Calmodulin- and protein phosphorylation-independent release of catecholamines from PC-12 cells

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Catecholamine secretion from PC-12 cells can be triggered by agents that increase intracellular Ca^{2+} and is enhanced by phorbol esters and agents that elevate intracellular cAMP concentrations. In mutant PC-12 cells lacking cAMP-dependent protein kinase (PK-A) in which protein kinase C (PK-C) was down-regulated, Ca^{2+}-dependent secretion occurred normally but was no longer enhanced by cAMP or phorbol esters. In digitonin-permeabilized PC-12 cells that lacked PK-C and PK-A, a range of calmodulin (CaM) inhibitors failed to block Ca^{2+}-triggered catecholamine release. Moreover, Mn^{2+}, a CaM activator, failed to trigger catecholamine release whereas Ba^{2+}, which does not activate CaM, supported secretion. These results indicate that the basic mechanism of stimulus/secretion coupling in PC-12 cells does not absolutely require a regulated protein phosphorylation- or calmodulin-dependent step.

Exocytosis; Protein kinase C; cAMP-dependent protein kinase; Calmodulin; (PC-12 cell)

1. INTRODUCTION

The evoked release of a neurotransmitter (stimulus/secretion coupling) is known to be triggered by an influx of Ca^{2+} into the nerve terminal (review [1]). Little is known about the intracellular site(s) at which Ca^{2+} exerts its effects though some investigators have proposed that calmodulin (CaM) is the intracellular receptor for Ca^{2+} during exocytosis (summarized in [1]). As secretion from many permeabilized cell systems is dependent on MgATP [2–8] and neurotransmitter release from intact cells is sensitive to metabolic poisons [9], it has also been suggested that a phosphorylation step is of central importance in the secretory process [1]. Indeed, activation of either cAMP dependent kinase (PK-A) or protein kinase C (PK-C) has been shown to modulate secretion from intact and permeabilized cell systems, including PC-12 cells [1,7,10] (for further refs see [11]), and injection of CaM-dependent protein kinase II into the squid

giant synapse promotes synaptic transmission [12]. However, it is not known whether the basic mechanism of secretion also requires the participation of (a) protein kinase(s) or whether regulated protein phosphorylation acts solely to modulate this process. We have investigated these hypotheses by using permeabilized PC-12 cells that can be induced to release catecholamines in response to increased Ca^{2+} in the presence of MgATP. We have examined secretion in cells lacking functional PK-A and PK-C to which CaM inhibitors were also added. As exocytosis proceeds normally under these conditions, we conclude that the basic mechanism does not involve PK-A, PK-C, or a CaM-mediated event.

2. MATERIALS AND METHODS

2.1. Cells

PC-12 cells were grown as described [11]. The PK-A deficient A126-1B2 line [13] and its wild-type counterpart were generously provided by Dr J.A. Wagner (Dana Farber Cancer Institute, Boston). Down-regulation of PK-C was produced by treating cells with 2 μM phorbol dibutyrate (PDBu, Sigma) for 24–30 h followed by extensive washing in buffers containing 1% fatty acid-free bovine serum albumin to remove the phorbol ester [11].

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2.2. Protein kinase activity measurements

Enzyme activities in PC-12 cell homogenates using the appropriate exogenous substrates were determined as described [11].

2.3. Secretion from intact cells

Release of catecholamine was determined as described [11] with the exception that [^{3}H]dopamine (NEN; spec. act. 41.9 Ci/mmol) was used to load the cells. Ahnert-Hilger et al. [14] have shown that [^{3}H]dopamine is released from PC-12 cells in an exocytotic manner justifying the use of [^{3}H]dopamine as an exocytotic marker (see [8] for another demonstration of exocytosis in PC-12 cells). Cultures were labeled for 60 min with 1 μM [^{3}H]dopamine (2 μCi/well) in 0.35 ml DMEM, 1% horse serum, 0.5 mM Na ascorbate, 10 μM pargyline with or without 2 μM PDBu. Addition of pargyline enhances percent released in these cells by 10–40% compared to untreated cells. In the experiments reported here, secretion was induced in all cases by the addition of ionomycin (5 μg/ml). When present, tetradecanoyl 12-myristate 13-acetate (TPA; 100 nM) or 2-chloroadenosine (2-CAD; 10 μM), a potent activator of adenylate cyclase in these cells [10], were included in the last two 6 min rinses prior to a 6 min ionomycin challenge (that also contained these same activators at the same concentrations).

