DPH), its cationic trimethylammonium derivative (TMA-DPH) and its anionic propionic acid derivative (DPH-PA). These alcohols decreased the fluorescence anisotropy of DPH, which is thought to be located within the hydrophobic core of the membrane, in concentration ranges that inhibited platelet aggregation. On the other hand, they had little or no effects on the fluorescence anisotropy of DPH-PA which is thought to be located in the interfacial region of the lipid bilayer. Likewise, they had little or no effects on the fluorescence anisotropy of TMA-DPH, which is also thought to be located in the interfacial region of the lipid bilayer, either when the probe was located in the outer layer of the plasma membrane or when the probe was located in the inner membrane compartment. These results suggest that alcohols mainly increase the fluidity in the central region of the lipid bilayer. Consistent with their effects on the fluorescence anisotropy of DPH, these alcohols increased the intracellular cyclic AMP concentration. Thus alcohols may inhibit platelet function due to stimulation of adenylate cyclase, which is mediated by perturbation of the central region of the membrane lipid bilayer.

Introduction

Various fluorescent probes have been employed to examine the fluidity of cellular membranes. The fluorophores of these probes are suggested to be present at different depths in the membrane due to differences in their chemical structures [1–3]. Moreover, these probes are thought to be located in different regions of cellular membranes in heterogeneous biological membranes. These differences in their localizations seem to depend on differences in their electric charges and chemical structures. Recently, we examined the localizations of diphenylhexatrine (DPH) and its cationic trimethylammonium derivative (TMA-DPH) and anionic propionic acid derivative (DPH-PA) in platelet membranes [4]. Cationic TMA-DPH first binds to the outer layer of the plasma membrane and then gradually penetrates to the cytoplasmic side by a flip process, finally binding to the negatively charged cytoplasmic surface of the membrane [4,5]. Anionic DPH-PA is mainly located in the outer layer as judged by its electronic expulsion from the cytoplasmic layer. The depths of ionic amphiphilic fluorescent probes differ from those of nonionic hydrophobic probes in membrane lipid bilayers; namely, ionic TMA-DPH and DPH-PA seem to be anchored in close proximity to the bilayer surface, and so provide information on the bilayer lipid environment rather near the surface [2,6], whereas neutral DPH is located within the hydrophobic core of the membrane and so provides information on the membrane fluidity in this deeper region [2].

Many drugs such as alcohols have been suggested to modify platelet functions by changing membrane fluidity [7–10]. However, to clarify the mechanism by which changes in membrane fluidity modify platelet functions, it is necessary to determine the parts of the

Correspondence to: S. Kitagawa, Niigata College of Pharmacy, Kamishin'ei-cho 5-13-2, Niigata 950-21, Japan.

membrane lipid bilayer in which fluidity is changed by the drugs. Therefore, in this work we investigated the effects of three short-chain alkyl alcohols and benzyl alcohol on membrane fluidity of bovine blood platelets using DPH and its derivatives (TMA-DPH and DPH-PA) to determine the relationships of the depth-dependent membrane fluidity changes by these alcohols with their inhibitory effects on platelet aggregation. We used TMA-DPH not only as a reporter of the outer leaflet after short-term incubation with the platelets, but also as a reporter of the inner leaflet after longer incubation and removing the probe in the outer leaflet. We also investigated the effects of these alcohols on the intracellular cyclic AMP level, because benzyl alcohol is known to stimulate adenylate cyclase [11–13], which regulates platelet functions [14,15], and we recently found that *cis*-polyunsaturated fatty acids inhibit platelet function by stimulating adenylate cyclase mediated by membrane perturbation [10]. We examined the relationship between the effects of the alcohols on adenylate cyclase and their effects on the fluorescence anisotropies of DPH and its two derivatives to determine the mechanisms of inhibition of platelet function by these alcohols.

Materials and Methods

Materials

TMA-DPH and DPH-PA were purchased from Molecular Probe (Junction City, OR). DPH and bovine serum albumin (essentially fatty acid free) were from Sigma (St. Louis, MO). ADP was purchased from Oriental Yeast (Tokyo, Japan). Alkyl alcohols, benzyl alcohol and other reagents were all from Wako Pure Chemical Industries (Osaka, Japan). A cyclic AMP radioimmunoassay kit was purchased from Yamasa (Tokyo, Japan).

