The relationship between intracellular second messengers and platelet secretion

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Cellular responses caused by interaction of agonists with specific cell-surface receptors are typically mediated by changes in the concentrations of several intracellular second messengers (Rasmussen, 1981). The relationships between receptor occupancy, changes in second messenger concentrations and the extent of response have been examined for many cells, although no system is as yet completely understood. Platelets are particularly suitable for such studies since they respond to a wide range of agonists including collagen, thrombin, ADP, PAF, thromboxane A2, etc., and exhibit a range of responses such as aggregation and secretion of the contents of the amine storage granules (e.g. ADP and serotonin), of the protein storage granules and, for some agonists, of the lysosomes (Gordon & Milner, 1970; Brydon et al., 1976). The ease with which such responses may be studied, together with the availability of apparently homogeneous preparations, has led to the widespread use of the platelet as a model for investigations into stimulus-secretion coupling.

Much evidence suggests that an increase in [Ca2+]i results from stimulation of platelets by agonists such as thrombin (Rink et al., 1982), PAF (Hallam et al., 1984) and ADP (Brydon et al., 1984), and is a prerequisite for the secretory response. The increase in [Ca2+]i appears to result both from an increased influx of extracellular Ca2+ and also from a release of Ca2+ from intracellular stores (Rink et al., 1982). However, the pattern and the extent of the secretory response cannot be explained solely on the basis of an increase in [Ca2+]i, and more recent studies have indicated a role for 1,2-diacylglycerol as an additional intracellular messenger (Kaibuchi et al., 1981; Rink et al., 1983). Platelets contain high concentrations of protein kinase c which is activated by both Ca2+ and 1,2-diacylglycerol (Takai et al., 1982). This kinase has been implicated in the secretory response since the synthetic activators of this enzyme, e.g. OAG and TPA, also induce secretion from platelets (Kaibuchi et al., 1981; Rink et al., 1983). Furthermore, many agonists which induce secretion, e.g. thrombin and collagen, also induce phospholipase C breakdown and lead to a transient increase in cellular 1,2-diacylglycerol (Rittenhouse-Simmons & Deykin, 1981; Kaibuchi et al., 1981; Nishizuka, 1983). Thus it has been proposed that Ca2+ and 1,2-diacylglycerol act as coordinate second messengers in the platelet secretory response (Castaagna et al., 1982; Kaibuchi et al., 1981; Rink et al., 1983). Some agonists, e.g. thrombin and PAF, cause increases in both [Ca2+]i and [1,2-diacylglycerol] (Rink et al., 1982; Hallam et al., 1984; Brydon et al., 1984) whereas others, e.g. collagen, release only [1,2-diacylglycerol] and have no apparent effect on [Ca2+]i, as measured by using quin2 (Rink et al., 1983). The absence of a [Ca2+]i transient is also observed when secretion is induced by exogenous addition of 1,2-diacylglycerol (or TPA) increases the sensitivity of cellular [3H]serotonin is released if [Ca2+] is increased to 0.01 μM in the presence of millimolar MgATP2− (Knight & Scrutton, 1980; Knight et al., 1982). The pattern of secretory responses observed in intact platelets is not, however, fully mimicked by the permeabilized preparations challenged with Ca2+ alone since (i) identical EC10 values for Ca2+ (2 μM) are observed for Ca2+-induced amine storage granule and lysosomal secretion and (ii) the maximal extent of lysosomal secretion is decreased 3-4-fold (Knight et al., 1982). Properties of the secretory response closely resemble those of intact platelets can be obtained in the permeabilized preparation if the Ca2+ concentration is increased in the presence of thrombin (Knight & Bartnik, 1983, 1984). Thus as shown in Figs. 1(a) and 1(b), the addition of thrombin enhances the sensitivity to Ca2+ of serotonin secretion, the EC10 for Ca2+ in the presence of thrombin being close to 0.5 μM. Figs 1(a) and 1(b) also show that addition of thrombin causes no significant increase in serotonin secretion at low [Ca2+]i, e.g. 0.01 μM. No secretion is observed at 10 μM levels of Ca2+ in the presence or absence of this agonist if MgATP2− is omitted. If a non-saturating concentration of MgATP2− is used, the maximal extent of serotonin secretion is reduced in both the presence or absence of thrombin without any significant effects on the respective EC10 values for Ca2+ (Figs. 1a and 1b). We have also shown that addition of thrombin to the permeabilized platelet preparation increases the maximal extent of lysosomal secretion to a level approximating that observed in intact platelets, but without altering the EC10 for Ca2+ (Knight et al., 1984). These effects of thrombin on permeabilised platelets appear to result from action of the agonist at its cell-surface receptor as is the case with intact platelets, since the stimulation of lysosomal secretion in both cases can be rapidly terminated by addition of hirudin (Knight et al., 1984). Thrombin inhibition of the release of intracellular markers as noted above suggests that thrombin (molecular mass 33 kDa) would be unable to enter the permeabilized cell.

