TMB-8 inhibits secretion evoked by phorbol ester at basal cytoplasmic free calcium in quin2-loaded platelets much more effectively than it inhibits thrombin-induced calcium mobilisation

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TMB-8 is widely regarded as an 'intracellular calcium antagonist', supposedly inhibiting the mobilisation of intracellular calcium. Rarely, however, have the effects of this compound on Ca^{2+} movements been measured. We report here that TMB-8 is not very effective in inhibiting thrombin-induced Ca^{2+} influx or internal release in human platelets judged from the fluorescent signal of cytoplasmic quin2. Only approx. 40% inhibition was seen at 500 μm TMB-8. Somewhat lower concentrations blocked the secretory response to thrombin and also the secretion evoked at basal $[Ca^{2+}]_i$ by phorbol ester and collagen. It is suggested that one target for TMB-8 may be the C-kinase pathway.

TMB-8 *Phorbol ester* *Platelet* *Calcium* *Secretion*

1. INTRODUCTION

8-(*N*,*N*-Diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8) was first reported as a non-selective inhibitor of contraction in skeletal and smooth muscle, reducing the responses to a wide range of stimuli (see, e.g., [1]). It was reasonably proposed that TMB-8 acted at a late step in excitation–contraction coupling, perhaps by 'interfering with the availability of Ca^{2+}'. It was found that TMB-8 reduced $^{45}Ca^{2+}$ influx and efflux in guinea pig ileum, and caffeine-induced release of $^{45}Ca^{2+}$ from sarcoplasmic reticulum vesicles [1]. Since then, TMB-8 has been widely described as an 'intracellular Ca^{2+} antagonist' and used in several preparations as a probe for Ca^{2+}-mediated processes. Some slight justification for the idea of Ca^{2+} antagonism has come from the finding that the inhibitory effects of this compound are sometimes counteracted by elevating the external Ca^{2+} concentration. However, in most studies there has been no experimental test for any effect on Ca^{2+} mobilisation, whether influx or internal release. Indeed, it is often not clear whether investigators have supposed that TMB-8 is acting to prevent Ca^{2+} movements or to inhibit the effect of Ca^{2+} on intracellular targets. Several groups have used this compound to investigate activation processes in blood platelets [2–7] and it was even claimed that the inhibition by TMB-8 of secretion induced by thrombin or A23187 was the most direct evidence then available that Ca^{2+} is required for platelet secretion [2].

However, TMB-8 has been shown to have several effects in platelets, not necessarily or even easily attributable to Ca^{2+} antagonism, which is perhaps not surprising in view of the very high concentrations of the compound often needed, e.g., 50–1000 μM. For example, TMB-8 interferes with the formation of thromboxane [3,7] by apparently inhibiting cyclooxygenase [4]. At concentrations that inhibit secretion, TMB-8 causes discoid platelets to

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become spherical, and also causes a leak of serotonin [4].

The introduction of the fluorescent Ca^{2+} indicator quin2, which can be trapped in the cytoplasm of intact cells [8], has allowed more direct testing of the effects of TMB-8 on Ca^{2+} mobilisation. Authors in [9] report that 50 μM TMB-8 does indeed inhibit increases in quin2 fluorescence evoked in clutured human fibroblasts by growth factors and mellitin. The effects of external Ca^{2+} were not examined so it is unclear if the effect was on Ca^{2+} influx or internal release. Nor was it reported whether Ca^{2+} ionophore could bypass the suppression of the quin2 signal. We report here the effects of TMB-8 in quin2-loaded human platelets which indicate only a weak inhibitory effect on Ca^{2+} movements, but a more effective inhibition of secretion including that evoked by TPA (12-*O*-tetradecanoylphorbol-13-acetate), a direct activator of protein kinase-C [10] that does not cause or require any change in cytoplasmic-free calcium, $[Ca^{2+}]_i$, measurable by quin2 fluorescence [11].

