Vol. 131, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1985 Pages 370-377

CALMODULIN ANTAGONISTS ELEVATE THE LEVELS OF ^{32}P-LABELED POLYPHOSPHOINOSITIDES IN HUMAN PLATELETS

E. Ann Tallant and Robert W. Wallace*

Department of Pharmacology
University of Alabama at Birmingham
Birmingham, AL 35294

Received July 15, 1985

SUMMARY: The calmodulin antagonists trifluoperazine, chlorpromazine, perphenazine, promazine, tamoxifen and the naphthalene sulfonamide derivatives W7 and W13 increased the level of ^{32}P-incorporation into human platelet PIP and PIP_2. Various drugs with poor anti-calmodulin activity were ineffective. The increase in ^{32}P-PIP and ^{32}P-PIP_2 required micromolar concentrations of trifluoperazine and was time-dependent, reaching half-maximal within two minutes of the addition of the drug. These results indicate that the calmodulin antagonists perturb polyphosphoinositide metabolism, probably at the level of the PI- and PIP-kinases and/or the PIP_2- and PIP-phosphomonoesterases.

The turnover of membrane phosphoinositides is thought to be a key event connecting receptor occupancy and the elevation of intracellular Ca^{2+} in many cell types (1,2), including platelets (3,4). Activation of a receptor by an agonist results in the hydrolytic cleavage of membrane PIP_2 by phospholipase C, yielding diacylglycerol and inositol triphosphate; diacylglycerol subsequently functions within the membrane to activate a Ca^{2+}/phospholipid-dependent protein kinase (5) and inositol triphosphate is released into the cytoplasm to mobilize intracellular Ca^{2+} (6). Polyphosphoinositide metabolism is regulated by several different enzymes; PIP and PIP_2 are formed by the stepwise phosphorylation of PI by PI- and PIP-kinases, and are degraded either by hydrolytic cleavage by phospholipase C as indicated above, or by dephosphorylation to PIP and subsequently to PI by phosphomonoesterases (1,2,5,6).

*To whom correspondence should be addressed.

Abbreviations used: phosphatidylinositol 4,5-bisphosphate, PIP_2; phosphatidylinositol 4-phosphate, PIP; phosphatidylinositol, PI; trifluoperazine, TFP; and phorbol myristate acetate, PMA.

0006-291X/85 $1.50

The Ca^{2+}-binding protein calmodulin is a major intracellular Ca^{2+}-receptor protein, which mediates many of the regulatory effects of Ca^{2+} upon stimulus-receptor coupling. In human platelets, calmodulin regulates a variety of enzymatic processes (7-10); various drugs which are potent calmodulin antagonists inhibit various aspects of platelet activation (9,11-14). In this paper, we show that these calmodulin-antagonistic drugs also cause increased ^{32}P incorporation into polyphosphoinositides which suggests the presence of a feed-back mechanism linking the level of intracellular Ca^{2+} with the level of polyphosphoinositides through a calmodulin-dependent process.

MATERIALS AND METHODS

Chemicals and Reagents: Carrier-free [^{32}P]orthophosphate was purchased from Amersham, Silica gel G plates (Redi-plate, 20 x 20 cm, 0.5 mm thickness) from Fisher Scientific Company and cellulose thin layer plates (Eastman Chromagram Cellulose 13255, 20 x 20 cm, 0.1 mm thickness) from Eastman Kodak. Chlorpromazine, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7), 4β-phorbol 12-myristate 13-acetate (PMA), propranolol, apyrase, heparin, pyruvate, nicotinamide adenine dinucleotide and phospholipid standards were purchased from Sigma Chemical Co. Various drugs and calmodulin antagonists were obtained from the following sources: trifluoperazine and trifluoperazine sulfoxide, Smith-Kline Laboratories; N-(4-aminobutyl)-2-naphthalenesulfonamide hydrochloride (W12) and N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide hydrochloride (W13), Seikagaka Kogyo Co.; promazine hydrochloride, Wyeth Laboratories Inc.; perphenazine hydrochloride, Schering Corp.; tamoxifen, Stuart Pharmaceuticals; verapamil, Knoll Pharmaceuticals Co.; lidocaine, K. & K. Laboratories Inc.; Haldol, McNiel; Trazadone hydrochloride, Mead Johnson & Co.; Mianserin hydrochloride, Organon (OSS-Holland); Merital (HOE 984, nomifensine maleate), Hoechst-Roussel Pharmaceuticals Inc.; and salicylic acid, a local drug dispensory. All other reagents were of highest analytical grade.