Preparation of platelet suspension

Platelet-rich plasma was prepared from bovine blood as described previously [16]. The plasma, which contained about 10% by volume of ACD anticoagulant solution (122 mM dextrose, 74.8 mM sodium citrate and 38.1 mM citric acid), was centrifuged, and the platelets were washed and suspended in a solution of Na,K-Tris medium (137 mM NaCl, 5.4 mM KCl, 11 mM dextrose and 25 mM Tris-HCl adjusted to pH 7.4). The final platelet concentration was about $2.7 \cdot 10^5/\mu l$. Spontaneous platelet aggregation during preservation was prevented by adding 129 mM citrate (adjusted to pH 7.4) to this suspension in a ratio of 1 : 9 (vol/vol) in order to minimize Ca^{2+} in the platelet suspension. Experiments were finished within five hours after preparation of the platelet suspension without significant deterioration of platelet functions.

Measurement of fluorescence anisotropy

The fluorescence anisotropies of DPH and its derivatives in platelets were measured as described previously [4]. The platelet suspension described above was mixed with Na,K-Tris medium. For fluorescence measurements, the final platelet concentration was adjusted to $9 \cdot 10^4/\mu l$ to avoid the effect of light scattering in the presence of a high concentration of platelets. TMA-DPH, DPH-PA and DPH were prepared as solutions in dimethylformamide. Platelets were incubated with final concentrations of 0.5 μM DPH-PA at 37 °C for 2 min or with 0.5 μM DPH for 10 min before the measurements when these probes are used to monitor membrane fluidity. Platelets were incubated with 0.5 μM TMA-DPH at 37°C for 2 min when the probe was used as a reporter of the outer leaflet. When the probe was used as a reporter of the inner leaflet, platelets were incubated with it at 37°C for 120 min and they were washed twice with 1% albumin to remove the probe in the outer leaflet [4]. Then the platelets were resuspended in Na,K-Tris medium. The amount of dimethylformamide solution of DPH derivatives added to the platelet suspension was 0.033% by volume to avoid the effect of the solvent on platelet membranes. The fluorescence in platelets was measured in an F-4010 spectrofluorometer (Hitachi Seisakusho, Tokyo, Japan) at 37°C. The excitation and emission wavelengths used with DPH, TMA-DPH and DPH-PA were 363 and 428 nm, 365 and 428 nm and 366 and 430 nm, respectively. Fluorescence anisotropy was calculated as described previously [17].

Measurement of platelet aggregation

Platelet aggregation induced by 10 μM ADP was measured as reported previously [18]. The platelet suspension was mixed with 9 vol. of Na,K-Tris medium containing 1 mg/ml fibrinogen. After preincubation with alcohols for 2 min, $CaCl_2$ at a final concentration of 0.5 mM and ADP were added and aggregation was measured at 37°C in an RAM-11 aggregometer (Rikadenki Kogyo Tokyo, Japan). The effects of alcohols were expressed as maximum aggregations relative to that without alcohol, as described previously [18].

Measurement of cyclic AMP level

Cyclic AMP levels were assayed as described previously [10] according to the method of Honma et al. [19]. A platelet suspension was mixed with 9 vol. of Na,K-Tris medium and incubated with alcohols at 37°C for 3 min in the presence of 7 mM theophylline. Then the reaction was terminated by adding cold trichloroacetic acid. After centrifugation and removal of trichloroacetic acid, the cyclic AMP concentration in the supernatant was measured with a radioimmunoassay kit.

