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Relationship between arachidonic acid release and Ca^{2+}-dependent exocytosis in digitonin-permeabilized bovine adrenal chromaffin cells

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The relationship between Ca^{2+}-dependent arachidonic acid release and exocytosis from digitonin-permeabilized bovine adrenal chromaffin cells was investigated. The phospholipase A_2 inhibitors mepacrine, nordihydroguaiaretic acid and indomethacin had no effect on either arachidonic acid release or secretion. The phospholipase A_2 activator melittin had no effect on secretion. The specific diacylglycerol lipase inhibitor RG80267 had no effect on secretion, but decreased basal arachidonic acid release to such an extent that the level of arachidonic acid in treated cells in response to 10 μM-Ca^{2+} was equivalent to that of control cells in the absence of Ca^{2+}. Staurosporine, a protein kinase C inhibitor, was found to abolish Ca^{2+}-dependent arachidonic acid release completely, but had only a slight inhibitory effect on Ca^{2+}-dependent secretion. It is concluded that arachidonic acid is not essential for Ca^{2+}-dependent exocytosis in adrenal chromaffin cells.

INTRODUCTION

Ca^{2+} is the major signal leading to exocytosis in many cell types. This has been shown to be the case in bovine adrenal chromaffin cells, since nicotinic-agonist-induced secretion is completely dependent on Ca^{2+} influx [1–3], and since exocytosis can be elicited by micromolar Ca^{2+} alone in permeabilized cells [4,5]. Exactly how Ca^{2+} acts to bring about exocytosis is still unknown, although various possible sites of action have been proposed, including the cortical cytoskeleton [6,7], protein kinase C [8], calmodulin [9] and calpactin [10]. In addition to initiating exocytosis, Ca^{2+} also acts to increase the cellular level of arachidonic acid in many cell types, including bovine adrenal chromaffin cells [11]. It seems possible that this could be the mechanism by which Ca^{2+} induces exocytosis, since arachidonic acid stimulates secretion from anterior pituitary [12] and GH_3 cells [13] and is capable of interacting with other second-messenger systems by stimulating phospholipase C [14], guanylate cyclase [15] and protein kinase C [16]. Furthermore, arachidonic acid has been shown to induce membrane–membrane fusion between isolated chromaffin granules [17], and so could be directly involved in the exocytotic fusion of granule and plasma membranes.

Release of arachidonic acid accompanies Ca^{2+}-dependent exocytosis in both intact [18] and permeabilized [11] bovine adrenal chromaffin cells. Moreover, inhibition of arachidonic acid release by phospholipase A_2 inhibitors also results in an inhibition of exocytosis [11]. Taken together, these results are suggestive of an obligate role for arachidonic acid in Ca^{2+}-dependent exocytosis in this cell type. Further support for this theory comes from a recent publication [19] on intact chromaffin cells, where secretion was inhibited by treatment with the diacylglycerol lipase inhibitor, RG80267, but could be restored by addition of exogenous arachidonic acid. By contrast, we have previously shown that increased arachidonic acid release is not responsible for GTP-analogue-induced exocytosis [20], and we report here that arachidonic acid is also not required for Ca^{2+}-dependent exocytosis from digitonin-permeabilized bovine adrenal chromaffin cells.

MATERIALS AND METHODS

Materials

Digitonin was obtained from Calbiochem. Foetal-calf serum and Dulbecco's modified Eagle's medium with 25 mM-Hepes were obtained from Gibco. [5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (200 Ci/mmol) was obtained from Amersham. RG80267 was generously given by Rorer Group, Fort Washington, Philadelphia, PA, U.S.A. Staurosporine was generously given by Dr. H. Nakano, of Tokyo Research Laboratories, Tokyo, Japan. All other chemicals were from Sigma.

Isolation and culture of chromaffin cells

Chromaffin cells were isolated from bovine adrenal medullae by enzymic digestion as described by Greenberg & Zinder [21] with modifications [8]. Cells were washed in Ca^{2+}-free Krebs–Ringer buffer, consisting of 145 mM-NaCl, 5 mM-KCl, 1.3 mM-$MgCl_2$, 1.2 mM-NaH_2PO_4, 10 mM-glucose and 20 mM-Hepes at pH 7.4 (Buffer A), resuspended in culture medium (Dulbecco's modified Eagle's medium with 25 mM-Hepes, 10% foetal-calf serum, 8 μM-fluorodeoxyuridine, 50 μg of gentamycin/ml, 10 μM-cytosine arabinofuranoside, 2.5 μg of fungizone/ml, 25 units of penicillin/ml, 25 μg of streptomycin/ml), plated in 24-well trays at a density of $(0.7–1) \times 10^6$ cells per well and maintained in culture for 2–8 days before use.