Methods: Venous blood was collected from healthy human volunteers and platelets were prepared by the method of Mustard et al. (15), as modified by Haslam and Lynham (16). Washed platelets were resuspended in Tyrode's buffer containing 0.1 M HEPES, (pH 7.5), at 1 x 10^9 platelets/ml and incubated for 90 min at 37° with 0.4 mCi of carrier-free [^{32}P] Pi/ml. The labeled platelets were washed in Tyrode's buffer and finally resuspended at 1 x 10^9 platelets/ml in albumin-free Tyrode's solution containing 6 μg/ml apyrase and 10 mM EDTA (16). The platelets were then incubated in the presence of various drugs and calmodulin antagonists, as indicated, and the reactions were stopped by the addition of 4.67 ml of chloroform:methanol:concentrated HCl (100:200:2, v/v/v) per ml of reaction mixture. Lipid extracts were prepared according to the procedure of Billah and Lapetina (3) and phospholipids were separated by thin layer chromatography (3,17). Phospholipids were identified by comparison with authentic standards after either staining with iodine vapor, charring with 30% sulfuric acid or autoradiography. Following autoradiography, spots were scraped into scintillation vials and counted for radioactivity by liquid scintillation spectrometry. Cell lysis was determined by measuring the release of lactate dehydrogenase from intact platelets; lactate dehydrogenase activity was determined according to Bergmeyer et al. (18).

RESULTS AND DISCUSSION

Table 1 shows the effect of various drugs on the level of ^{32}P-incorporation into platelet phosphoinositides. The addition of any of the drugs in Group 1 resulted in a significant increase in ^{32}P-incorporation into PIP and PIP_2 and a concomitant decrease in PI. The drugs in this group are all calmodulin antagonists: trifluoperazine, chlorpromazine, perphenazine and promazine are antipsychotics (19,20); tamoxifen is a triphenylethylene antiestrogen (21); and W7 and W13 are naphthalene sulfonamide derivatives (22,23). Trifluoperazine has been shown to increase the formation of PIP and

Table 1
Effect of Various Drugs on the ^{32}P-Incorporation into Platelet Phosphoinositides

Addition	Concentration (μM)	PIP_2	PIP (% Control)	PI
None	-	100	100	100
GROUP 1				
Trifluoperazine	100	205	213	72
Chlorpromazine	100	275	279	74
Perphenazine	100	292	283	85
Tamoxifen	100	189	265	89
Promazine	100	162	162	86
W7	50	158	163	81
W13	50	113	140	72
GROUP 2				
Trifluoperazine-sulfoxide	100	101	109	93
W12	50	96	92	94
Verapamil	100	98	83	76
Propranolol	100	109	111	86
Lidocaine	100	82	75	84
Salicylic Acid	100	87	96	96
Haloperidol	100	105	112	89
Trazadone	100	84	94	99
Mianserin	100	115	115	96
Merital	100	92	87	94
GROUP 3				
PMA	10 ng/ml	144	127	89

Human platelets were prelabeled with ^{32}Pi and incubated for 5 min at 37° in the presence of the indicated concentration of drug. The lipids were subsequently extracted and separated by thin layer chromatography as described in "Materials and Methods". ^{32}P-incorporation is presented as the % of the control value, i.e., with no addition.

PIP_2 in human platelets (24) and in guinea pig macrophages (25). The drugs in Group 2 showed no effect on ^{32}P-incorporation into PI, PIP or PIP_2. All the drugs in Group 2 have been shown to have poor anti-calmodulin activity at the indicated concentration (20). This group includes the sulfoxide derivative of trifluoperazine, the naphthalene sulfonamide derivative W12, a local anesthetic, a Ca^{2+}-channel blocker, an anti-adrenergic, an antipsychotic, an anti-inflammatory agent and various antidepressants. The one drug in Group 3, PMA, a tumor promoting phorbol ester, showed an effect similar to the calmodulin antagonists, causing an increase in ^{32}P-incorporation into PIP and PIP_2 and a decrease into PI. A similar effect of PMA on platelet phosphoinositide metabolism was shown by de Courcelles et al. (26) and by Halenda and Feinstein (27). Halenda and Feinstein (27) also showed that the increased ^{32}P-labeling of PIP and PIP_2 represented an actual increase in the mass of the polyphosphoinositides.