2. EXPERIMENTAL

Human platelets were isolated from freshly drawn blood and loaded with approx. 1 mM quin2 as in [11]. The cells were resuspended in a physiological saline containing: 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM Hepes, 10 mM dextrose (pH 7.4) at 37°C. All preparations were preincubated in 100 μM aspirin to eliminate the formation of thromboxane and avoid any effects of TMB-8 on this pathway. Measurement of quin2 fluorescence and calibration of the signal was done in a cuvette thermostatted to 37°C in a Perkin Elmer MPF44A spectrophotometer, and secretion from amine storage granules was measured from the luminescence of a luciferase preparation (Chronolume reagent) in a Chronologue lumiaggrometer, as in [11]. Human thrombin (Calbiochem) was added from a stock solution of 100 units/ml in Ca^{2+}-free buffer. TPA (Sigma) was added from 20 μM stock dissolved in dimethylsulphoxide. Serotonin (5-hydroxytryptamine, Sigma) and cyproheptadine (Sigma) were added from 10 mM or 100 mM stocks, respectively, in physiological saline. Collagen (Hormon-Chemie) was added from a 1 mg/ml stock solution. TMB-8 was added from a methanol stock solution of sufficient strength so that the methanol concentrations did not exceed 0.2%. Higher concentrations of TMB-8 produced a small fluorescence artefact which was subtracted from the records.

3. RESULTS AND DISCUSSION

Fig.1 shows typical records of the effects of 500 μM TMB-8 on the response to thrombin. In the presence or absence of external Ca^{2+} the secretion was substantially reduced. Aggregation was also blocked (not shown); this concentration of TMB-8 itself produced the optical signals associated with shape-change. The increase in $[Ca^{2+}]_i$ reported by quin2 fluorescence was much less inhibited than the secretion both in the presence and absence of external Ca^{2+}, suggesting that neither Ca^{2+} influx nor the internal release of Ca^{2+} was much affected by TMB-8. Fig.2 shows the collected results of experiments of this type for a range of TMB-8 concentrations confirming that secretion is more effectively inhibited than is the rise in $[Ca^{2+}]_i$. The secretion evoked by thrombin in the absence of Ca^{2+} may be somewhat more susceptible with an IC_{50} of 300 μM TMB-8, than the secretion evoked in the presence of calcium, IC_{50} approx. 400 μM.

Protein kinase-C appears to play an important part in stimulus-secretion coupling in platelets [11–13]. The phosphorylation of a protein of between 40 and 47 kDa by this enzyme is correlated with secretion (see, e.g., [12,13]) and direct activation of protein kinase-C by TPA can evoke a secretory response that neither causes nor requires a rise in $[Ca^{2+}]_i$ judged from the quin2 signal [11]. We have therefore tested the effect of TMB-8 on the secretion evoked by TPA. As shown in fig.3A, 500 μM TMB-8 completely suppressed the secretion. The collected data from this type of experiment is shown in fig.3B. The IC_{50} for TMB-8 is here distinctly lower, at 150 μM, than that seen for thrombin-evoked responses. The reason for this difference is not known, but it may reflect a partially protective effect of elevated $[Ca^{2+}]_i$ against inhibition by TMB-8. A similar effect was seen with the inhibitory effects of trifluoperazine and chlorpromazine [14]. These results suggest that TMB-8 blocks at some point in the C-kinase pathway but they do not say whether the phosphorylation itself, or the actions of one of the phosphorylated products, is blocked. The results in [16] hint