Cell permeabilization and assay of catecholamine secretion

The protocol here was in five stages. (1) Cultures were incubated for 15 min in Buffer A. (2) Cultures were washed once with Buffer A containing 0.5% fatty-acid-free BSA. (3) Cultures were pre-permeabilized for 6 min by addition of Buffer B (20 μM-digitonin in 139 mM-potassium glutamate, 2 mM-ATP, 2 mM-$MgCl_2$, 5 mM-EGTA, 0.5% fatty-acid-free BSA, 20 mM-Pipes at

Abbreviations used: RG80267, 1,6-bis(cyclohexyloximinocarbonylamino)hexane; PMA, phorbol 12-myristate 13-acetate; NDGA, nordihydroguaiaretic acid; NEM, *N*-ethylmaleimide; BPB, *p*-bromophenacyl bromide.

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pH 6.5). (4) Cultures were then challenged by replacement of buffer with Buffer B or with Buffer B containing added $CaCl_2$ to give the indicated calculated free Ca^{2+} concentration. (5) After 15 min the buffer was removed, centrifuged at 16000 *g* for 2 min, and samples were taken for assay of released catecholamine.

For those experiments using staurosporine, nordihydroguaiaretic acid (NDGA) and indomethacin, the drugs (or vehicles for controls) were present in stages 1 and 4. Where RG80267 and melittin were used, the drugs or vehicles were present in stages 3 and 4, whereas with mepacrine the cells were incubated in the drug or vehicle in stage 1 only.

Assay of released catecholamine was performed by a fluorimetric method [22]. Total catecholamine content of the cells was determined after release of catecholamines with 1% Triton X-100. Catecholamine secretion was calculated as a percentage of total cellular catecholamine. All experiments were performed at room temperature.

Assay of arachidonic acid release

This was based on a previously described method [11]. Cells were incubated with 250 μl (per well) of culture medium containing 0.5% fatty-acid-free BSA and 10 μCi of [^{3}H]arachidonic acid/ml for 3 h at 37 °C before being subjected to the protocol described above. After centrifugation in stage 5, samples of supernatant were applied to t.l.c. plates along with non-radioactive arachidonic acid. The plates were developed in ethyl acetate/acetic acid (100:1, v/v), and the arachidonic acid spots were made visible with iodine vapour and then scraped directly into vials for scintillation counting. Total radioactivity in the cells was determined by solubilizing cells with 1% Triton X-100 and taking samples for scintillation counting.

RESULTS

In a previous study [11], it was reported that the non-specific protein-alkylating agents *p*-bromophenacyl bromide (BPB) and *N*-ethylmaleimide (NEM) inhibited both catecholamine release and arachidonic acid release in permeabilized chromaffin cells. In order to test whether the observed inhibition of secretion was due to the inhibition of arachidonic acid release, cells were treated with NEM as previously described [11] and stimulated to secrete in the presence of exogenous arachidonic acid (Table 1). NEM did indeed inhibit catecholamine secretion, but, although arachidonic acid potentiated secretion slightly in both control and NEM-treated cells, it did not reverse the NEM-induced inhibition of exocytosis.

The effects of non-protein-alkylating inhibitors of phospholipase A_2 are shown in Table 2. The well-documented phospholipase A_2 inhibitors mepacrine [23,24] and indomethacin [24] had no effect on either secretion or arachidonic acid release (although mepacrine increased basal secretion and decreased basal arachidonic acid release, this is believed to be an artefact due to the fluorescent [23] and quenching [23] properties, respectively, of mepacrine). As well as inhibiting lipoxygenase, NDGA has been shown to inhibit phospholipase A_2 *in vitro* [25], and was found here to inhibit partly both basal and Ca^{2+}-dependent arachidonic acid release. NDGA increased basal secretion, but had no effect on Ca^{2+}-dependent secretion. The effects of NDGA on arachidonic acid release were assumed to be unrelated to a decrease in phospholipase A_2 activity, since these effects were not exhibited by the more established inhibitors, mepacrine and indomethacin. We also examined the effect of melittin, a peptide activator of phospholipase A_2 [26], on secretion. It was found that this drug had little effect on secretion in either the presence or the absence of Ca^{2+} at concentrations up to 10 μg/ml.