Since thrombin increases the concentration of 1,2-diacylglycerol in both intact and permeabilized platelets (Rittenhouse-Simmons & Deykin, 1981; R. J. Haslam, personal communication), and is possibly a result of activation of phospholipase C, it was reasonable to consider that the effects of this agonist on Ca2+-induced secretion in

Abbreviations used: PAF, platelet-activating factor; [Ca], cytosolic Ca2+ concentration; OAG, 1-octyl-2-acetylglceral; TPA, 1,2-tetradecanoylphorbol-13-acetate; [1,2-diacylglycerol], cytosolic 1,2-diacylglycerol concentration; BAPTA, aminophenoxylthetane-N,N,N',N'-tetra-acetic acid; IMBX, 3-isobutil-1-methylxanthine.

Vol. 12
Platelets were rendered permeable in a medium containing 2mM-EGTA as described by Knight & Scrutton (1984), diluted to give 4mM-MgATP\textsuperscript{2-} (●), 0.8mM-MgATP\textsuperscript{2-} (▲) and 0mM-MgATP\textsuperscript{2-} (◇), and after 10min challenged with 15mM-CaEGTA buffers either (a) alone, or (b) with 0.7 units of thrombin/ml, or (c) with 7.5μg of OAG/ml. The \[^{14}C\]serotonin in the supernatant was determined 5min later. Temperature 20°C.

Fig. 1. MgATP\textsuperscript{2-} and Ca\textsuperscript{2+} dependence of secretion in the presence and absence of thrombin and OAG

Permeabilized platelets could result from its effects on membrane phosphoinositide metabolism and hence activation of protein kinase c. This postulate is supported by the finding that addition of synthetic activators of protein kinase c, e.g. OAG (Fig. 1c) or TPA (Knight & Scrutton 1984), mimic the effect of thrombin on Ca\textsuperscript{2+}-induced serotonin secretion in permeabilized platelets. A similar mimicking by OAG or TPA of the effect of thrombin on lysosomal secretion is observed (Knight et al., 1984). However, if high concentrations of OAG or TPA are used very significant secretion of serotonin is observed at low calculated (0.05μM) [Ca\textsuperscript{2+}] (Figs. 2a and 2b) in contrast to the situation observed on addition of thrombin (Fig. 1b). This seemingly Ca\textsuperscript{2+}-independent’ release of serotonin by OAG and TPA does not appear to result from non-specific membrane fusion since it shows a MgATP\textsuperscript{2-} dependence similar to that which characterizes Ca\textsuperscript{2+}-induced serotonin secretion (Fig. 2a). The extent of the response can also be markedly reduced if the Ca\textsuperscript{2+}-buffering capacity of the system is enhanced by increasing the concentration of BAPTA (Fig. 2b) or EGTA. The apparent lack of dependence on Ca\textsuperscript{2+} of the response therefore may be explained in terms of inadequate control of Ca\textsuperscript{2+} concent-
determined $S_{\text{min}}$ later. The ordinate is expressed as the
TPA/ml
cells, open symbols. Data points reflect means and
four determinations.

and various concentrations of \(3'\)-

Ca\(_{2+}\) for thrombin, 11% and 66% for OAG, and
approx. 1 nM and 0.6\(\mu\)M-Ca\(_{2+}\), together with either 0.6 units
incubated for 4 min with