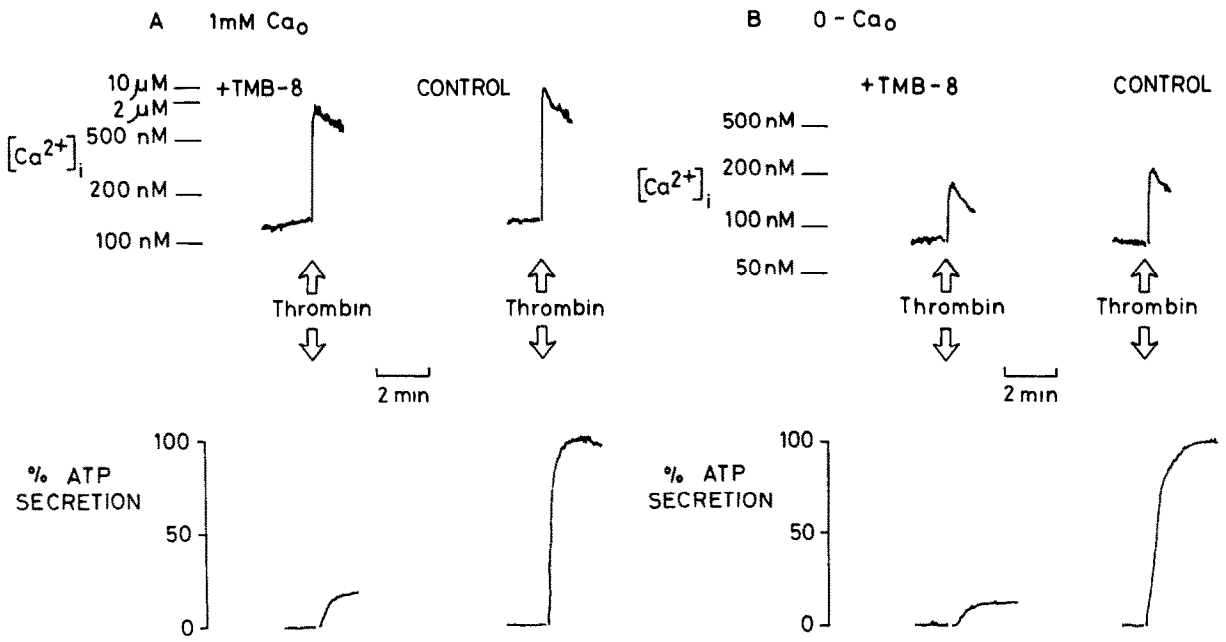

Fig.1. Effect of 500μM TMB-8 on thrombin-evoked rises in $[Ca^{2+}]_i$ and secretion of ATP. (A) In the presence of 1 mM external Ca^{2+}; (B) in the absence of added Ca^{2+}, and with 1 mM EGTA. 0.5 units/ml thrombin were added where indicated. ATP secretion is expressed as the percentage of the amount maximally releasable by thrombin in the presence of 1 mM Ca^{2+} (A) or in the absence of Ca^{2+} (B).

