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EFFECTS OF PROSTAGLANDIN D_2 ON MEMBRANE POTENTIAL IN NEUROBLASTOMA X GLIOMA HYBRID CELLS AS DETERMINED WITH A CYANINE DYE*

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SUMMARY: A cyanine dye, diS-C_3-(5) was used to determine the effects of prostaglandins on the membrane potential in neuroblastoma X glioma cells (NG 108-15). The largest depolarization was seen with prostaglandin D_2 (ED_{50} = 1.5 μM), and relative potencies of various prostaglandins (3 μM) were: D_2, 100; I_2, 41; E_1, 17; E_2, 7; and $F_{2\alpha}$, 7. 5-Hydroxytryptamine in a dose over 100 μM also depolarized the membrane. The effect of prostaglandin D_2 was observed in a Na^+-free medium or when Ca^{2+} was replaced by Sr^{2+}. The addition of 3 mM ethylene-glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid or 5 mM Co^{2+} partially inhibited the effects. These observations suggest that the depolarization of membrane by prostaglandin D_2 may primarily be related to alteration of Ca^{2+} permeability in the cell membrane.

Recent investigations in our laboratory revealed that prostaglandin D_2 (PG[1/] D_2) was actively biosynthesized and metabo-

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1/ Abbreviations used are: PG, prostaglandin; diS-C_3-(5), 3,3'-dipropyl-2,2'-thiadicarbocyanine iodide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FI, fluorescence intensity; and EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

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lized in neural tissues (1-4) and that it activated the adenylate cyclase system of cultured neuroblastoma cells (3, 4). In addition, the neuromodulatory function of PGD_2 as related to autonomic neurotransmission has been reported (5). In an attempt to determine whether or not this compound alters the membrane potential of neural cells, we used a nondestructive fluorescent probe that interacted with intact cell membrane, as has been reported by various investigators (6-10). In the present communication, we report the effects of various PGs on the membrane potential in neuroblastoma X glioma hybrid cells. The possible role of Ca ion in this process is briefly discussed.

Materials and Methods

Chemicals. Commercial sources of reagents and materials were as follows: Cyanine dye diS-C_3-(5), from Nippon Kankoh Shikiso Kenkyusho; valinomycin and Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), from Nakarai Chemicals; ionophore A23187, from Calbiochem; Dulbecco's modified Eagle medium (DME-medium), from Nissui Seiyaku Co.; penicillin G potassium and streptomycin sulfate, from Meiji Seika Co.; hypoxanthine, aminopterin, thymidine, $SrCl_2$, and $CoCl_2$, from Wako Pure Chemicals; and 5-hydroxytryptamine creatinine sulfate, from Sigma. PGs were kindly donated by Dr. M. Hayashi of the Ono Central Research Institute.

Cells. Neuroblastoma X glioma hybrid cells (NG 108-15) were generous gifts of Dr. T. Amano of Mitsubishi-Kasei Institute of Life Sciences and were grown in petri dishes (Nunc) in 90% DME-medium containing 10% fetal bovine serum (Grand Island Biological Company) and 44 mM $NaHCO_3$ supplemented with 0.1 mM hypoxanthine, 0.25 µM aminopterin, 16 µM thymidine, 100 units/ml penicillin G potassium and 100 µg/ml streptomycin sulfate. Cells were grown in a humidified atmosphere of 10% CO_2-90% air at 37°C. The culture medium was changed every 1 or 2 days. Cells in confluent cultures were washed twice with a medium A (140 mM NaCl, 3 mM KCl, 2.4 mM $CaCl_2$, 1.3 mM $MgCl_2$, 10 mM glucose, and 25 mM Hepes/NaOH pH 7.4), scraped out with a rubber policeman and suspended in a medium A. The washed cells were resuspended in the same medium at a density of 5 X 10^6 cells/ml, and kept in an ice bath until use. Cell viability was 80 $\pm$ 5% as determined by dye exclusion test (trypan blue).

Fluorescence measurements with the intact cells. Fluorescence intensities were measured using a Shimadzu spectrofluorometer model RF 501 with a 3-ml quartz cuvette. The fluorescence at 670 nm was determined on excitation at 622 nm. All recordings were made with both excitation and emission slits of 5 nm. The temperature was maintained constant at 37°C by water circulating

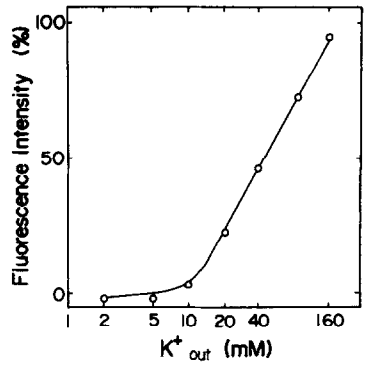

Fig. 1. Effects of KCl on FI in the presence of 3 μM valinomycin. To the cell suspension (3 X 10^5 cells in 3 ml) equilibrated with 1 μM diS-C_3-(5) and 3 μM valinomycin, aliquots of 3 M KCl were successively added. FI change was expressed according to the equation described under "Materials and Methods".