2.4. Secretion from digitonin-permeabilized cells

Wild type or A126-1B2 cells were prelabeled with [^{3}H]dopamine as described above. The medium was then removed and the cells washed twice with 1 ml of buffer A (148 mM NaCl, 2.6 mM KCl, 0.5 mM CaCl$_2$, 1 mM MgCl$_2$, 5.6 mM glucose, 15 mM N-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (Tes) (pH 7.4), 0.5 mM Na ascorbate, 10 μM pargyline and 0.5% fatty acid-free bovine serum albumin). Cells were then incubated for 2 h in 1 ml buffer A in an air incubator at 37°C followed by 5 successive rinses with 0.35 ml buffer A to remove PDBu from chronically treated cells [11]. Finally, the cultures were washed for 6 min in a Ca^{2+}-free buffer A containing 100 μM K$_2$ EGTA. Permeabilization was achieved by incubation in buffer B (85 mM K glutamate, 30 mM Tes (pH 7.1), 4 mM free MgATP, 1 mM free MgCl$_2$ and 10 mM K$_2$ EGTA) with 14 μM digitonin for 6 min. Permeabilized cells were then induced to release [^{3}H]dopamine by incubation buffer B containing various Ca^{2+} concentrations for 18 min. The supernatants were collected and analyzed for [^{3}H]dopamine content by liquid scintillation. The CaCl$_2$, MgCl$_2$ and MgATP concentrations were adjusted according to Fabiato and Fabiato [15] to give free [Ca^{2+}] ranging from 1 nM to 30 μM. ATP was added as the Na$_2$ salt yielding approx. 9 mM Na$^+$ in the buffer depending on the Ca^{2+} concentration. The pH was adjusted using KOH, so that the final K concentration became 120 mM and the ionic strength approx. 145 mM. When present, TPA (100 nM) was included for 20 min prior to digitonin treatment and in all subsequent steps.

3. RESULTS AND DISCUSSION

The levels of PK-A, PK-C and Ca/CaM-dependent kinase II (CaM-PK II) in wild-type PC-12 cells and in the mutant cell line A126-1B2

[13] are listed in table 1. PK-A is present at very low levels in the mutant cells, as previously reported [13]; however, the levels of PK-C and CaM-PK II are similar in A126-1B2 to wild-type cells. We have previously demonstrated that long-term treatment of PC-12 cells with active phorbol esters results in the down-regulation of PK-C [11]. After 24 h treatment with 1 μM TPA, enzymatically detectable PK-C is completely absent in both wild-type and mutant PC-12 cells (table 1). Thus in phorbol ester-treated A126-1B2 cells both PK-A and PK-C are virtually undetectable.

Next, we examined the Ca^{2+}-dependent release of [^{3}H]dopamine from both wild-type and mutant PC-12 cells (fig.1). Release was stimulated with the Ca^{2+}-ionophore ionomycin to bypass plasma membrane Ca^{2+} channels, thus avoiding complications that may arise from possible effects of the agents used here on the activity of these channels. In wild-type PC-12 cells, ionomycin-induced release was enhanced by acute exposure to either phorbol esters (e.g. TPA) or 2-CAD as previously reported [10,11]. By contrast, in mutant PC-12 cells phorbol esters were still effective, but 2-CAD was not, confirming that the effects of the adenosine analogue on secretion are mediated by activation of PK-A. In wild-type PC-12 cells in which PK-C had been down-regulated, 2-CAD still enhanced release but phorbol esters were no longer effective. In fact, a significant reduction in ionomycin-induced secretion was seen following down-regulation of PK-C, agreeing with previous results [11]. After PK-C down-regulation in A126-1B2 cells neither phorbol esters nor 2-CAD enhanced release but basal release was unaffected. The mutant cells differed from the wild-type cells in that down-regulation of PK-C did not reduce

