Trifluoperazine and chlorpromazine block secretion from human platelets evoked at basal cytoplasmic free calcium by activators of C-kinase

A. Sanchez, T.J. Hallam and T.J. Rink*

Physiological Laboratory, Downing Street, Cambridge CB2 3EG, England

Received 27 September 1983

Trifluoperazine, chlorpromazine and other drugs known to inhibit calmodulin-dependent processes are also known to inhibit protein kinase C. The effect of these agents on secretion evoked by known activators of C-kinase has been studied in human platelets loaded with the fluorescent Ca indicator, quin2 and preincubated with aspirin. The secretory response stimulated by phorbol ester and exogenous diacylglycerol, at basal levels of cytoplasmic free Ca^{2+}, $[Ca^{2+}]_i$, was suppressed by trifluoperazine, chlorpromazine and W-7, as was the secretion evoked by collagen that occurs without a change in $[Ca^{2+}]_i$. The response to thrombin, which is accompanied by elevated $[Ca^{2+}]_i$ was barely affected. Modest elevation of $[Ca^{2+}]_i$ by Ca ionophore was able to overcome the inhibitory effect of these drugs on the response to phorbol ester, diacylglycerol, and collagen.

Trifluoperazine *Phorbol ester* *Diacylglycerol* *C-kinase* *Calcium* *Platelet*

1. INTRODUCTION

Trifluoperazine, chlorpromazine and a number of other antipsychotic drugs are known to inhibit calmodulin-dependent processes [1], and the inhibition of a cellular function by trifluoperazine has been proposed as a criterion for assigning a role for calmodulin in the activation of that process [2]. However, it is also known that these drugs have many other activities including powerful inhibitory effects on C-kinase both isolated in the test-tube [3,4] and in intact cells [5,6]. Activation of C-kinase by diacylglycerol, formed as a result of stimulus-evoked hydrolysis of phosphoinositides [7], is thought to be a key event in coupling receptor occupancy to cell activation [5]. We have recently shown that activators of C-kinase, exogenous diacylglycerol and phorbol ester (TPA) [8], promote secretion from platelets in the absence of changes of cytoplasmic free calcium, $[Ca^{2+}]_i$, as can natural agonists under suitable experimental conditions [10,11]. We report here experiments in which trifluoperazine, chlorpromazine and W7 (a calmodulin antagonist also known to inhibit isolated C-kinase [4]) suppress this 'calcium-independent' secretion in human platelets and show that this inhibition is actually relieved by elevation of $[Ca^{2+}]_i$.

2. EXPERIMENTAL

Platelet-rich plasma (PRP) was prepared from freshly drawn blood anticoagulated with acid–citrate dextrose. The cells were loaded with the fluorescent calcium-indicator, quin2 (Lancaster Synthesis) by incubating the PRP with 20 μM quin2 acetoxymethyl ester for 30 min at 37°C. The platelets were then centrifuged at 350 × *g* for 20 min and resuspended in a medium containing 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM Hepes, 10 mM glucose (pH 7.4). 1 mM $CaCl_2$ or 1 mM K_2H_2EGTA were added as required. $[Ca^{2+}]_i$ was measured from the quin2 fluorescence of a continuously stirred suspension in a Perkin Elmer

* To whom correspondence should be addressed

Published by Elsevier Science Publishers B.V.
001457593/83/$3.00

MPF44A fluorimeter as in [9,10]. Excitation and emission wavelengths were 339 and 500 nm respectively, with 10 and 20 nm bandwidths. The release of ATP, as a marker for dense granule secretion, was continuously monitored by luciferin-luminescence in a Chronolog Lumiaggrometer. Secretion of 5-[^{14}C]hydroxytryptamine and β-thromboglobulin (RIA, Amersham) were measured in some experiments as in [9,10]. Trifluoperazine (Smith, Kline & French), chlorpromazine (Sigma) and W7 were added from methanol stock solutions. 12-*O*-Tetradecanoylphorbol 13-acetate (TPA) and 1-oleoyl-2-acetyl-glycerol (OAG) were added from DMSO stock. The vehicle concentration did not exceed 0.2% (v/v) which by itself had no obvious effect. For all experiments reported the cells were preincubated with 100 μM acetylsalicylic acid to block formation of prostaglandin endoperoxides and thromboxane A_2.