through the cuvette holder. The medium in a cuvette was mixed gently with a magnetic stirrer throughout the measurements. To a 3-ml suspension medium in a cuvette, 3 μl of diS-C_3-(5) (final concentration, 1 μM) were added. After equilibration, 60 μl of cell suspensions were added to a final concentration of 10^5 cells/ml. After 8 min the steady level of fluorescence was obtained and then 4.5 μl of 2 mM valinomycin dissolved in ethanol were added. Test reagents were added 4 min after the addition of valinomycin. Change in fluorescence intensities (FI) was defined as follows (6): Change in FI = $(FI_{t2} - FI_{t1})/(FI_{t0} - FI_{t1})$: Where FI_{t0} is FI of a cyanine dye, FI_{t1} is FI of cell/dye suspensions, and FI_{t2} is FI 4 min after the addition of a test reagent.

Results and Discussion

Measurements of Membrane Potentials of NG 108-15 Cells with a Cyanine Dye diS-C_3-(5). The membrane potential is dependent on the extracellular potassium concentration in the presence of valinomycin, a K^+ selective carrier (6). As shown in Fig. 1, the fluorescence intensities (FI) of cell/dye suspensions were related to the log concentrations of potassium in the medium (log (K_{out})). There was a linear correlationship between FI and log (K_{out}) with concentrations over 10 mM. When the extracellular K^+ concentration was 160 mM, FI was 100% (all dye was excluded from cells) indicating the lack of transmembrane potential caused by

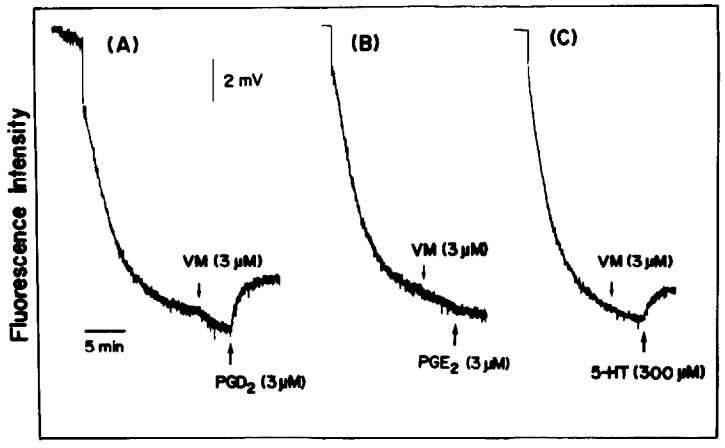

Fig. 2. Effects of PGD_2 (A), PGE_2 (B), and 5-hydroxytryptamine (5-HT) (C) on changes in FI in the presence of 3 μM valinomycin (VM). PGs were added as 30 μl aliquots of 300 μM solutions in 90% medium A-10% ethanol, and 5-hydroxytryptamine was added as 45 μl aliquots of 20 mM solution in medium A. The pH was adjusted to 7.4.

K^+ gradient. The effects of KCl solution were not attributable to Cl^- or osmolarity because neither NaCl nor sucrose at the same concentrations had any effects. The results indicate that the changes in FI were due to a membrane potential-sensitive partition of the dye between NG 108-15 cells and the suspension medium, and the relative changes of membrane potential could be determined using the changes in FI as have been demonstrated in other types of cells (6-10).

Effects of Various PGs and 5-Hydroxytryptamine on the Changes in FI. When PGD_2 (3 μM) was added to the cell suspensions previously equilibrated with 1 μM diS-C_3-(5) and 3 μM valinomycin, a rapid increase in FI was observed and lasted about 2 min (Fig. 2A). The change could not be detected when cells were omitted from the medium or when 30 μl of 10% ethanol (used as a solvent for various PGs) were added to the cell/dye suspensions. PGE_2 in a dose of 3 μM had little effect (Fig. 2B). Among the various types of PG tested, PGD_2 was the most effective in depolarizing

Table I.

Effects of various PGs on the changes in FI with NG 108-15 cells

	0.3 μM	3 μM	30 μM
		(%)	
PGD_2	22.1	100[a]	115
PGI_2 (methyl ester)	26.0	40.7	61.4
PGE_1	12.1	17.0	21.0
PGE_2	-5	7.2	11.0
$PGF_{2\alpha}$	0	7.4	8.0

a/ All values are expressed as percentages of the change in FI obtained with 3 μM PGD_2 (=100).

the membrane (Table I). PGI_2 was as effective as PGD_2 at 0.3 μM, but above 3 μM it was half as active as PGD_2. The depolarization with PGE_1 was 20-50% of that with PGD_2, and PGE_2 and $F_{2\alpha}$ were almost ineffective. 5-Hydroxytryptamine which was shown by other investigators to depolarize the membrane of NG 108-15 cells (11) was also effective at higher concentrations (Fig. 2C). Fig. 3

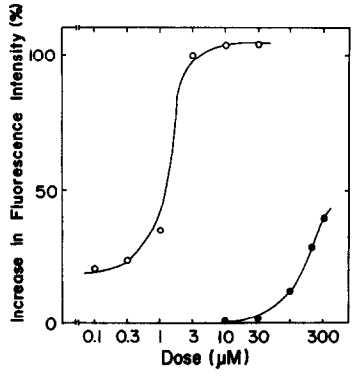

Fig. 3. Dose dependencies of PGD_2 (o) and 5-hydroxytryptamine (●). To cell suspensions, PGD_2 (30 μl) and 5-hydroxytryptamine (45 μl) were added to obtain the final concentrations as shown in the abscissa. One hundred percent is defined as the change in FI obtained with 3 μM PGD_2.

