Guanine nucleotides and Ca-dependent exocytosis

Studies on two adrenal cell preparations

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Exposure of 'leaky' adrenal medullary cells to GTP-γ-S inhibits Ca-dependent exocytosis in bovine cells, but stimulates exocytosis in chicken cells. The inhibitory action on bovine cells persists in the presence of TPA suggesting that in this tissue an inhibitory GTP-binding protein may modulate the action of protein kinase C on exocytosis.

Calcium *Exocytosis* *GTP-binding protein* *GTP-γ-S* *Phorbol ester* *Protein kinase C*

1. INTRODUCTION

A transient increase in free Ca is probably the primary trigger for initiating exocytosis in neuronal cells, but there is growing evidence for the involvement of other factors [1]. The analysis of such factors in intact cells is hampered by the large number of possible ways in which they might influence the secretory process. We recently described a cell preparation, permeabilized by exposure to brief high-voltage electric fields, which permits many of the physiological control steps that fashion the Ca transient to be by-passed thereby providing rather direct access to the intracellular sites at which exocytosis takes place [2,3]. We have used this technique to examine the properties of exocytosis in adrenal medullary cells of cattle and chicken, representatives of two vertebrate classes. These were chosen because physiological control by acetylcholine involves nicotinic receptors in cattle and muscarinic receptors in the chicken. In both preparations secretion from intact cells is absolutely dependent on external Ca and in permeabilized cells, provided Mg-ATP is present, is half-maximally activated at a free Ca of about 1 μM. The apparent affinity for Ca in permeabilized cells is, however, subject to modulation [4]. Most notably, in both animals, the phorbol ester TPA (12-*O*-tetradecanoyl-13-acetate) increases the apparent affinity of exocytosis for intracellular calcium.

The reactions underlying the phorbol effect remain unclear although an action on protein kinase C is consistent with the known properties of this enzyme [5,6] and the molecular specificity of the phorbol effect. Involvement of protein kinase C is, however, not immediately consistent with the finding that TPA is largely without effect on freshly isolated intact bovine cells [4] although it does enhance secretion in intact chicken cells. As TPA seems to replace diacylglycerol (DAG) in activating protein kinase C [7], it should be possible to mimic the TPA effect by DAG. We have, accordingly, examined the effects of the diacylglycerol analogue 1-oleoyl-2-acetylglycerol on Ca-dependent exocytosis in permeable cells. Additionally, as there is some evidence that diacylglycerol production may be regulated by a GTP-dependent reaction [8], we have investigated the effects of GTP and various GTP analogues on Ca-dependent exocytosis in our permeable preparations.

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Our results point rather unexpectedly to a direct involvement of GTP-binding sites in the control of exocytosis and lead to a new working hypothesis of the early events in membrane fusion.

2. MATERIALS AND METHODS

Bovine cells were prepared by enzyme digestion as described [9]. Chicken cells were obtained in an essentially similar manner except that the initial protease digestion steps required for bovine material were omitted. Permeabilization was achieved by exposure to 10 brief high voltage pulses (2 kV/cm, $\tau \sim 200\ \mu s$) in a medium containing 160 mM potassium glutamate, 20 mM K-Pipes (pH 6.6), 5 mM glucose, 2 mM free Mg, 0.5 mM EGTA or BAPTA, 5 mM Mg-ATP. Permeable cells were exposed to nucleotides and other agents for 10 min before exposure to a range of free Ca concentrations buffered with EGTA.

Catecholamine release was assayed fluorimetrically.

1-Oleoyl-2-acetylglycerol was a gift from Professor Y. Nishizuka; BAPTA was from BDH, Poole, Dorset, England; GTP-γ-S was from Boehringer and all other chemicals were from Sigma.

3. RESULTS AND DISCUSSION

In both permeable bovine and permeable chicken cells, the diacylglycerol analogue, 1-oleoyl-2-acetylglycerol has no detectable effect on the Ca-activation curve for exocytosis although in these same cells TPA increases the apparent affinity of exocytosis for Ca (fig.1). This observation makes it difficult to argue that some phospholipid – perhaps derived from the breakdown of phosphatidylinositol – is involved in exocytosis. It is, of course, possible that some other lipid is active under physiological conditions and we do not know the correct molecular species; but it is also possible that TPA acts in a different way.