Results

Effects of alcohols on fluorescence anisotropies of DPH and its analogs in platelet membranes and their relationship with inhibitory effects on platelet aggregation

First we investigated the effects of three alkyl alcohols, n-amyl, n-hexyl and n-heptyl alcohols, and benzyl alcohol, on the fluorescence anisotropies of DPH, TMA-DPH and DPH-PA to determine the effects of these alcohols on membrane fluidity at different depths in the lipid bilayer. These alcohols did not cause lysis of platelets within the concentrations tested in this experiment according to the assay of lactate dehydrogenase activity liberated into the medium (data not shown). Intactness of the platelets was also ascertained by the result that initial binding of TMA-DPH to the platelets was not changed by the alcohols because lysis should increase the binding [4] (data not shown). Previously we found that n-amyl and n-hexyl alcohols inhibit platelet aggregation in the same concentration ranges in which they decrease the fluorescence polarization of DPH [7]. The dose-dependent effect of n-hexyl alcohol on the fluorescence anisotropy of DPH is shown in Fig.

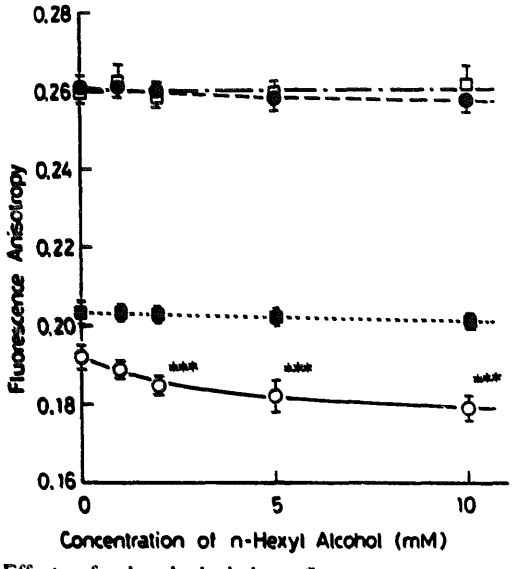

Fig. 1. Effects of n-hexyl alcohol on fluorescence anisotropies of DPH (○——○) DPH-PA (●- - -●) TMA-DPH in the outer leaflet (□- - -□) and TMA-DPH in the inner membrane compartment (■·····■) in bovine blood platelets. Platelets were incubated with DPH-PA for 2 min or with DPH for 10 min at 37°C before measuring fluorescence anisotropy. When TMA-DPH was used as a reporter of the outer leaflet, platelets were incubated with TMA-DPH at 37°C for 2 min. When the probe was used as a reporter of the inner leaflet, platelets were incubated with it at 37°C for 120 min and they were washed with albumin to remove the probe in the outer leaflet and then resuspended in Na,K-Tris medium. Data are means ±S.D. for eight experiments. Statistically significant differences between control values and those in the presence of n-hexyl alcohol were determined by Student's *t*-test: * $P < 0.05$, *** $P < 0.001$.

TABLE I

Effects of alcohols on fluorescence anisotropies of DPH, DPH-PA and TMA-DPH

The experimental procedures were as for Fig. 1. Data are means ± S.D. Numbers in parentheses indicate numbers of replicate experiments. The statistical significance was determined by Student's *t*-test: * $P < 0.05$, *** $P < 0.001$.

Probe	Alcohol	Concn. (mM)	Fluorescence anisotropy
DPH	Control		0.192 ± 0.003 (8)
	n-Amyl	25	0.179 ± 0.003 (8) ***
	n-Hexyl	10	0.179 ± 0.003 (6) ***
	n-Heptyl	2.5	0.182 ± 0.005 (7) ***
	Benzyl	20	0.181 ± 0.003 (7) ***
DPH-PA	Control		0.261 ± 0.003 (8)
	n-Amyl	25	0.257 ± 0.004 (6)
	n-Hexyl	10	0.257 ± 0.003 (8) *
	n-Heptyl	2.5	0.257 ± 0.003 (8) *
	Benzyl	20	0.258 ± 0.004 (9)
TMA-DPH (outer layer)			
	Control		0.260 ± 0.003 (8)
	n-Amyl	25	0.259 ± 0.004 (6)
	n-Hexyl	10	0.261 ± 0.005 (6)
	n-Heptyl	2.5	0.257 ± 0.005 (7)
	Benzyl	20	0.261 ± 0.004 (8)
TMA-DPH (inner layer)			
	Control		0.203 ± 0.002 (8)
	n-Amyl	25	0.200 ± 0.002 (8) *
	n-Hexyl	10	0.201 ± 0.002 (8)
	n-Heptyl	2.5	0.201 ± 0.002 (8)
	Benzyl	20	0.199 ± 0.002 (8) *

1 and the effects of other alcohols at the concentrations that significantly inhibit platelet aggregation are shown in Table I. The decreases in fluorescence anisotropy of DPH by these alcohols corresponded with their inhibitory effects on aggregation, which are shown in Fig. 2 and Table II. These findings are consistent with previous results [7].

