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Identification of a key domain in annexin and 14-3-3 proteins that stimulate calcium-dependent exocytosis in permeabilized adrenal chromaffin cells

Dagmar Roth, Alan Morgan and Robert D. Burgoyne

The Physiological Laboratory, University of Liverpool, PO Box 147, Liverpool, L69 3BX, UK

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Calcium-dependent secretion in digitonin-permeabilized adrenal chromaffin cells is stimulated by exogenous annexin II and 14-3-3 proteins. These proteins share a conserved domain that has been suggested to be involved in specific protein–protein interactions. We examined whether this domain was involved in secretion by using a synthetic peptide (P16) of sequence KGDYQKALLYLCGGDD corresponding to the C-terminus of annexin II. P16, but not truncated peptides, prevented the stimulation of secretion by 14-3-3 proteins and produced a partial inhibition of control secretion. These data suggest that the shared annexin/14-3-3 domain is important in the mechanisms controlling Ca^{2+}-dependent secretion and may play a key role in protein–protein interactions during exocytosis.

Exocytosis; Calcium; Secretion; Annexin; 14-3-3 Protein; Chromaffin cell

1. INTRODUCTION

Calcium-dependent exocytosis is likely to involve a number of specific proteins but we have only limited information on the possible proteins involved [1,2]. Soluble proteins (cytosolic and extrinsic membrane proteins) appear to be required for Ca^{2+}-dependent exocytosis [3], and a number of proteins that regulate exocytosis in permeabilized cells have been identified recently using assays based on recovery of secretory run-down following protein leakage after cell permeabilization. These proteins include annexin II [4,5], 14-3-3 proteins (Exo1; [6–8]), p145 [9,10] and several uncharacterised activities [6,11]. Annexin II, 14-3-3 proteins (Exo1) and p145 possess properties that suggest that they play key roles in the regulation of the exocytotic response but their exact site of action remains to be determined. The 14-3-3 protein family have a conserved internal domain which has homology to the C-terminus of the annexins [12]. The possession of this domain by both classes of proteins that stimulate exocytosis in permeabilized adrenal chromaffin cells suggests that it could be involved in protein–protein interactions in exocytosis. In support of the idea that this domain mediates such interactions, it was found that a synthetic peptide corresponding to the C-terminus of annexin I inhibited protein kinase C (PKC) binding to cytoskeletal protein receptors known as the RACKs (receptors for activated C kinase; [13,14]). In addition, the cytoplasmic domain of the synaptic vesicle protein, p65 [15], binds to the RACKs and its binding was also inhibited by the synthetic peptide [16]. Recently, it has been suggested that p65 (synaptotagmin) may be involved in exocytosis by docking secretory vesicles to the plasma membrane [17–19]. In the light of these observations we have tested whether the conserved domain in annexins and 14-3-3 proteins is important for the stimulation of exocytosis in digitonin-permeabilized chromaffin cells.

Correspondence address: R.D. Burgoyne, The Physiological Laboratory, University of Liverpool, PO Box 147, Liverpool, L69 3BX, UK. Fax: (44) (51) 794-5337.

Abbreviations: PKC, protein kinase C; RACK, receptor for activated C-kinase; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid.

2. MATERIALS AND METHODS

The synthetic peptides, P4, P9 and P16 (Fig. 1), were synthesised by Multiple Peptide Systems, San Diego, CA, and analysed by mass spectroscopy to confirm their molecular mass. Peptide purification was by reverse-phase HPLC. A second batch of P16 was synthesised in the Department of Biochemistry, University of Liverpool, and peptide purification carried out by ion-exchange chromatography on an FPLC Mono Q column (Pharmacia). All peptide solutions were freshly prepared and used the same day. Exo1 (sheep brain 14-3-3 proteins) was purified and dialysed for use as previously described [6,7].