Figs 2 and 3 examine the sensitivity to Ca in the presence of GTP-γ-S. Exposure of permeable bovine and chicken cells to this molecule has quite different effects: Ca-dependent exocytosis in bovine cells is inhibited whereas that in chicken cells is stimulated. Half-maximal inhibition of bovine secretion is seen at about 5 μM GTP-γ-S

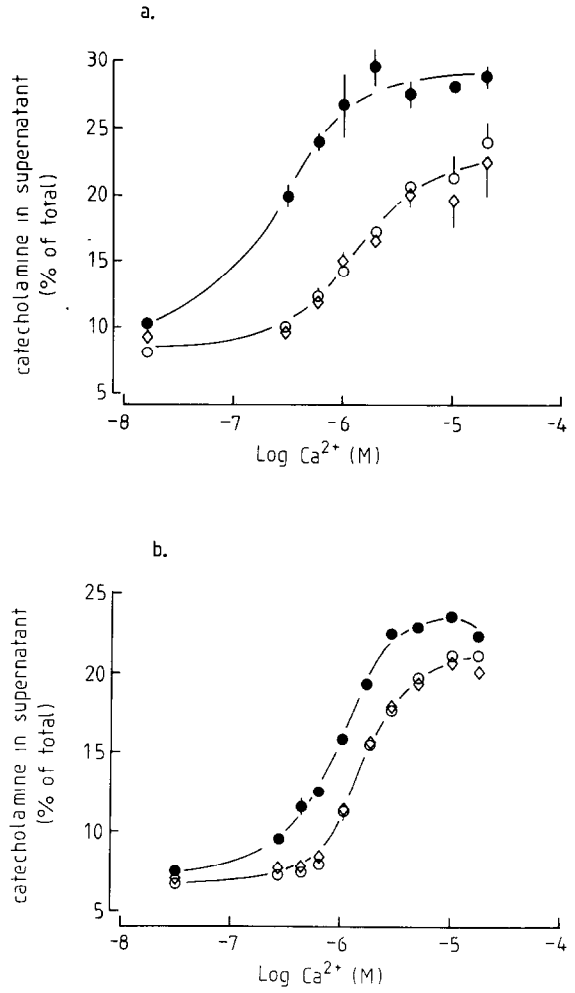

Fig.1. Effect of 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) and 1-oleoyl-2-acetylglycerol (OAG) on Ca-dependent catecholamine secretion from leaky chicken (a) and bovine (b) adrenal cells. Cells were isolated, suspended in K glutamate based medium containing 5 mM Mg-ATP and either (a) 1 mM EGTA or (b) 0.4 mM EGTA before being rendered leaky and diluted into buffer to give 0.2% (v/v) dimethylsulphoxide (DMSO) (○), and either 30 nM TPA (●) or 30 μM OAG (◇). After 5 min incubation aliquots of the cell suspensions were challenged with 18 mM Ca·EGTA buffers, and the catecholamine in the supernatant determined 15 min later. Temperature 26°C.

and half-maximal stimulation of chicken secretion at 50 μM. GTP-γ-S seems to act inside the cell because it is without effect, at concentrations up to 200 μM, on the response of intact cells to acetylcholine or high potassium. In permeable