Table II.

Changes in FI with various modified media

medium A	100%
Na^+-free medium[a]	90
Na^+-free[a], $SrCl_2$ medium[b]	91
Na^+-free[a] medium + 5 mM $CoCl_2$	54
Na^+-free[a] medium + 3 mM EGTA	50

One hundred percent is defined as the change caused by PGD_2 in a medium A. The composition of medium A was described under "Materials and Methods".

a/ NaCl was replaced by 140 mM choline chloride.

b/ $CaCl_2$ was replaced by the same concentration of $SrCl_2$.

shows dose response curves for PGD_2 and 5-hydroxytryptamine. PGD_2 was about two orders of magnitude more potent than 5-hydroxytryptamine in depolarizing the membrane. Effects of PGD_2 were not observed when human erythrocytes were equilibrated with the same dye (data not shown), whereas KCl at 50 mM did increase FI under the same conditions. These results, taken together, suggest that the depolarization caused by PGD_2 may be a receptor-mediated process, rather than a nonspecific interaction of PGD_2 with cells or the dye, or both. This interpretation was further substantiated by the observation that the effect of PGD_2 was saturable (ED_{50} = 1.5 μM) (Fig. 3). Even in the absence of valinomycin, PGD_2 as well as 5-hydroxytryptamine caused membrane depolarization. Although the relative potencies of various agents remained unchanged, the degrees of depolarization decreased to about 25%. This is probably due to the fact that the electrical excitability of neuroblastoma cells is enhanced in the presence of valinomycin as suggested before (12).

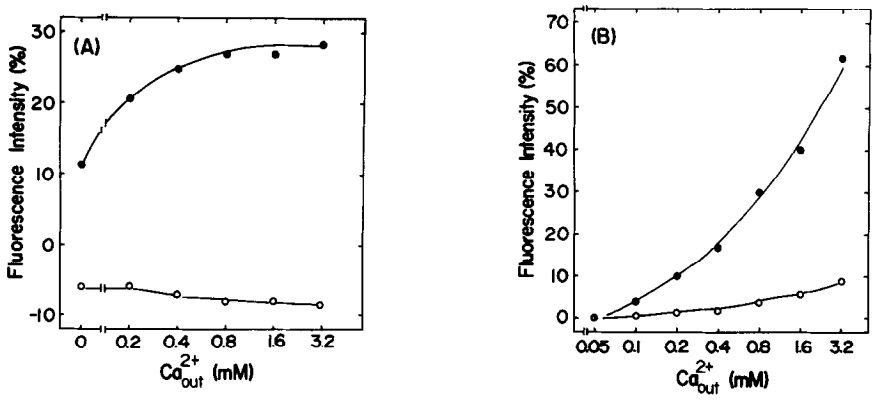

Fig. 4A. Effects of extracellular Ca^{2+} concentrations on the change in FI caused by 3 μM PGD_2 (●), or ethanol alone (o). B. Effects of Ca^{2+} concentrations on the change in FI either in the presence (●) or absence (o) of 2 μM A23187.

Roles of Various Ions and Effects of Inhibitors on PGD_2-elicited Change. As previously reported, NG 108-15 cells had at least two types of ion (Na^+ and Ca^{2+}) channels (13). To determine which type of ion channels is involved in the membrane depolarization caused by PGD_2, we replaced Na^+ by choline$^+$. As shown in Table II, the effect of PGD_2 was also observed in a Na^+-free medium to the same extent. Ca^{2+} could for the greater part be replaced by Sr^{2+}. Co^{2+} (5 mM) and EGTA (3 mM) inhibited the depolarization by about 50%. Fig. 4A illustrates the depolarization by PGD_2 as measured with various concentrations of Ca^{2+} in the medium. Changes in FI increased depending on the concentrations of extracellular Ca^{2+} up to 0.8 mM, but not K^+ from 3 mM to 20 mM. These findings suggest that the depolarization of membrane potential by PGD_2 is at least in part due to the participation of Ca^{2+}. In fact, the influx of Ca^{2+} could cause membrane depolarization, as proven by the experiment with a divalent cation ionophore A23187 (Fig. 4B).

PGD_2, PGE_1 (3) and I_2[2/] are all activators of the adenylate cyclase system in cultured neuroblastoma cells. It remains to be

2/ Shimizu, T. and Hayaishi, O. Manuscript in preparation.

elucidated in what manner the activation of the adenylate cyclase correlates with the change in Ca^{2+} permeability and membrane potential.

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