For cell permeabilization and catecholamine release, dissociated bovine adrenal medullary chromaffin cells were maintained in culture for 3–7 days in 24-well trays as described previously [6,7]. For digitonin-permeabilization [20,21], a variety of protocols were used and defined in the text but in general the cells were washed, permeabilized in buffer A (139 mM potassium glutamate, 2 mM ATP, 2 mM $MgCl_2$, 5 mM EGTA, 20 mM PIPES, pH 6.5) containing 20 μM digitonin and incubated further in buffer A prior to challenge by replacement of buffer with fresh buffer A either without Ca^{2+} or with $CaCl_2$ added

to a free Ca^{2+} concentration of 10 μM. After 10–20 min, buffer was removed, centrifuged at 16,000 × g for 2 min and aliquots assayed fluorimetrically for released endogenous catecholamine [22]. Total catecholamine left in the cells was determined by releasing it with 1% Triton X-100. Data for catecholamine release was expressed as a percentage of total cellular catecholamine. Data was calculated as mean ± S.E.M., but in some cases errors were smaller than the symbols used and are not shown in the figures. All experiments were carried out at 22–25°C.

3. RESULTS

Fig. 1 shows the conserved domain in 14-3-3 proteins showing sequence homology to the C-terminal domain of the annexins [12,23] and the three peptides, P4, P9 and P16, used in this study. The sequence of the 16-mer was based on the C-terminus of annexin II. The effect of P16 on control secretion and secretion stimulated by Exo1 (brain 14-3-3 proteins) in digitonin-permeabilized chromaffin cells was first examined. As shown in Fig. 2, P16 at 60 μM produced a partial inhibition of control secretion and abolished the stimulation due to a low (5 μM) concentration of Exo1. In the experiment shown P16 was absent during the stimulation period. In an attempt to increase the efficiency of the inhibition of secretion by P16 it was included in both pre-incubation and stimulation steps and the effects of a range of concentrations of the peptide examined. Under these conditions P16 never produced more than a partial inhibition of secretion which was essentially maximal at 100 μM (Fig. 3A). From a series of experiments the maximal inhibition at 250 or 500 μM of P16 was 39.0 ± 4.1% (n = 14). Also shown in Fig. 3 is that P4 did not inhibit secretion at any concentration. Instead, both P4 and P9 produced a small but reproducible increase in catecholamine secretion at 10 μM Ca^{2+}. The inhibition of secretion observed appeared, therefore, to be specific for the longer peptide. None of the peptides had any effect on

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K	G	D	Y	F	R	Y	L	S	E	V	A	S	G	D	N	β
K	G	D	Y	Y	R	Y	L	A	E	V	A	T	G	E	K	γ
K	G	D	Y	H	R	Y	L	A	E	F	A	T	G	N	D	ε
K	G	D	Y	Y	R	Y	L	A	E	V	A	A	G	D	D	ζ
K	G	D	Y	Y	R	Y	L	A	E	V	A	S	G	E	K	η
K	G	D	Y	F	R	Y	L	A	E	V	A	C	G	D	D	T cell
K	G	D	Y	H	R	Y	L	A	E	F	K	T	G	A	E	Plant (Oenothera)
K	G	D	Y	Y	R	Y	L	A	E	V	A	T	G	D	A	Drosophila
K	G	D	Y	Y	R	Y	L	S	E	V	A	S	G	D	S	Xenopus
K	G	D	Y	H	H	Y	L	A	E	F	S	S	G	D	A	Yeast
K	G	D	Y	Q	K	A	L	L	Y	L	C	G	G	D	D	Annexin II
K	G	D	Y	Q	K	A	L	L	Y	L	C	G	G	D	D	P16
K	G	D	Y	Q	K	A	L	L								P9
K	G	D	Y													P4

Fig. 1. Comparison of the common conserved domain in 14-3-3 proteins and annexin II and sequences of the synthetic peptides used. Sequences are derived from the various known 14-3-3 proteins [23] and correspond to residues 127–142 of the eta form. The extreme C-terminal residues, 323–338, of annexin II and the 16-mer, 9-mer and 4-mer synthetic peptides used in this study are shown below. Identical amino acids are boxed and conservative substitutions marked by asterisks.