Table 1

Protein kinase activities in wild type PC-12 and mutant A126-1B2 cells

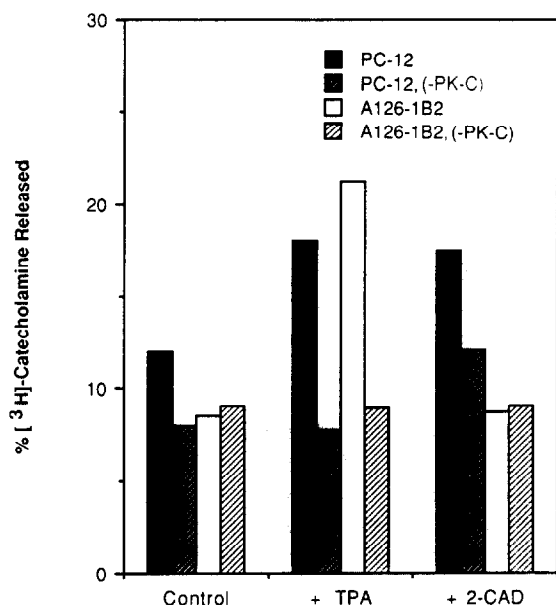| Kinase	Phosphate incorporated (pmol/mg per min)	
	PC-12	A126-1B2
PK-C	1012	1289
PK-C following down-regulation	0	0
Ca/CaM-PK II	779	747
PK-A	220	27

Fig.1. TPA and 2-chloroadenosine (2-CAD) enhanced release of catecholamines from wild type or mutant (A126-1B2) PC-12 cells in response to an ionomycin challenge. Cells were either untreated or PK-C down-regulated (-PK-C) prior to the experiment.

Fig.2. Ca^{2+}-dependence of catecholamine release from digitonin permeabilized A126-1B2 cells prior to ($\square$, $\triangle$) or after down-regulation of protein kinase C ($\blacksquare$). Control secretion ($\square$); effect of TPA (100 nM) in control cells ($\triangle$); effect of TPA (100 nM) in cells lacking PK-C ($\blacksquare$). Standard errors are <10% of means.

the stimulation of secretion by ionomycin. These results indicate that the basic mechanism of catecholamine exocytosis in PC-12 cells remains intact in the functional absence of both PK-A and PK-C.

We investigated the situation further by using digitonin-permeabilized PC-12 cells that have been shown by Peppers and Holz [7,8] to secrete catecholamines in a Ca^{2+}- and MgATP-dependent fashion. This secretion is associated in several cell types with the Ca^{2+}-dependent release of vesicular but not cytosolic proteins [4,7], thus such permeabilized cell systems are being evaluated as valid models for exploring the molecular basis of exocytosis [4–8]. We found that digitonin-permeabilized A126-1B2 cells secreted [^{3}H]dopamine in a Ca^{2+}-dependent manner as described for wild-type cells (fig.2, cf. [7,8]). In agreement with the results in fig.1 for intact cells, the Ca^{2+}-dependent release of [^{3}H]catecholamine from permeabilized mutant PC-12 cells was enhanced by phorbol esters but not by cAMP. However, phorbol esters were also ineffective in enhancing Ca^{2+}-dependent release from permeabilized
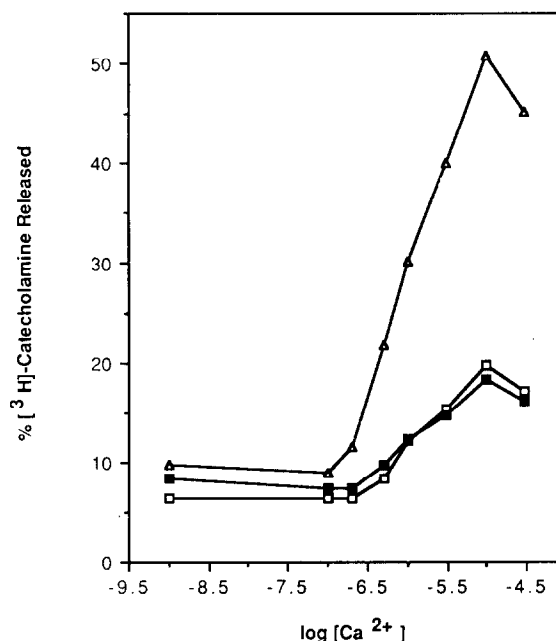
A126-1B2 cells after down-regulation of PK-C (fig.2). Thus the behavior of the permeabilized mutant cells resembles that of the intact cells in that neither PK-A nor PK-C are involved in the basic mechanism of catecholamine secretion.