The effects of RG80267, a specific inhibitor of diacylglycerol lipases [27], on secretion and arachidonic acid release are shown in Table 3. With regard to secretion, RG80267 had no effect on either basal or Ca^{2+}-dependent exocytosis. However, although the drug was without effect on Ca^{2+}-dependent arachidonic acid release, it decreased the basal level of arachidonic acid by 44%. Furthermore, this decrease in the basal concentration of arachidonic acid meant that in RG80267-treated cells the Ca^{2+}-induced release of arachidonic acid was no different from that observed in control cells in the absence of Ca^{2+} (0.334±0.013% and 0.327±0.004% of total incorporated radioactivity respectively).

Figs. 1(*a*) and 1(*b*) illustrate the effects of increasing doses of

Table 1. Effect of exogenous arachidonic acid on catecholamine secretion from control and NEM-treated permeabilized chromaffin cells

Cells preincubated in the absence or presence of 100 μM-NEM were challenged with Buffer B or with Buffer B containing 10 μM-Ca^{2+} in the absence or presence of 100 μM-arachidonic acid (AA). The data are shown as a percentage of total cellular catecholamine. Data shown are means±S.E.M. ($n = 6$).

Condition	Catecholamine released (%)	
	0 Ca^{2+}	10 μM-Ca^{2+}
Control	2.6±0.1	27.7±0.3
+AA	4.4±0.2	29.0±0.4
NEM	3.5±0.1	8.4±0.2
NEM+AA	4.8±0.1	9.7±0.3

Table 2. Effects of phospholipase A_2 inhibitors on catecholamine secretion and arachidonic acid release from permeabilized chromaffin cells

Cells were treated with the various drugs as described in the Materials and methods section and then challenged with Buffer B or Buffer B containing 10 μM-Ca^{2+} in the absence or presence of drug, as appropriate. Data are expressed as a percentage of control secretion or arachidonic acid release. Data are means [$n = 6$ for catecholamine secretion, $n = 2$ for arachidonic acid (AA) release].

Drug	Secretion (%)		AA release (%)	
	Basal	Ca^{2+}-dependent	Basal	Ca^{2+}-dependent
1 mM-mepacrine	151	103	82	108
100 μM-indomethacin	108	105	102	101
20 μM-NDGA	128	108	86	68

Table 3. Effect of RG80267 on catecholamine secretion and arachidonic acid release from permeabilized chromaffin cells

Cells were challenged with Buffer B or Buffer B containing 10 μM-Ca^{2+} in the absence or presence of 250 μM-RG80267. The data are shown as a percentage of control catecholamine secretion or arachidonic acid release induced by 10 μM-Ca^{2+}. Data shown are means±S.E.M. ($n = 4$ for catecholamine secretion, $n = 2$ for arachidonic acid release).

Drug	Secretion (%)		AA release (%)	
	0 Ca^{2+}	10 μM-Ca^{2+}	0 Ca^{2+}	10 μM-Ca^{2+}
Control	3±0.1	100±1	71±1	100±4
250 μM-RG80267	4±0.2	97.5±2	40±2	72±3