3. RESULTS AND DISCUSSION

Fig. 1 shows the inhibitory effects of a near maximally effective concentration, 20 μM, of trifluoperazine (Smith, Kline and French). The control responses to OAG, TPA (Sigma), collagen (Hormon-Chemie) and thrombin (Calbiochem) are similar to those in [9]. OAG, TPA and collagen gave a characteristic delayed secretion of ATP with virtually no alteration of the measured $[Ca^{2+}]_i$. This secretion is thought to be initiated by activation of C-kinase, directly by the first 2 agents, and by the diacylglycerol, produced endogenously as a result of agonist-evoked breakdown of phosphoinositide, in the case of collagen. As can be seen in the figure, 20 μM trifluoperazine completely blocked the secretory responses to these agents. The $[Ca^{2+}]_i$ was not altered by the drug. Closely similar records, under the same experimental conditions, were obtained with chlorpromazine (Sigma) (20–40 μM) and W7 (60 μM). We also confirmed that trifluoperazine blocked the TPA-evoked secretion of another constituent of the dense granule, 5-hydroxytryptamine, and a marker for α-granule exocytosis, β-thromboglobulin. The apparent EC_{50} for trifluoperazine and chlorpromazine to inhibit TPA evoked secretion was 9 μM and 14 μM respectively. These values are slightly lower than those reported for these drugs acting on isolated C kinase [3,4]. The EC_{50} for W7 was 25 μM.

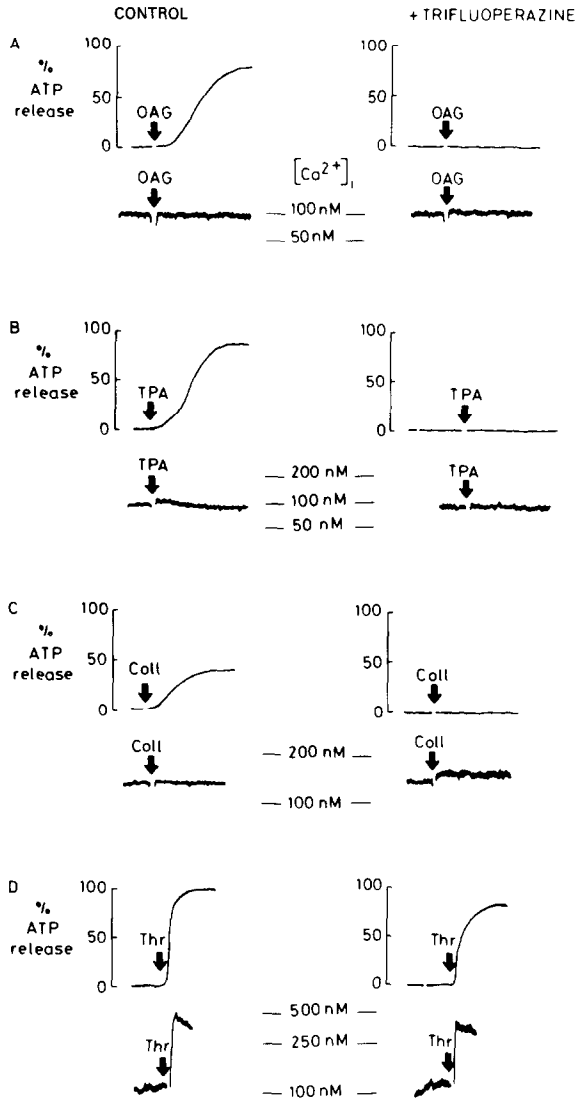

Fig. 1. Effects of 20 μM trifluoperazine on responses to 60 μg/ml OAG, 20 nM TPA, 20 μg/ml collagen and 0.5 U/ml thrombin. The upper trace of each pair shows the secretion of ATP, expressed as a % of the maximum released by thrombin. The lower trace shows $[Ca^{2+}]_i$ reported by fluorescence of intracellular quin2. The addition of trifluoperazine (or chlorpromazine) produced a steep increase in signal due to its native fluorescence. This increase merely added to the cell autofluorescence and has been substracted from retraced records. In (a) and (b) 1 mM EGTA was added and in (c) and (d) 1 mM $CaCl_2$. In other experiments we examined the responses to OAG and TPA in 1 mM Ca medium and obtained results like those shown here. Comparable results were also found in cells prepared similarly, but not loaded with quin2.