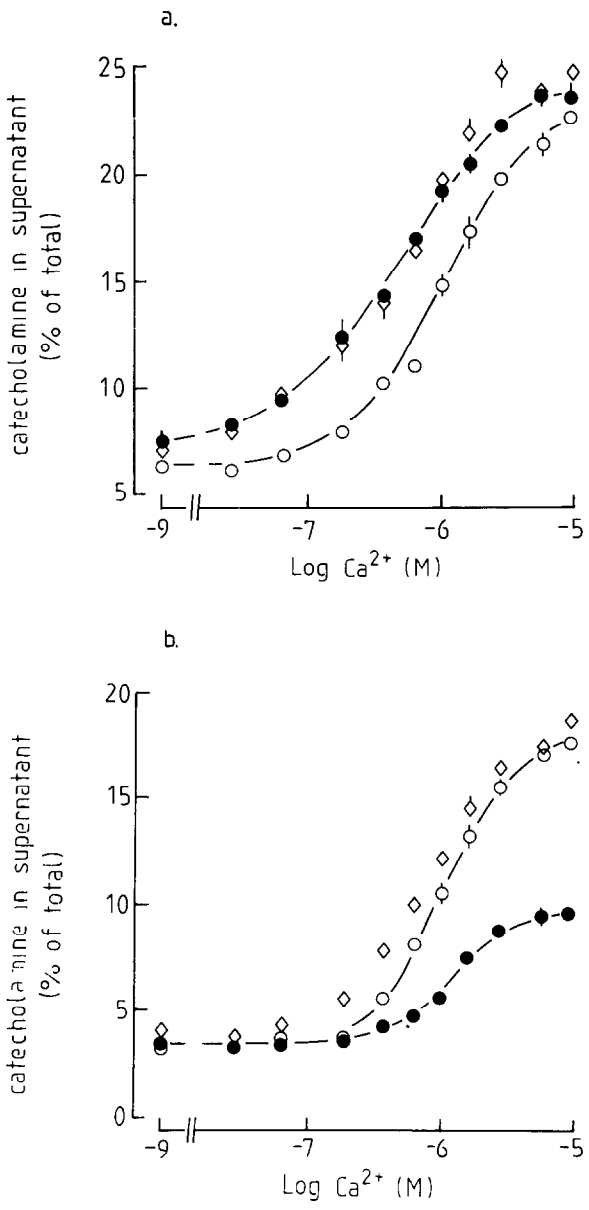

Fig.2. Calcium activation curve in the presence of the guanine nucleotides GTP-γ-S and GppNp. Chicken (a) and bovine (b) adrenal medullary cells were suspended in K glutamate buffer containing 5 mM Mg-ATP, 30 mM Pipes and 2 mM BAPTA, rendered leaky, and diluted into buffer alone (○), or with 80 μM GTP-γ-S (●) or 80 μM GppNp (◇) for 2 min before being challenged with 18 mM Ca-EGTA buffers for a further 18 min. Temperature 24°C. Data points are mean ± SE of 3 determinations. Addition of buffer to Ca changed the pH from 6.6 to 6.75 and the free Ca^{2+} concentrations have been corrected for this.

bovine cells GppNp does not mimic the effects of GTP-γ-S: it either has no effect or causes some stimulation of secretion. In permeabilized chicken cells both GppNp and GTP-γ-S are stimulatory. In confirmation of previous work, GTP and cGMP have no detectable effects on the Ca-activation curve although high concentrations of GTP protect against the inhibitory actions of GTP-γ-S.

In the chicken, GTP-γ-S and TPA exert qualitatively similar actions on exocytosis, whereas in bovine preparations their actions are opposite. The inhibitory effect in bovine material is still seen in the presence of TPA: the extent of secretion is reduced by GTP-γ-S but a TPA shift remains.

Where TPA and GTP-γ-S exert similar effects it is possible to argue that they may be acting on a common underlying mechanism – for instance GTP-γ-S may favour endogenous diacylglycerol production by breakdown of phosphatidylinositol; but this seems very unlikely when their effects are opposite and where GTP-γ-S can reduce secretion even in the presence of TPA. For the bovine adrenal it seems hard to escape the conclusion that, irrespective of the mechanism of TPA stimulation, there is a separate inhibitory site at which GTP-γ-S can act. It is highly unlikely that this inhibitory site is concerned with cyclic nucleotide levels because both cAMP and cGMP are without effect on Ca-dependent exocytosis in 'leaky' bovine adrenal medullary cells [3,10]. No evidence for a comparable inhibitory site is apparent in the chicken where only stimulation is seen.