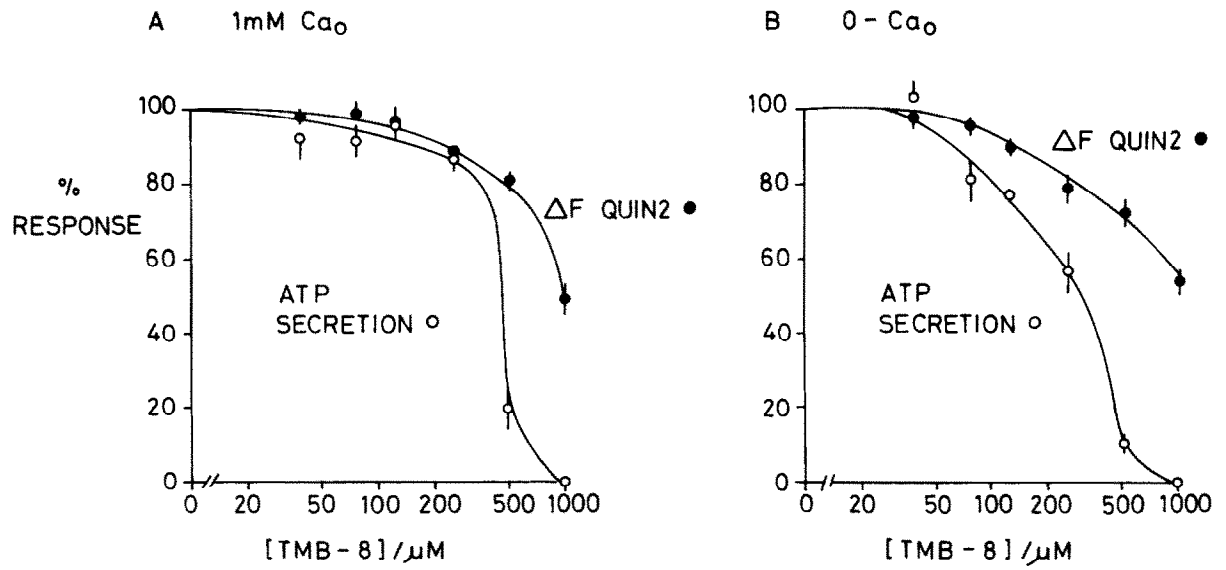

Fig.2. Dose-effect relation for TMB-8 on thrombin-evoked secretion and increase in quin2 fluorescence. (A) In the presence of 1 mM Ca^{2+}; (B) in the absence of added Ca^{2+} and with 1 mM EGTA. The bars show the SE of 3–5 determinations. Here, the secretion is expressed as a percentage of the amount seen in the absence of TMB-8. The quin2 signal was not converted to $[Ca^{2+}]_i$; the results are expressed as peak fluorescence after addition of thrombin, minus resting fluorescence (Δquin2 fluorescence) and expressed as a percentage of that seen in the absence of TMB-8. The curves were fitted by eye.

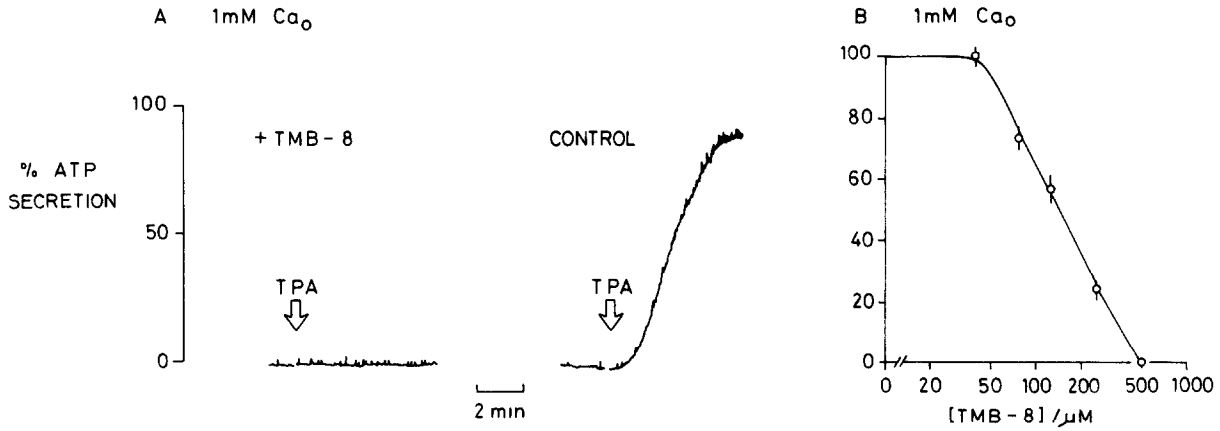

Fig.3. (A) Effect of 500 μM TMB-8 on the secretion evoked by 20 nM TPA. (B) Dose-effect relation for TMB-8 on secretion evoked by 5 min exposure to 20 nM TPA. Conditions as for fig.1,2.

that TMB-8 may act to inhibit the action of protein kinase-C. They found that a relatively low concentration of TMB-8 (75 μM) substantially reduced both the secretion of serotonin and the phosphorylation of a 41-kDa protein evoked by platelet-activating factor. Interestingly, the phosphorylation of the 20-kDa protein (presumably myosin light chain [17]) was inhibited less than that of the 41-kDa protein.