means and

shifts in the
Ca\(_{2+}\) dose-response curves are not far enough to the left to
be induced by exogenous 1,2-diacylglycerol at the resting
levels of [Ca\(^{2+}\)] of about 0.1 \(\mu\)M or less (Rink et al., 1983).
Addition of thrombin or OAG alone to permeabilized
platelets has little effect on the extent of serotonin secretion
if [Ca\(^{2+}\)] is buffered to a concentration approximating to
these reported (Rink et al., 1982, 1983) resting [Ca\(^{2+}\)] levels
(Figs. 1a and 1b; Fig. 3a). Such a discrepancy can however be
explained in part by the finding that cyclic nucleotides modulate the effect of thrombin on the Ca\(^{2+}\)-sensitive
secretory process. Studies using the permeabilized prepara-
tion have shown that addition of micromolar levels of 3':5'-
cyclic GMP enhance the Ca\(^{2+}\) sensitivity of serotonin
secretion in the presence of thrombin by shifting the Ca\(^{2+}\)
dose-response curve further to the left (Knight & Scrutton 1984).
Such an effect could resolve the dilemma indicated above since at a fixed Ca\(^{2+}\) concentration
(0.17 \(\mu\)M), which is close to [Ca\(^{2+}\)], in resting platelets,
addition of 3':5'-cyclic GMP induces marked serotonin
secretion in the presence of thrombin (Fig. 3a).
The secretory process is still Ca\(^{2+}\)-dependent, at as low as [Ca\(^{2+}\)]
e.g. 0.01 \(\mu\)M, insignificant serotonin release occurs when cells are challenged with 3':5'-cyclic GMP and thrombin.
The effect of 3':5'-cyclic GMP is selective to the thrombin-
induced response since addition of this cyclic nucleotide
causes no change in the Ca\(^{2+}\) sensitivity of secretion
observed when cells are challenged with Ca\(^{2+}\) alone, or in
the presence of TPA or OAG. It is therefore likely to result
from an action at the level of phospholipase C.

In contrast, addition of micromolar levels of 3':5'-cyclic AMP
to the permeabilized preparation decreases the sensitivity of secretion to Ca\(^{2+}\) in the presence of thrombin
by shifting the dose-response curve to the right (Knight & Scrutton, 1984). Inhibition by 3':5'-cyclic AMP is primarily
exerted on the thrombin-induced response since addition of
this nucleotide causes no change in the secretory response to
Ca\(^{2+}\) alone (Knight & Scrutton, 1984), and only inhibits
the Ca\(^{2+}\)-dependent effects of OAG and TPA at much higher
3':5'-cyclic AMP concentrations (Fig. 3b). Such an effect of
3':5'-cyclic AMP is direct evidence in support of the well-
established postulate that this cyclic nucleotide acts as an
inhibitory intracellular second messenger in the platelet
(Mills & Smith, 1971; Steer & Salzman, 1980), and suggests
that the effect primarily results from an action at the level of
phospholipase C in accord with some earlier studies on
intact platelets (Rittenhouse-Simmons, 1979; Billah et al.,
1979). The data here suggest that whereas a second site for
the action of 3':5'-cyclic AMP exists at, or beyond, the level
of protein kinase c in the stimulus–secretion coupling but
that the affinity for this site is relatively low.

Our present understanding of the intracellular mecha-
nisms responsible for control of platelet secretion therefore
suggest that a complex network of factors may be
responsible for regulating the sensitivity of the secretory
process to Ca\(^{2+}\) but provide no evidence to suggest that such
factors operate in a strictly Ca\(^{2+}\)-independent manner.

We thank Dr Y. Nishizuka for the gift of OAG, Professor P. F.
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Fig. 3. The effect of cyclic nucleotides on secretion

(a) A suspension of leaky platelets were incubated in 20 mM-
CaEGTA buffers corresponding to 0.17 \(\mu\)M-Ca\(^{2+}\) ( ), 0.6 \(\mu\)M-Ca\(^{2+}\) for thrombin, closed symbols. Data points refer to
means and S.E.M.S of four determinations.

(b) Platelets rendered permeable were incubated for 4 min with 1 mM-BAPTA and 0.2 \(\mu\)M-IBMX and various concentrations of 3':5'-cyclic AMP, before being challenged with 20 mM-CaEGTA corresponding to
1M and 0.6 \(\mu\)M-Ca\(^{2+}\), together with either 0.6 units of thrombin/ml ( ), 10 \(\mu\)g of OAG/ml ( ), or 5 ng of
TPA/ml ( ). The \([\text{IC}_{50}]\) serotonin in the supernatant determined 5 min later.