The results shown in fig. 1D, 2 suggest that these drugs were not inhibiting secretion by generalised toxic or non-specific effects. First, at concentrations that blocked the secretory responses to diacylglycerol, TPA and collagen, these drugs had only a small effect on either the increase in $[Ca^{2+}]_i$ or the secretion evoked by thrombin. This is shown for trifluoperazine in fig. 1D and closely similar results were obtained with chlorpromazine and W7. Second, the addition of low concentrations of calcium ionophore, that increased $[Ca^{2+}]_i$ to about 700 nM, below that threshold for $[Ca^{2+}]_i$ alone to evoke secretion, could largely overcome the inhibitory effects of trifluoperazine, chlorpromazine and W7. This effect is illustrated in fig. 2. In panel A application of 40 nM A23187 to chloropromazine-treated cells raised $[Ca^{2+}]_i$ to approximately 700 nM without causing any secretion. (This elevation of $[Ca^{2+}]_i$ equally produces no secretion in cells not inhibited with chlorpromazine.) Subsequent application of diacylglycerol, which by itself is quite ineffective in chlorpromazine-treated cells (fig. 2B) now evokes a substantial secretory response. In fig. 2B diacylglycerol failed to elicit a response in the chlorpromazine-treated cells. A normally subthreshold elevation of $[Ca^{2+}]_i$ caused by subsequent addition of A23187 now stimulates secretion. Again, very similar results were obtained with the other drugs, trifluoperazine and W7, and also when TPA or collagen was used as the stimulus. The secretion evoked from the drug-treated cells by thrombin, or by a subthreshold increase in $[Ca^{2+}]_i$ together with diacylglycerol or TPA, may reflect the ability of calcium to counteract the inhibition of C-kinase, as has been seen in experiments with the isolated enzyme [3,4].

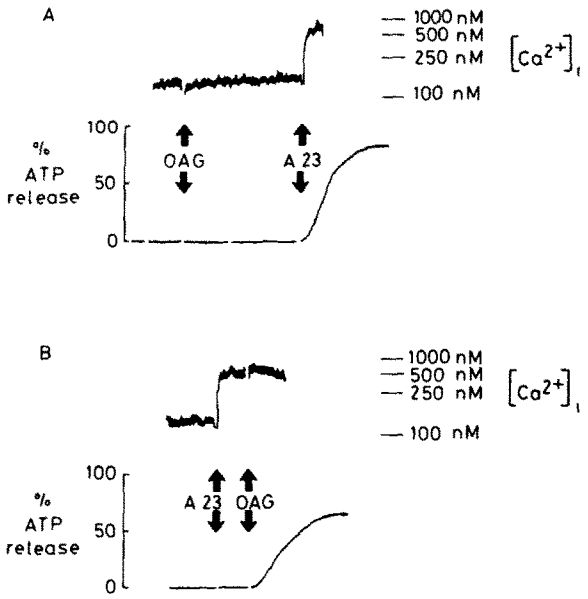

Fig. 2. Experimental conditions were as stated in the legend to fig. 1. The cells were pretreated with 30 μM chlorpromazine. In (a) 60 μg/ml OAG was applied followed by 40 nM A23187 (Calbiochem) added from 20 μM methanol stock. In (b) these agents were applied in reverse order. The $[Ca^{2+}]_i$ records are cut short because the fluorescence signal is spuriously lost as the cells aggregate [8].

The data suggest that the most prominent effect of this group of drugs on platelets may reflect their interference with C-kinase rather than with calmodulin-dependent processes. The finding that the response to collagen, in aspirin-treated cells, is inhibited in the same manner as the response to known activators of C-kinase supports our previous arguments [8] that the response to collagen under these conditions reflects activation of this enzyme independent of changes in $[Ca^{2+}]_i$. It is interesting to note that in the earliest reports of inhibition of platelet action by chlorpromazine the agonist apparently most susceptible was collagen [12], and that in a more recent study trifluoperazine appeared to be more effective in inhibiting phosphorylation of the 40 kDa substrate of C-kinase than the 20 kDa substrate of calmodulin-dependent protein kinase [6].