On the other hand, as shown in Fig. 1 and Table I, in the concentration ranges that decreased the fluorescence anisotropy of DPH, the alcohols had scarcely any effects on the fluorescence anisotropies of TMA-DPH and DPH-PA which are located in the interfacial region of the lipid bilayer. That is, no significant effects were found on the fluorescence anisotropy of TMA-DPH when the probe was located in the outer layer of the plasma membrane and only slight effects of 25 mM n-amyl alcohol and 20 mM benzyl alcohol were found when the probe was located in the inner membrane compartment. Likewise, only slight effects of 10 mM n-hexyl alcohol and 2.5 mM n-heptyl alcohol were found for the fluorescence anisotropy of DPH-PA which was mainly located in the outer layer [4]. These results suggest that alcohols increase membrane fluidity mainly in the hydrophobic core of the lipid bilayer.

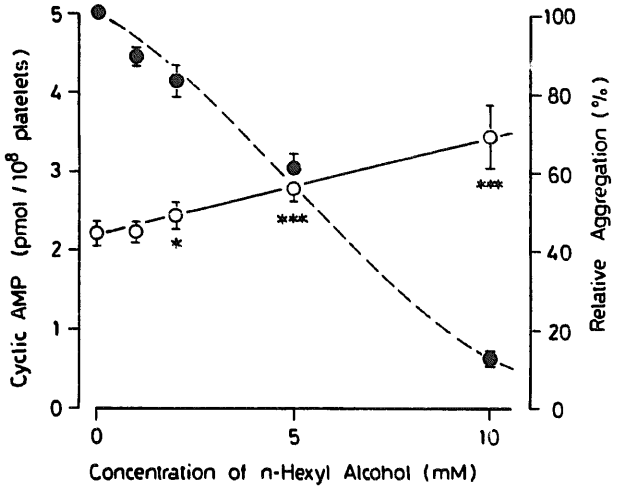

Fig. 2. Effects of n-hexyl alcohol on intracellular cyclic AMP levels (○———○) and 10 μM ADP-induced aggregation (●- - -●). In assay of cyclic AMP levels, the platelet suspension described above was incubated with n-hexyl alcohol at 37°C for 3 min in the presence of 7 mM theophylline. The reaction was terminated with trichloroacetic acid, and cyclic AMP concentrations were measured with a radioimmunoassay kit. ADP-induced aggregation was measured as described previously [18]. Data on cyclic AMP are means ± S.D. for six experiments and data on aggregation are those for three experiments. Statistical significance of data on cyclic AMP levels was determined by Student's *t*-test: * $P < 0.05$, *** $P < 0.001$.

This possibility is consistent with the findings with DPH and TMA-DPH in phosphatidylcholine liposomes reported by Zavoico et al. [20].

Effects of alcohols on intracellular cyclic AMP levels

Next we examined the mechanism by which a change in fluidity in the central region of the lipid bilayer modifies platelet functions. Change in membrane fluidity seems to modify some key enzymes regulating platelet functions. A likely candidate is adenylate cyclase, which is involved in cyclic AMP production. This enzyme is believed to be modulated by the lipid environment of the membrane [11,12,21]; for example, decrease in the cholesterol level enhances the adenylate cyclase activity [21]. Several reagents are known to inhibit platelet functions by activating this enzyme system with consequent inhibition of increase in cytoplasmic Ca^{2+} [14,15]. Moreover, benzyl alcohol, one of the alcohols tested in this study, is known to stimulate adenylate cyclase by increasing membrane fluidity [11,12].