The amount of ^{32}P-incorporated into the phosphoinositides is dependent upon the amount of trifluoperazine added, as shown by the dose-response curves in Figure 1. Half-maximal increases in ^{32}P-PIP and ^{32}P-PIP_2 were obtained at 110 and 84 μM, respectively. This concentration of drug is similar to that required for inhibition of various aspects of platelet aggregation (9,12,13). The lower curve in Figure 1 shows the percent platelet lysis as a function of trifluoperazine concentration, measured as the release of the soluble enzyme lactate dehydrogenase. Only a small amount of platelet lysis (less than 8%) was found at the concentrations of trifluoperazine which cause increases in the ^{32}P-labeled phospholipids, indicating that the majority of the platelets were still intact. This contrasts with the results of Holmsen et al. (28), who found 30 to 40% platelet lysis in the presence of 50 to 100 μM trifluoperazine; however, their measurements were conducted in the presence of thrombin, a condition which they indicate may make the platelet more susceptible to lysis.

The time course of the effect of trifluoperazine on the ^{32}P-incorporation into the phosphoinositides is shown in Figure 2. ^{32}P-labeled PIP and PIP_2

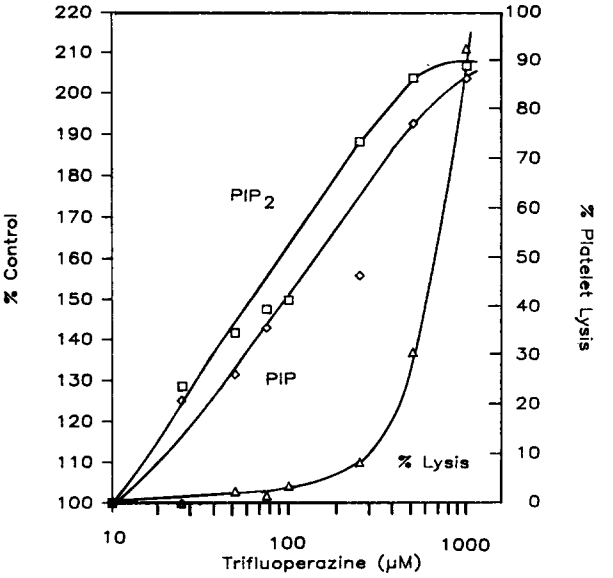

Figure 1. The effect of trifluoperazine on ^{32}P-incorporation into platelet PIP and PIP_2 and on platelet lysis. Washed platelets were prelabeled with ^{32}Pi and incubated for 5 min with various concentrations of trifluoperazine at 37°. Platelet lipids were extracted and analyzed by thin layer chromatography, as described in "Materials and Methods". Data are presented as % Control, i.e. in the absence of drug. Washed platelets were also incubated for 5 min with various concentrations of trifluoperazine and rapidly pelleted by centrifugation at 4000 xg for 5 min. The amount of lactate dehydrogenase activity in the incubation medium was determined as an indication of platelet lysis, as described in "Materials and Methods". Data are presented as the % of lactate dehydrogenase activity in a platelet sample lysed by freeze-thawing. (◇—◇), PIP; (□—□), PIP_2; (△—△), platelet lysis.