It seems that drugs often used as probes for calmodulin actions can be very effective against processes in intact cells mediated by C-kinase even though the activity of C-kinase is apparently not influenced by calmodulin [15]. Indeed, the inhibitory effects of these agents were seen best at basal $[Ca^{2+}]_i$ and were markedly counteracted by elevation of $[Ca^{2+}]_i$ whether by an agonist, thrombin, or by calcium ionophores. Our results point particularly clearly to the hazards of deducing from the observation that trifluoperazine, W7 or related compounds inhibit a cellular function, that this function is mediated by Ca-calmodulin (e.g. [12,16,17,18]. In particular the present results suggest the need for reappraisal of those studies in which 'calmodulin antagonists' were found to inhibit secretion, and those findings taken to confirm the role of calcium and calmodulin in the trig-

gering of secretory exocytosis. Such experiments may well prove to be pointers to the importance of C-kinase in initiating exocytosis. The concentrations of trifluoperazine and/or chlorpromazine needed to inhibit platelet secretion are about an order of magnitude greater than high therapeutic levels, but the possibility exists that part of the antipsychotic action of these drugs might be due to interference with C-kinase at critical sites in the brain.

ACKNOWLEDGEMENTS

This work was funded by the SERC, and A.S. held a long-term EMBO fellowship. We thank Ms B.N. O'Connor for her assistance. The W7 was a kind gift from Dr Alan Higgins, Pfizer, UK and the OAG was synthesised by Dr Roger Tsien.

REFERENCES

[1] Weiss, B. and Levin, R.M. (1982) Adv. Cyclic Nucleotide Res. 9, 285–304.
[2] Cheung, W.G. (1980) Science 207, 20–27.
[3] Wise, B.C. and Kuo, J.F. (1983) Biochem. Pharmacol. 32, 1259–1265.
[4] Mori, T., Takai, Y., Minakuchi, B.Y. and Nishizuka, Y. (1980) J. Biol. Chem. 225, 8378–8380.
[5] Nishizuka, Y. (1983) Phil. Trans. R. Soc. Lond. 302, 101–112.
[6] Feinstein, M.B. and Hadjan, R.A. (1981) Mol. Pharmacol. 21, 422–431.
[7] Rittenhouse-Simmons, S. (1979) J. Clin. Invest. 63, 580–587.
[8] Castagna, M., Takai, T., Kaibuchi, K., Sano, K. Kikkawa, N. and Nishizuka, Y. (1981) J. Biol. Chem. 257, 7847–7851.
[9] Rink, T.J., Sanchez, A. and Hallam, T.J. (1983) Nature 305, 317–319.
[10] Rink, T.J., Smith, S.W. and Tsien, R.Y. (1982) FEBS Lett. 148, 21–26.
[11] Hallam, T.J., Rink, T.J. and Sanchez, A. (1983) Br. J. Pharmacol. 79, 351P.
[12] Nishikawa, M., Tanaka, T. and Hidaka, H. (1980) Nature 287, 863–865.
[13] Tsien, R.Y., Pozzan, T. and Rink, T.J. (1980) J. Cell Biol. 94, 325–334.
[14] Mills, D.C.B. and Roberts, G.C.K. (1967) Nature 217, 35–58.
[15] Kuo, J.F., Andersson, R.G.G., Wise, B.C., Mackerlova, L., Sabmonsson, I., Brackett, N.L., Katoh, N., Shoji, M. and Wren, R.W. (1980) Proc. Natl. Acad. Sci USA 77, 7039–7044.
[16] Naccache, P.H., Molski, T.F.P., Alobaidi, T., Becker, E.L., Showell, H.J. and Sha'afi (1980) Biochem. Biophys. Res. Commun. 97, 62–68.
[17] Satir, B.H., Garofalo, R.S., Gilligan, D.M. and Maihle, N.J. (1980) Ann. N.Y. Acad. Sci. 356, 83–91.
[18] Douglas, W.W. and Nemeth, E.F. (1981) J. Physiol. 323, 299–344.