As shown in Fig. 2 for the dose-dependent effects of n-hexyl alcohol and in Table II for the effects of other alcohols at the concentrations that fully inhibit platelet aggregation, the increases in the cyclic AMP level by the alcohols corresponded well with their inhibitory effects on aggregation. At the concentrations of these alcohols that fully inhibited aggregation, the cyclic AMP level was increased to similar extents to 1.5–1.7-fold the basal level. The stimulating effects of these alcohols resemble those of long-chain *cis*-unsaturated fatty acids, which we recently reported [10].

The present results indicate that these alcohols activate the basal activity of adenylate cyclase by increasing membrane fluidity, mainly in the central region of the membrane lipid bilayer. These results are consistent with the report by Spence and Houslay that benzyl alcohol stimulates the basal activity of adenylate cyclase in human platelets but has no effect on the detergent-solubilized enzyme [13]. The present results revealed that the inhibition of platelet aggregation by the alcohols is at least partly due to activation of the enzyme induced by perturbation of the central region of the lipid bilayer.

TABLE II

Effects of alcohols on intracellular cyclic AMP levels in the presence of theophylline and on 10 μM ADP-induced aggregation

The experimental procedures were as for Fig. 2. Data on cyclic AMP are means ± S.D. for four experiments and data on aggregation are those for three experiments. Experiments were made on the different samples from those shown in Fig. 2. Statistical significances of data on cyclic AMP levels were determined by Student's *t*-test: *** $P < 0.001$.

Alcohol	Concn. (mM)	Cyclic AMP (pmol/10^8 platelets)	Relative aggregation %
Control		2.11 ± 0.11	100
n-Amyl	25	3.38 ± 0.21 ***	18.6 ± 2.4
n-Hexyl	10	3.32 ± 0.14 ***	12.9 ± 1.6
n-Heptyl	2.5	3.16 ± 0.17 ***	17.3 ± 4.6
Benzyl	20	3.53 ± 0.24 ***	2.2 ± 1.4

Discussion

The results described in this paper suggest that alcohols affect the fluidity of a membrane lipid bilayer differentially depending on the depth. These results are consistent with previous reports. From findings in phosphatidylcholine liposomes with DPH and TMA-DPH, Zavoico et al. concluded that alkyl alcohols become located at the surface of a membrane lipid bilayer causing increase in the packing density at the surface resulting in disorder of the center of the membrane [20]. This possibility is consistent with the ESR data of Chin and Goldstein obtained with 5-, 12- and 16-doxylstearic acid on the effects of ethanol in phosphatidylcholine liposomes [22]. In studies on brain membranes with the fluorescent probe 1-aminopyrene, which monitors the membrane surface, the effects of ethanol were smaller than those observed in the hydrophobic region using DPH as a probe [23]. Similar results were observed with the surface probe 2-*p*-toluidinylnaphthalene-6-sulfonate for the effect of butanol on erythrocyte membranes [24].

However, the present results are not consistent with our previous ESR findings in blood platelet membranes using 5-doxylstearic acid, its methyl ester, 16-doxylmethylstearate and 4-(*N*,*N*-dimethyl-*N*-pentadecyl)ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl (CAT-15) as spin-labeled probes [25]. It is uncertain whether spin labels or fluorophores are more accurate for detecting fluidity changes in membranes. Since much lower concentrations of fluorescent probes than those of spin labels are required for detecting these changes, the possibility that probes and alcohols synergistically modify membrane fluidity must be less in fluorescence measurements. However, the possibility cannot be excluded that although alcohols affect membrane fluidity mainly in the central region, they also modify the fluidity in the interfacial region of the lipid bilayer in a different manner. The rotational movement of a large spin-labeled group such as a doxyl group attached to a long alkyl chains might be more sensitive to change in membrane fluidity than DPH derivatives. Moreover, in biological membranes with complicated lipid compositions, the localizations of DPH derivatives may be different from those of long-chain alkyl probes.