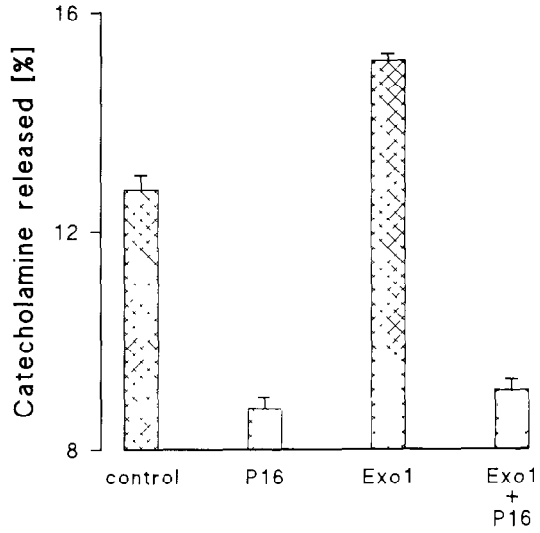

Fig. 2. Peptide P16 inhibits control secretion and that stimulated by 14-3-3 proteins (Exo1). Chromaffin cells were permeabilized with 20 μM digitonin for 10 min, pre-incubated for a further 15 min with or without peptide (60 μM) or Exo1 (brain 14-3-3 proteins; 5 μM) and then challenged with 10 μM Ca^{2+} in the absence of any additions. The catecholamine release over a 20 min period was expressed as a percentage of total cellular catecholamine (n = 4). The submaximal dose of Exo1 used produced a 20% increase in secretion ($P < 0.001$) which was abolished by P16.

catecholamine secretion in the absence of Ca^{2+} (Fig. 3B). Inhibition of secretion was found with two independently synthesised batches of P16 and with purification using two different techniques.

P16 would be expected to inhibit processes involving annexin II or Exo1. A proportion of these proteins leak from digitonin-permeabilized cells over a 25 min permeabilization period [8,24,25]. Therefore, we examined the possibility that the degree of inhibition by P16 may have been higher with shorter pre-permeabilization periods. Using a high concentration of p16 and a range of protocols with no pre-permeabilization (Fig. 4, condition A) to pre-permeabilization for 25 min prior to stimulation (Fig. 4, condition D), it was found that the percentage inhibition by P16 was essentially the same (around 35–45%) in all cases (Fig. 4).

It has been shown that secretion in permeabilized chromaffin cells can be dissected into distinct ATP-dependent and ATP-independent phases [26]. The degree of inhibition of secretion by P16 was found to be the same when cells were stimulated in the presence or absence of ATP (33.3 and 39.0%, respectively, means from two separate experiments) indicating that the partial nature of the inhibition by P16 was not due to a selective action on one or other of these phases. The inhibition by P16 was independent of PKC activity as it was also detected when cells were incubated in the presence of a high (10 μM) concentration of the PKC inhibitor staurosporine. The inhibition of secretion by P16 was not due to a change in the Ca^{2+} affinity for secretion and

was not overcome by increasing Ca^{2+} concentration (not shown).

4. DISCUSSION

The results presented here show that a 16-mer synthetic peptide based on the C-terminus of annexin II and covering a domain conserved in annexins and 14-3-3 proteins partially inhibits Ca^{2+}-dependent secretion in digitonin permeabilized chromaffin cells. The effect appears to be specific since truncated peptides had no inhibitory activity. This may be due to a lack of the correct sequence required in the shorter peptides or the inability of these peptides to fold correctly. In previous work we found that a synthetic peptide based on the N-terminus of annexin II had no effect on secretion [27], whereas a peptide (annexin consensus peptide) based on a conserved region within the annexin repeats also partially inhibited Ca^{2+}-dependent secretion in permeabilized chromaffin cells [4]. We have demonstrated that, following permeabilization and secretory run-down, exogenous annexin II or 14-3-3 proteins are able to stimulate secretion from chromaffin cells [4,6]. The results from the use of synthetic peptides which inhibit control secretion support a role for endogenous annexin and/or 14-3-3 proteins in secretion in chromaffin cells. The relative contribution of each of these classes of proteins cannot, however, be determined from this approach.