We next investigated the role of Ca/CaM in exocytosis from permeabilized cells. Cells were treated with a variety of different CaM antagonists including calmidazolium, 48/80 and mastoparan, all of which have been shown to potently inhibit CaM-dependent processes at low concentrations [16]. We confirmed this in PC-12 cells by showing that in A126-1B2 cell homogenates calmidazolium (0.5 μM), 48/80 (1.0 μM) and mastoparan (5.0 μM) blocked the phosphorylation of exogenously added synapsin I, mediated by CaM-PK II, by 94, 98 and 95%, respectively. In permeabilized, PK-C-deficient A126-1B2 cells, [^{3}H]catecholamine release was unaffected by calmidazolium, 48/80, and mastoparan (fig.3) at concentrations where these agents effectively blocked Ca/CaM-dependent protein kinase activi-
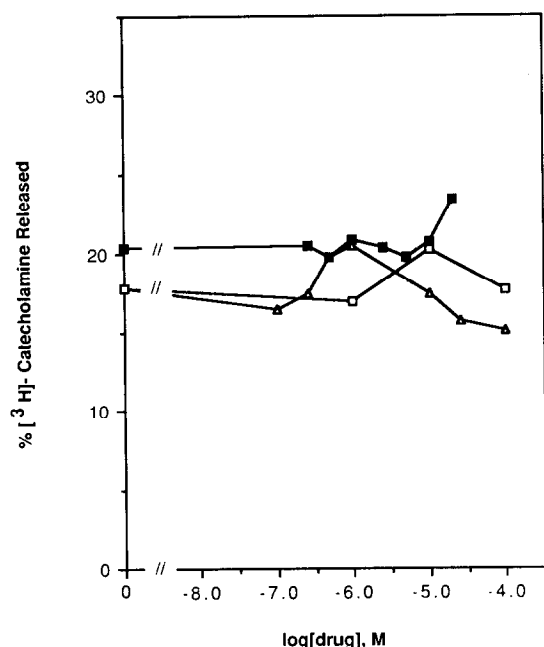
Fig.3. Influence of CaM antagonists on Ca-dependent catecholamine release from digitonin permeabilized A126-1B2 cells. Experimental procedures were as in fig.2. Cells were labeled for 60 min, rinsed, incubated for 120 min, permeabilized for 6 min in the presence of CaM antagonists 48/80 (△), calmidazolium (■) and mastoparan (□), and secretion was subsequently induced with 10 mM Ca EGTA buffers yielding 10 μM free Ca^{2+} [15]. The composition of the buffers used was as in fig.2. Calmidazolium was used in buffers containing 1% DMSO which alone had no effect on secretion. Points are means of triplicate cultures from one typical experiment which was repeated four times. Standard errors are <10% of means.

ty in homogenates. None of these agents altered the background release of catecholamine seen at 1 nM Ca^{2+}. As these digitonin-treated cells are permeable to fairly large proteins [7,8], there seems little doubt that such low molecular mass inhibitors can enter cells under these conditions. These results imply that Ca/CaM-regulated systems, such as a CaM-dependent protein kinase, are not essential for the basic mechanism of exocytosis in PC-12 cells. This hypothesis receives further support from an examination of the divalent cation specificity of [^{3}H]dopamine release. It has been shown that Mn^{2+} can compete with Ca^{2+} for binding to CaM and can activate CaM, whereas Ba^{2+} is ineffective [17,18]. For example, Kuret and Schulman [19] observed that