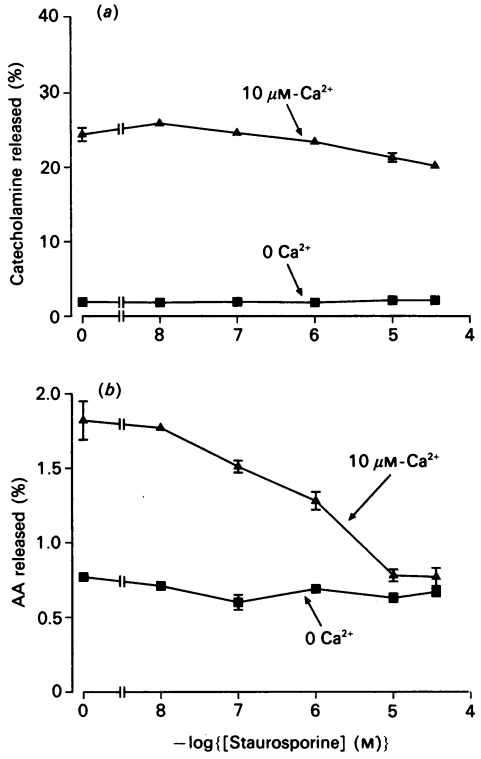

Fig. 1. Effect of increasing doses of staurosporine on catecholamine secretion and arachidonic acid release from permeabilized chromaffin cells

Cells were preincubated in various concentrations of staurosporine and then challenged with Buffer B or Buffer B containing 10 μM-Ca^{2+} in the presence of various concentrations of staurosporine. The data for secretion are shown as a percentage of total cellular catecholamine (*a*) and those for arachidonic acid (AA) release as a percentage of total cellular incorporated radioactivity (*b*). Data shown are means or means $\pm$ S.E.M. [$n = 4$ for secretion (*a*), $n = 2$ for arachidonic acid (AA) release (*b*)]. In some cases the S.E.M. was too small for inclusion.

staurosporine, a protein kinase C inhibitor [28], on secretion and arachidonic acid release. Although staurosporine was without effect in the absence of Ca^{2+}, it caused a weak dose-dependent inhibition of Ca^{2+}-dependent exocytosis, but a strong dose-dependent inhibition of Ca^{2+}-dependent arachidonic acid release. In a series of experiments, Ca^{2+}-dependent arachidonic acid release was inhibited by 77 ± 7% ($n = 6$) at 10 μM-staurosporine and by 102 ± 11% ($n = 4$) at 30 μM-staurosporine. The dissociation of exocytosis from arachidonic acid release can most clearly be seen in Fig. 1 at the highest concentration of drug used (30 μM), where Ca^{2+}-dependent secretion is still 83% of control, whereas Ca^{2+}-dependent arachidonic acid release is only 5% of control.

DISCUSSION

To date, the only published work on the relationship between arachidonic acid release and secretion from permeabilized bovine adrenal chromaffin cells is that of Frye & Holz [11]. The authors demonstrate a striking correlation between the two processes with regard to time course, Ca^{2+}-dependency and responses to various drugs. A key finding in their work is the observation that NEM and BPB inhibit both secretion and arachidonic acid release. This was interpreted by the authors as being suggestive of a role for arachidonic acid (and more specifically for phospholipase A_2) in exocytosis. However, we have shown in the present study that the NEM-induced inhibition of exocytosis is unconnected with a decrease in the level of free arachidonic acid, since addition of exogenous arachidonic acid failed to reverse this inhibition. Furthermore, the observation that an activator of phospholipase A_2 failed to enhance exocytosis significantly, coupled with the finding that inhibitors of phospholipase A_2 had no effect on either secretion or arachidonic acid release, provides compelling evidence that phospholipase A_2 is not involved either in exocytosis or in the regulation of free arachidonic acid levels in permeabilized bovine adrenal chromaffin cells. Support for this conclusion comes from the observation that isolated bovine chromaffin-cell plasma membranes contain no observable phospholipase A_2 activity [29]. Although our conclusions on the role of phospholipase A_2 in arachidonic acid release and exocytosis directly contradict those of Frye & Holz [11], the discrepancy can be explained by the different nature of phospholipase A_2 inhibitors used. The protein-alkylating agents NEM and BPB used by Frye & Holz [11] act by covalently binding to nucleophilic groups such as thiol and amine groups, and so would be expected to interfere with the function of a wide range of proteins in the permeabilized cell [24]. Thus, any conclusions on the role of phospholipase A_2 drawn from the use of these inhibitors alone can be misleading and should be regarded with scepticism, a point commendably raised by the authors [11].