The inhibitory effect of P16 on 14-3-3 protein-stimulated and on control secretion demonstrates a key role for this sequence in the events leading to exocytosis. Previous work has suggested that this domain may be involved in interactions between protein kinase C [14] or p65 [16] and a series of binding proteins known as RACKs. Amongst proteins that can act as RACKs are annexins I and II [13], and evidence that annexin V can inhibit PKC [28] and certain 14-3-3 proteins can inhibit [29] or stimulate [30] PKC suggests that they may also directly bind to PKC. The interaction of RACKs with

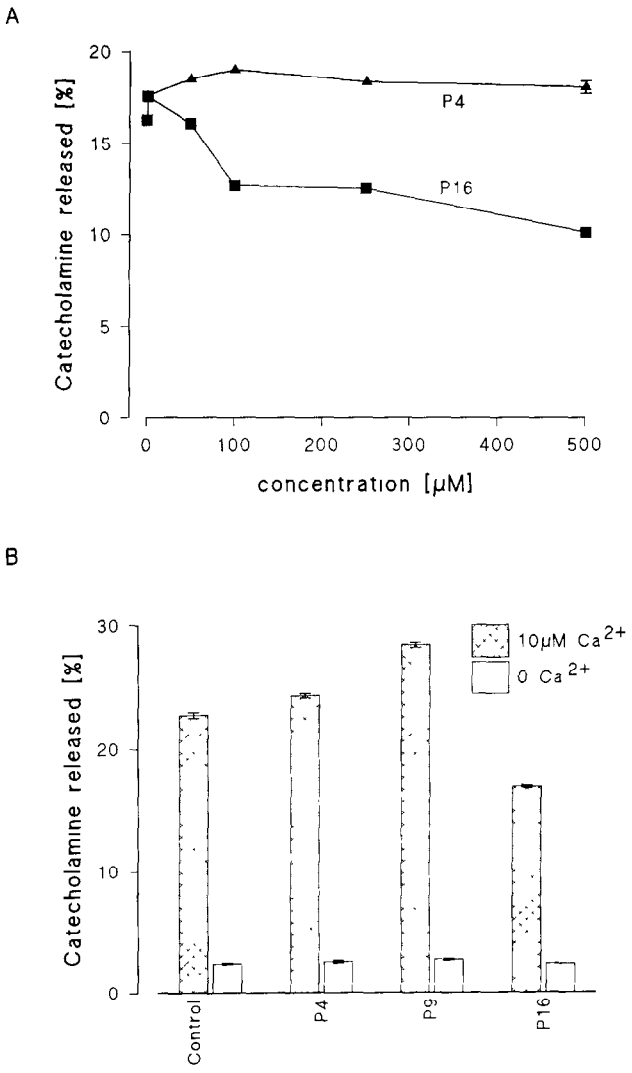

Fig. 3. P16 produces a specific inhibition of Ca^{2+}-dependent secretion. Chromaffin cells were permeabilized with 20 μM digitonin for 10 min, pre-incubated for a further 5 min with or without the indicated concentration of peptide and then challenged with 0 μM or 10 μM Ca^{2+} in the presence of peptide The catecholamine released over a 10 min period was expressed as a percentage of total cellular catecholamine (n = 4). (A) Response to varying concentrations of P4 and P16. (B) Effect of P4, P9 and P16 at 500 μM on secretion at 0 and 10 μM Ca^{2+}