By analogy with the control of adenylate cyclase [11,12], perhaps GTP analogues can act rather directly on the machinery of exocytosis to exert stimulatory or inhibitory control. One possibility worthy of close examination is that this action is exerted through the binding of protein kinase C to the plasma membrane. Thus the apparent lack of phorbol activation of exocytosis in intact cells may imply that some component of the exocytotic machinery in bovine cells can fully satisfy the phorbol binding site on protein kinase C. Permeabilization with brief, high voltage pulses may weaken this interaction in such a way that it can subsequently be reestablished in the presence of TPA (see [13]): but apparently not 1-oleoyl-2-acetylglycerol. The present results would be explained if the protein kinase C membrane interaction site is subject to inhibitory or

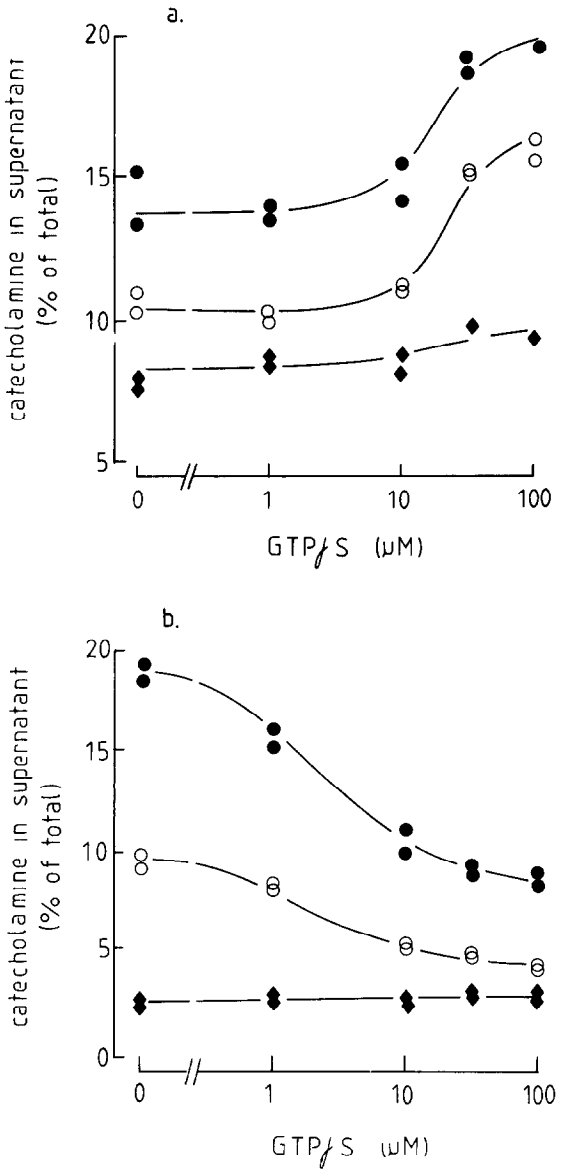

Fig.3. GTP-γ-S effects on Ca-dependent secretion. Chicken (a) and bovine (b) adrenal cells in buffer containing 2 mM Mg-ATP, 1 mM BAPTA, at pH 6.6, were rendered leaky, diluted into buffers containing GTP-γ-S to give a final concentration as shown in the figure, and 6 min later challenged with 13 mM Ca-EGTA buffers. The catecholamine in the supernatant was determined 15 min later. Temperature 26°C. The effect of adding the BAPTA-containing buffer to Ca buffers was to change the pH from 6.6 to 6.7 and this was taken into account in calculating the Ca^{2+} concentrations. Free Ca was: 6 μM (●), 0.7 μM (○) and close to 0.01 μM (◆).

stimulatory modulation by GTP-binding proteins thereby facilitating rather direct control over exocytosis by a variety of extracellular and intracellular agents. In this context two observations are of considerable interest: (i) membrane preparations from brain contain a remarkably high concentration of a GTP-γ-S-binding protein of, as yet, unknown function [14] and (ii) introduction of GTP-γ-S into mast cells also stimulates exocytosis [15,16]. As protein kinase C is known to bind to chromaffin vesicle membranes in a Ca-dependent fashion [17], it is possible that Ca may promote the binding of secretory vesicles to plasma membrane-associated protein kinase C thereby bringing vesicle and plasma membranes into close proximity [18]. This close apposition of membranes may facilitate membrane fusion and the initiation of exocytosis [19].

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