The changes in fluorescence anisotropy of DPH observed in this study correlated well with aggregation inhibition and the platelet cAMP level. Therefore, although alcohols may modify other enzymatic systems, such as the inositol phospholipid metabolizing system which also regulates platelet functions, stimulation of the basal activity of adenylate cyclase, which is probably induced by perturbation of the central region of the lipid bilayer, seems to be at least partly involved in the inhibition of platelet functions. Thus alcohols seem to inhibit platelet functions by the same mechanism as the long-chain *cis*-polyunsaturated fatty acids studied previously [10]. These compounds increased the cyclic AMP level to similar extents to 1.5–1.7-fold the basal level at the concentrations that fully inhibited aggregation. However, to which extent such increase of cAMP can explain the effects of these compounds on aggregation is still not clear. Moreover, the mechanism of the modification of the basal activity of the enzyme activity by these compounds is also not clear. Perturbation of the central region of the lipid bilayer may modify the conformation of the catalytic unit of the enzyme and change its activity.

References

1 Thulborn, K.R., Tilley, L.M., Sawyer, W.H. and Treloar, F.E. (1979) Biochim. Biophys. Acta 558, 166–178.
2 Trotter, P.J. and Storch, J. (1989) Biochim. Biophys. Acta 982, 131–139.
3 Collins, J.M. and Grogan, W.M. (1991) Biochim. Biophys. Acta 1067, 171–176.
4 Kitagawa, S., Matsubayashi, M., Kotani, K., Usui, K. and Kametani, F. (1991) J. Membr. Biol. 119, 221–227.
5 Bevers, E.M., Verhallen, P.F.J., Visser, A.J.W.G., Comfurius, P. and Zwaal, R.F.A. (1990) Biochemistry 29, 5132–5137.
6 Kuhry, J.-G., Fonteneau, P., Duportail, G., Maechling, C. and Laustriat, G. (1983) Cell Biophys. 5, 129–140.
7 Kitagawa, S., Shinohara, T. and Kametani, F. (1984) J. Membr. Biol. 79, 97–102.
8 Kitagawa, S., Endo, J. and Kametani, F. (1985) Biochim. Biophys. Acta 818, 391–397.
9 Nosál, R., Jančinová, V., Ondriaš, K., Jakubovský, J. and Balgavý, P. (1985) Biochim. Biophys. Acta 821, 217–228.
10 Kitagawa, S., Kotani, K. and Kametani, F. (1990) Biochim. Biophys. Acta 1054, 114–118.
11 Gordon, L.M., Sauerheber, R.D., Esgate, J.A., Dipple, I., Marchmont, R.J. and Houslay, M.D. (1980) J. Biol. Chem. 255, 4519–4527.
12 Friedlander, G., Le Grimellec, C., Giocondi, M.-C. and Amiel, C. (1987) Biochim. Biophys. Acta 903, 341–348.
13 Spence, S. and Houslay, M.D. (1989) Biochem. J. 264, 483–488.
14 Feinstein, M.B., Egan, J.J., Sha'afi, R.I. and White, J. (1983) Biochem. Biophys. Res. Commun. 113, 598–604.
15 Zavoico, G.B. and Feinstein, M.B. (1984) Biochem. Biophys. Res. Commun. 120, 579–585.
16 Kitagawa, S., Hongu, Y. and Kametani, F. (1982) Biochem. Biophys. Res. Commun. 104, 1371–1375.
17 Kubina, M., Lanza, F., Cazenave, J.-P., Laustriat, G., Kuhry, J.-G. (1987) Biochim. Biophys. Acta 901, 138–146.
18 Kitagawa, S., Nishitama, H. and Kametani, F. (1984) Biochim. Biophys. Acta 775, 197–202.
19 Honma, M., Satoh, T., Takezawa, J. and Ui, M. (1977) Biochem. Med. 18, 257–273.
20 Zavoico, G.B., Chandler, L. and Kutchai, H. (1985) Biochim. Biophys. Acta 812, 299–312.
21 Sinensky, M., Minneman, K.P. and Molinoff, P.B. (1979) J. Biol. Chem. 254, 9135–9141.
22 Chin, J.H. and Goldstein, D.B. (1981) Mol. Pharmacol. 19, 425–431.
23 Harris, R.A. and Schroeder, F. (1981) Mol. Pharmacol. 20, 128–137.
24 Kramer, P.A. and Li, T.-K. (1975) Biochem. Pharmacol. 24, 341–346.
25 Kitagawa, S., Kametani, F., Tsuchiya, K. and Sakurai, H. (1990) Biochim. Biophys. Acta 1027, 123–129.