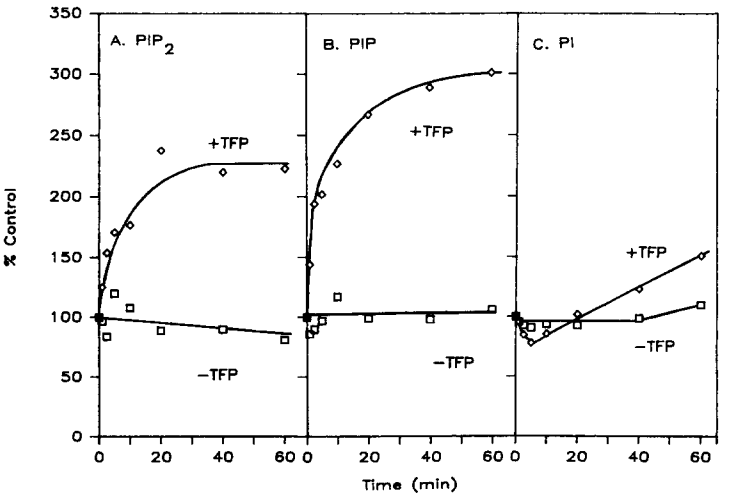

Figure 2. Time course of the effect of trifluoperazine on ^{32}P-incorporation into platelet phosphoinositides. Washed platelets were prelabeled with ^{32}Pi and incubated in the presence or the absence of 100 μM trifluoperazine for the indicated amount of time at 37°. Lipids were extracted and analyzed by thin layer chromatography, as described in "Materials and Methods". The data are presented as % Control, i.e. at zero time, either in the absence (□) or presence (◇) of 100 μM trifluoperazine. Panel A, PIP_2; Panel B, PIP; Panel C, PI.

increased with time of exposure to the drug, significantly increasing as early as 20 seconds after drug addition; half-maximal incorporation occurred after two minutes for both PIP and PIP_2. At the earlier times of drug exposure (up to 10 min), the increase in PIP and PIP_2 was accompanied by a decrease in ^{32}P-incorporation into PI; however, after 10 minutes, the level of ^{32}P-PI also increased and continued to rise throughout the time of incubation. This is probably due to a trifluoperazine-induced increase in the de novo synthesis of PI; trifluoperazine, as well as other cationic amphiphiles, have been shown to increase the de novo synthesis of PI in other tissues and cell types through inhibition of phosphatidate phosphohydrolase (25,29,30). In the absence of the drug, there was no significant change in the level of ^{32}P-incorporation into any of the phosphoinositides.

It has been clearly established that micromolar levels of trifluoperazine inhibit platelet aggregation (9,12,14) and the secretion of both ATP and Ca^{2+} from dense granules (9,14) as well as proteins stored in α-granules (14), presumably through inhibition of the Ca^{2+}/phospholipid-dependent protein kinase and Ca^{2+}/calmodulin-dependent reactions. However, micromolar levels of trifluoperazine have also been shown to actually increase the rate of Ca^{2+} mobilization in thrombin-stimulated platelets (14). This is consistent with our observation that trifluoperazine increases the level of PIP_2, which would facilitate the thrombin-induced release of inositol triphosphate and the subsequent mobilization of intracellular Ca^{2+}. Thus, even though treatment of platelets with trifluoperazine increases the level of PIP_2 and the rate of Ca^{2+} mobilization, the drug has an overall inhibitory effect on platelet activation due to the blockade of Ca^{2+}-dependent reactions distal to Ca^{2+} mobilization.

Elevations in the level of ^{32}P-incorporation into platelet PIP and PIP_2 induced by the calmodulin-antagonistic drugs could result from activation of the PI- and PIP-kinases which phosphorylate the phospholipids, inhibition of the PIP- and PIP_2-phosphomonoesterases which dephosphorylate the respective lipids, or decreased activity of the phospholipase C which hydrolyzes PIP

and/or PIP_2. The drug effects are probably not due to inhibition of phospholipase C since Halenda and Feinstein (27) showed that, in platelets incubated with PGD_2 and theophylline, a condition which inhibits phospholipase C, there was no significant increase in the level of PIP or PIP_2. Some of these drugs antagonize the activity of the Ca^{2+}/phospholipid-dependent protein kinase (20). However, the phorbol ester PMA, which activates the Ca^{2+}/phospholipid-dependent kinase, also caused an elevation in the phosphoinositide levels (this paper; 26,27); it therefore seems unlikely that drugs which inhibit this enzyme would also cause the same effects. Although it is clear that additional research is required to elucidate the mechanism of action of the drugs which elevate the levels of PIP and PIP_2, our data is consistent with the possibility that calmodulin may be involved, either directly or indirectly, in regulation of the polyphosphoinositide kinases or phosphomonoesterases. Such a regulatory mechanism would provide a feedback loop coupling intracellular processes which are regulated by Ca^{2+} with those that are involved in its release.