Ba^{2+} failed to activate purified rat brain CaM-PK II whereas Mn^{2+} was almost as effective as Ca^{2+}. In A126-1B2 cell homogenates, Mn^{2+} (0.1 mM) stimulated the phosphorylation of exogenously added synapsin I whereas Ba^{2+} (0.1 mM) did not. In permeabilized A126-1B2 cells in which PK-C had been down-regulated, Ba^{2+} was found to stimulate [^{3}H]catecholamine release whereas Mn^{2+} did not (fig.4). This effect is very unlikely to be due to Ba^{2+}-induced Ca^{2+} release from intracellular stores because cells were permeabilized in the presence of 25 mM EGTA and then induced to secrete in the presence of 25 mM EGTA, buffered to contain the appropriate amounts of Ba^{2+} or Mn^{2+}. Under these conditions, any Ca^{2+} released would displace Ba^{2+} from EGTA since it has a much higher affinity for the chelator. Mn^{2+}, on the other hand, has a greater affinity for EGTA than does Ca^{2+} and
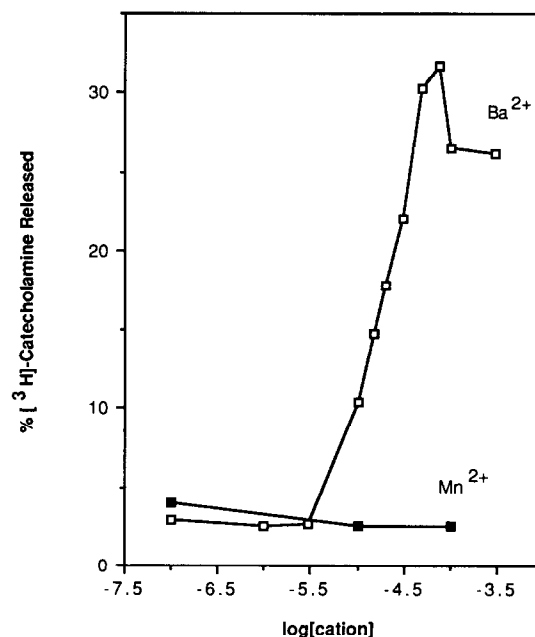

Fig.4. Effect of Ba^{2+} and Mn^{2+} on catecholamine release. Cells were treated as in other experiments (fig.2) but permeabilized in the presence of 25 mM K_2 EGTA without Mg^{2+} or ATP. During the release period, 25 mM Mn EGTA or Ba EGTA, with 1 mM free $MgCl_2$ and 4 mM free MgATP was added yielding the free divalent cation concentrations shown. Points are means of triplicate cultures from one experiment which was repeated twice. Standard errors are <10% of means.

would lead to a rise in free Ca^{2+}, if it could release Ca^{2+} from an internal site.

We conclude that in PC-12 cells which lack functional PK-A, PK-C or Ca/CaM (and thus Ca/CaM-regulated kinases), the basic mechanism of exocytosis in response to Ca^{2+} and MgATP is unimpaired. Nevertheless, PK-A and PK-C play a modulatory role in the process as indicated by the augmentation of secretion in PC-12 cells and many other systems by cAMP and phorbol esters [1]. These modulatory phenomena may be superimposed on a basic mechanism that requires the participation of Ca^{2+} and ATP in events that may not involve protein kinase activation. Phosphorylation may modulate at several potential steps prior to secretion, such as the regulation of plasma membrane Ca^{2+} channels (e.g. [20]), the positioning of exocytotic vesicles near the presynaptic terminal (e.g. [21]), or the regulation of protein binding to the surface of vesicles [22]. Additionally, different cells may exert distinct regulatory controls over the secretory process and this may even extend to different types of vesicles within one cell type (review [23]). Thus, caution should be exercised in extrapolating mechanisms of stimulus-secretion coupling from one cell type to another.

The question remains as to the Ca^{2+} receptor involved in the basic mechanism of stimulus-secretion coupling and the nature of the MgATP requirement. (It should be noted that contradictory results have been obtained using permeabilized PC-12 cells, as two groups have shown an ATP dependence [7,8] whereas another finds ATP independent release [14,24]. Our results suggest that neither PK-C nor CaM subserves the function of the Ca^{2+} receptor. The divalent cation specificity of secretion from permeabilized cells could mean that, in contrast to CaM, a receptor with affinity for both Ba^{2+} and Ca^{2+} is involved. Several possible sites for MgATP involvement have been proposed (review [25]) including roles in mechanochemical systems, ion pumps and in lipid and/or protein phosphorylation. Although it is possible that an as yet unidentified protein kinase could participate in the exocytotic mechanism, our results suggest that none of the well-known regulated protein kinases are central to this process.

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