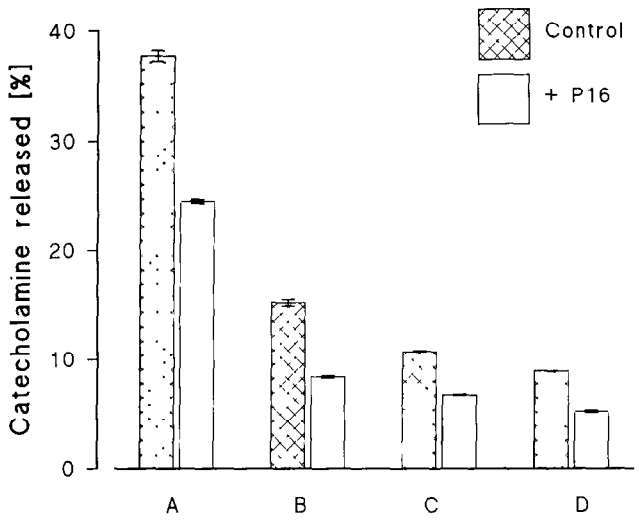

Fig. 4. P16 results in partial inhibition of secretion over a range of pre-permeabilization times. (A) Cells were permeabilized directly into 10 μM Ca^{2+} ± P16 and secretion measured after a 10 min period. (B) Cells were permeabilized in 0 Ca^{2+} for 10 min ± P16 and then challenged in 10 μM Ca^{2+} ± P16 and secretion measured after a further 10 min. (C) Cells were permeabilized in 0 Ca^{2+} for 10 min, pre-incubated for a further 5 min ± peptide and then challenged for 10 min in 10 μM Ca^{2+} ± peptide. (D) Cells were permeabilized in 0 Ca^{2+} for 10 min, pre-incubated for a further 15 min ± peptide and then challenged for 10 min in 10 μM Ca^{2+} ± peptide. In each case n = 4. Increasing permeabilization times prior to challenge leads to a decrease in the extent of secretion (secretory run-down), and P16 (500 μM) was inhibitory at all times of pre-permeabilization.

both PKC and p65 suggests that this may occur via the PKC C2 domain, which is also present in the cytoplasmic fragment of p65 [31]. PKC C2 domains are also present in other proteins that translocate to the plasma membrane on cell stimulation, including phospholipase Cγ [32], a cytosolic phospholipase A_2 [33] and GTPase activating protein [34]. It is possible that the common annexin/14-3-3 domain could be involved in a variety of specific protein–protein interactions, the consequence of which could be determined by the specificity of the annexin or 14-3-3 protein involved. It may be significant that the C-terminal domain in the annexins forms an exposed α-helical structure [35].

The finding that P16 produced no more than a partial inhibition of secretion under a variety of experimental conditions could be taken as suggesting that the annexin/14-3-3 proteins are not essential for exocytosis but merely regulate its extent. Alternatively, it is possible that assembly of a fusion complex involving these proteins is required for exocytosis, in which case any preformed complexes may not be inhibitable by the peptide. It is also possible that there are two parallel pathways leading to exocytosis, only one of which involves annexin/14-3-3 proteins. It is not clear which of these is the correct explanation. P16 was inhibitory even after permeabilisation for 25 min, during which time considerable leakage of annexin II and 14-3-3 proteins occurs [8,24,25]. The effect of P16 may have been exerted on residual annexin II since it is known that some annexin II is tightly associated in a Ca^{2+}-independent manner with membranes, including chromaffin granule membranes [36], and the annexin consensus peptide also produced partial inhibition of secretion after this permeabilisation time [4].

The stimulatory effect of 14-3-3 proteins on exocytosis in chromaffin cells is potentiated by PKC activation [6,7]. It is unlikely that the stimulatory effect of 14-3-3 proteins on secretion is due to a direct interaction with PKC [7]. In addition, the inhibition by P16 does not appear to be mediated through an effect on PKC-related processes since the peptide was inhibitory on secretion in the absence of ATP and in the presence of staurosporine where PKC activity would not occur. The availability of this inhibitory peptide should allow further investigation of the protein–protein interactions involving annexin II and 14-3-3 that lead to stimulation of Ca^{2+}-dependent secretion.

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