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Vol. 12
The calelectrins: new and ubiquitous Ca\textsuperscript{2+}-regulated proteins with a possible role in cytotic processes

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Ca\textsuperscript{2+} is a universal and possibly the most important second messenger in eukaryotic cells, regulating such diverse processes as muscle contraction, secretion, proteolysis and mitogenesis. While some of its actions are understood at the molecular level (e.g. excitation-contraction coupling by Ca\textsuperscript{2+}-binding to troponin C), many regulatory effects of Ca\textsuperscript{2+} have escaped clarification up to now. The most notable of which might be Ca\textsuperscript{2+}-triggered exocytosis in neurons and other secretory cells. This is an especially challenging biological problem because virtually all cells have constitutive pathways for the exo- and endo-cytosis of macromolecules which operate independently of stimulation. Cells capable of Ca\textsuperscript{2+}-triggered exocytosis must therefore have two independent pathways, suggesting that a cell is not only capable of sorting proteins and membranes, but also of classifying membrane fusion and budding events into regulated and non-regulated processes (Moore et al., 1983).

Although membrane fusion is a ubiquitous and constant process in cells, it has been extremely hard to reconstitute either Ca\textsuperscript{2+}-regulated or non-regulated fusion events in vitro. As a consequence of this difficulty the mechanism of membrane fusion remains unknown. In view of the triviality of membrane fusion in cells therefore the problem of Ca\textsuperscript{2+}-triggered exocytosis could be equally well stated as: (1) what inhibits membrane fusion in the absence of Ca\textsuperscript{2+}, and (2) which Ca\textsuperscript{2+}-dependent component enhances membrane fusion?

Secretory vesicles are specialized organelles with structural features important for exocytosis. Chromaffin granules for example, the best studied of these, are slightly hyperosmolar and contain high concentrations of core molecules bound in an osmotically inactive form (Südhof, 1982, 1983). These findings are relevant in view of the fact that an osmotic gradient has been shown to facilitate fusion (Cohen et al., 1980). Nevertheless, isolated chromaffin granules have not been shown to undergo fusion reactions in the presence of Ca\textsuperscript{2+} under physiological conditions (Morris et al., 1982, 1983). This finding suggests that Ca\textsuperscript{2+}-triggered exocytosis is not a property of the secretory vesicles per se, and that it may require the participation of other proteins such as cytosolic Ca\textsuperscript{2+}-binding proteins. Certain requirements can be postulated for these: they should interact with membranes in a Ca\textsuperscript{2+}-dependent manner, they should be evolutionarily conserved in view of the universality of secretion, and they should be distributed in a tissue- or cell-specific manner. None of these postulates although we have not yet demonstrated a direct role of these proteins in exocytosis.

In 1982, J. H. Walker purified a protein from the electric organ of Torpedo marmorata, a protein which binds to membranes in a Ca\textsuperscript{2+}-dependent manner (Walker, 1982). This protein has some interesting properties: it not only binds to membranes as a function of Ca\textsuperscript{2+}, it also binds Ca\textsuperscript{2+} in solution, self-aggregates and promotes membrane aggregation as a function of Ca\textsuperscript{2+} (Südhof et al., 1982, 1983). We called this protein calelectrin because of its tissue source and regulation by Ca\textsuperscript{2+}. Torpedo calelectrin is a 34 kDa protein with an acidic pI of 4.9–5.2; monospecific antibodies against it do not demonstrate the presence of a single cross-reactive protein in Torpedo with a distinctive tissue distribution (Walker, 1982). In mammalian tissue extracts, however, we detected three immunologically cross-reactive proteins with molecular masses of 32.5, 35 and 67 kDa. Immunofluorescence on mammalian cells again demonstrated a distinctive distribution in that calelectrin was present in bovine and rat chromaffin cells and in human white blood cells but not detectable in fibroblasts, erythrocytes and thymocytes (Südhof et al., 1983, 1983, Walker et al., 1983). We then tried to purify the mammalian calelectrins and found that they could be isolated like calmodulin by Ca\textsuperscript{2+}-dependent hydrophobic affinity chromatography, followed by conventional column chromatography. The three mammalian calelectrins are very similar proteins. They have almost identical pI values (between 5.6 and 5.9) and amino acid compositions, and in Ca\textsuperscript{2+}-free solutions occur as globular monomers. The calelectrins are Ca\textsuperscript{2+}-binding proteins in solution and also bind to membranes in a Ca\textsuperscript{2+}-dependent fashion with half-maximal binding at 5 μM.Ca\textsuperscript{2+}. They cause membrane aggregation at higher Ca\textsuperscript{2+} concentrations. All three calelectrins are present in native tissue as judged by immunoblots of total extracts. They are abundant tissue proteins. Several milligrams of protein can be isolated from 100 g of tissue. However, there are also distinctive differences between the low and high molecular mass.