ACKNOWLEDGMENTS:

This work was supported by NIH grant HL29766 and by grant support from the Cystic Fibrosis Foundation.

REFERENCES

(1) Downes, P. and Michell, R.H. (1982) Cell Calcium 3, 467-502.
(2) Berridge, M.J. (1984) Biochem. J. 220, 345-360.
(3) Billah, M.M. and Lapetina, E.G. (1982) J. Biol. Chem. 257, 12705-12708.
(4) Agranoff, B.W., Murthy, P. and Seguin, E.B. (1983) J. Biol. Chem. 258, 2076-2078.
(5) Nishizuka, Y. (1984) Nature 308, 693-698.
(6) Berridge, M.J. and Irvine, R.F. (1984) Nature 312, 315-321.
(7) Hathaway, D.R. and Adelstein, R.S. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 1653-1657.
(8) Gergley, P., Castle, A.G. and Crawford, N. (1980) Biochim. Biophys. Acta 612, 50-55.
(9) Feinstein, M.B. (1982) In Progress in Hemostasis and Thrombosis, (Spaet, T.H., ed.) Volume 6, Grune and Stratton, Inc., New York, pp. 25-61.
(10) Tallant, E.A. and Wallace, R.W. (1985) J. Biol. Chem. 260, 7744-7751.
(11) Nishikawa, M., Tanaka, T. and Hidaka, H. (1980) Nature 287, 863-865.
(12) White, G.C. and Raynor, S.T. (1980) Thrombosis Res. 18, 279-284.
(13) Suda, J. and Aoki, N. (1981) Thrombosis Res. 21, 447-455.
(14) Feinstein, M.D. and Hadjian, R.A. (1982) Mol. Pharmacol. 22, 422-431.

(15) Mustard, J.F., Perry, D.W., Ardlie, N.G. and Packham, M.A. (1972) British J. Haematology 22, 193-204.
(16) Haslam, R.J. and Lynham, J.A. (1977) Biochem. Biophys. Res. Commun. 77, 714-722.
(17) Marche, P., Koutouzov, S. and Meyer, P. (1982) Biochim. Biophys. Acta 710, 332-340.
(18) Bergmeyer, H.-U., Bernt, E. and Hess, B. (1965) In Methods of Enzymatic Analysis (Bergmeyer, H.-U., ed.), Academic Press, New York, pp. 736-741.
(19) Weiss, B. and Levin, R.M. (1978) In Advances in Cyclic Nucleotide Research, (George, W.J. and Ignarro, L.J., eds.) Volume 9 , Raven Press, New York, pp. 285-303.
(20) Roufogalis, B.D. (1982) In Calcium and Cell Function (Cheung, W.Y., editor) Volume 3, Academic Press, New York, pp. 129-159.
(21) Lam, H.-Y. P. (1984) Biochem. Biophys. Res. Commun. 118, 27-32.
(22) Hidaka, H., Yamaki, T., Totsuka, T. and Asano, M. (1979) Mol. Pharmacol. 15, 49-59.
(23) Chafouleas, J.G., Bolton, W.E., Hidaka, H., Boyd, A.E., III, and Means, A.R. (1982) Cell 28, 41-50.
(24) Billah, M.M. and Lapetina, E.G. (1982) Biochem. Biophys. Res. Commun. 109, 217-222.
(25) Takenawa, T., Homma, Y. and Nagai, Y. (1982) Biochem. J. 208, 549-558.
(26) deCourcelles, D. deC., Roevens, P. and Van Belle, H. (1984) FEBS Lett. 173, 389-393.
(27) Halenda, S.P. and Feinstein, M.B. (1984) Biochem. Biophys. Res. Commun. 124, 507-513.
(28) Holmsen, H., Daniel, J.L., Dangelmaier, C.A., Molish, I., Rigmaiden, M. and Smith, J.B. (1984) Thrombosis Res. 36, 419-428.
(29) Brindley, D.N. and Bowley, M. (1975) Biochem. J. 148, 461-469.
(30) Allan, D. and Michell, R.H. (1975) Biochem. J. 148, 471-478.