In conditions like those in fig.3 (i.e., quin2-loaded cells preincubated with aspirin) collagen evokes secretion while $[Ca^{2+}]_i$ remains at or near the basal level. We have proposed that diacylglycerol formed from the result of collagen-evoked phospholipid hydrolysis, may activate protein kinase-C and thereby initiate the secretory response [11,14,15]. This collagen-induced secretion was blocked by concentrations of trifluoperazine that blocked the response to TPA [14]. We therefore tested the effect of TMB-8 on the secretion evoked by 20 μg/ml collagen added to aspirin-treated cells in 1 mM Ca^{2+} medium. This secretion was blocked by 200 μM TMB-8, consistent with an action on the C-kinase pathway. Since platelets are very powerfully inhibited by agents, such as prostaglandin I_2, that raise cAMP levels one should consider whether TMB-8 works in this way. The pattern of inhibitory effects argues strongly against this mechanism. Prostaglandin I_2 very effectively suppresses the quin2 signals produced by thrombin, but only weakly inhibits the secretion evoked by TPA or collagen (in aspirin-treated cells) [18], the reverse of what one finds with TMB-8.

Our data add to those of others in showing that TMB-8 is a poor investigative tool. Relatively high concentrations are often needed to obtain inhibition and the compound affects many different processes. According to our results it is not very effective, at least in human platelets, in preventing Ca^{2+} movements even at concentrations that inhibit secretion. We see no evidence to suggest any specific interference with internal Ca^{2+} release. At concentrations that do partly inhibit Ca^{2+} movements, TMB-8 inhibits thromboxane production and causes leakage of serotonin and nucleotides. Furthermore, TMB-8 produces a change in absorbance indicative of shape-change. Electron micrographs show TMB-8-treated platelets to be spherical [19]. As there is no increase in quin2 fluorescence on addition of TMB-8, the 'shape-change' cannot be attributed to a rise in $[Ca^{2+}]_i$. One possible explanation was that the change in shape was due to the leakage of serotonin. However, after desensitization with serotonin or treatment with the receptor blocker cyproheptadine, such that no shape-change could be obtained by the addition of serotonin, the change in absorbance still occurred on addition of TMB-8.

TMB-8 was more effective in preventing responses to TPA than in preventing calcium movements, suggesting that it acts to inhibit the C-kinase pathway. Such an action of TMB-8 on protein kinase-C may explain some of the observations in the literature. For instance, it was reported that TMB-8 could prevent TPA-induced secretion of lysozyme in human polymorphs incubated in the absence of

external Ca^{2+} [20,21]. This result was interpreted as indicating that TPA acted to mobilise internal Ca^{2+}, TMB-8 being assumed to antagonise Ca^{2+} mobilisation. On the basis of the present data, and knowing that TPA can activate quin2-loaded polymorphs without any apparent rise in $[Ca^{2+}]_i$ [22], we might now guess that the inhibitory effect of TMB-8 reflected interference with the protein kinase-C pathway rather than with Ca^{2+} mobilisation.

Two recent papers report effects of relatively low concentrations of TMB-8 that seem unlikely to rely on calcium antagonism. At only 10 μM, TMB-8 greatly enhanced the release of insulin from rat islets incubated with 1 mM isobutylmethylxanthine [23]. No measurements of Ca^{2+} movements were made, but the authors hypothesised that this effect reflected inhibition of Ca^{2+} mobilisation from endoplasmic reticulum, the mechanism usually invoked to explain inhibition of secretion. In another study, 15 μM TMB-8 inhibited, by 30%, the $(H^+ + K^+)$-ATPase of hog gastric mucosa [24]. This appeared to be due to interaction with the K^+ site. Clearly, TMB-8 has different actions in different preparations and one has to find out, rather than assume, its mode of action in particular conditions.

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