Although it is often assumed that the release of arachidonic acid is mediated by phospholipase A_2 activation, an alternative pathway exists involving activation of phospholipase C, whereby released diacylglycerols are sequentially deacylated by diacylglycerol lipases and monoacylglycerol lipases to yield non-esterified fatty acids [30]. The published work on chromaffin cells suggests that the latter pathway plays a major role in the release of arachidonic acid, since phospholipase C is activated by micromolar Ca^{2+} in digitonin-permeabilized cells [31] and the generation of arachidonic acid in isolated plasma membranes is dependent on the activity of both diacylglycerol and monoacylglycerol lipases [27]. In the present study, the diacylglycerol lipase inhibitor RG80267 was found to affect only the basal level of arachidonic acid. Thus it can be concluded that the breakdown of diacylglycerol plays a major role in determining the level of free arachidonic acid in permeabilized chromaffin cells in the absence of Ca^{2+}, but that micromolar Ca^{2+} acts in some way to modulate yet another pathway for arachidonic acid mobilization. Although RG80267 was without effect on Ca^{2+}-dependent arachidonic acid release, it inhibited basal release to such an extent that the level of arachidonic acid in treated cells in response to 10 μM-Ca^{2+} was equivalent to that seen in control cells in the absence of Ca^{2+}. This demonstrates that the increased level of free arachidonic acid in response to Ca^{2+} is not essential for exocytosis, since under identical conditions 10 μM-Ca^{2+}-induced secretion was unaffected. These data alone do not rule out the possibility that the increase in free arachidonic acid seen in response to Ca^{2+} is itself essential for exocytosis. Our results using staurosporine, however, rule out any such possibility.

Previous reports have demonstrated that activators of protein kinase C, such as phorbol esters and diacylglycerol, enhance the release of arachidonic acid due to Ca^{2+} in platelets [32], neutrophils [33], HL60 cells [34] and RBL-2H3 cells [35]. Indeed, the phorbol ester PMA has been shown to potentiate both Ca^{2+}-dependent secretion and arachidonic acid release in digitonin-permeabilized chromaffin cells [11]. In the present study, the effects of inhibitors, as opposed to activators, of protein kinase C were investigated. It was found that 30 μM-staurosporine completely abolished Ca^{2+}-dependent arachidonic acid release, whereas Ca^{2+}-dependent exocytosis was inhibited by only 17%. This result indicates that the increase in arachidonic acid release which accompanies secretion is not essential for Ca^{2+}-dependent

exocytosis, and thus represents the first dissociation of the processes of exocytosis and arachidonic acid release in chromaffin cells. We have previously shown that the stimulatory effects of GTP analogues on secretion from permeabilized chromaffin cells are not mediated by increased arachidonic acid release [20]. Therefore, arachidonic acid release can at most have only a minor role in the regulation of exocytosis from digitonin-permeabilized bovine adrenal chromaffin cells. However, a recent publication [19] has reached the opposite conclusion, i.e. that arachidonic acid is essential for secretion in bovine adrenal chromaffin cells. However, that work used intact cells, which are dependent on a nicotinic-agonist-mediated Ca^{2+} influx for exocytosis to occur. Since those authors have not monitored the effects of their drugs on cytosolic Ca^{2+} levels, their results may indicate an inhibition of Ca^{2+} entry rather than an effect distal to this step, i.e. exocytosis. This problem is not encountered in the digitonin-permeabilized cell system used in our study, where receptors and ion channels are by-passed and secretion is elicited by micromolar Ca^{2+} alone, thus allowing direct access to the exocytotic machinery.

Inhibitors of protein kinase C are notoriously non-specific, and staurosporine is no exception, in that it is known to be capable of inhibiting cyclic AMP-dependent protein kinase [28]. The effect of this drug on arachidonic acid release seems unlikely to be due to an inhibition of cyclic AMP-dependent protein kinase, however, since addition of exogenous cyclic AMP had no effect on arachidonic acid release (results not shown). Further evidence that staurosporine acts via protein kinase C inhibition comes from the observation that down-regulation of protein kinase C by overnight incubation with 1 μM-PMA results in a total inhibition of Ca^{2+}-dependent arachidonic acid release, but only a 50% inhibition of exocytosis (results not shown). Thus Ca^{2+}-dependent arachidonic acid release is completely dependent on protein kinase C activation, but is not essential for exocytosis.

In addition to dissociating the processes of arachidonic acid release and exocytosis, the results of this study provide clues to the pathway(s) involved in the generation of arachidonic acid in this cell type. Phospholipase A_2 can be ruled out, since its inhibitors had no effect on arachidonic acid release. Metabolism of diacylglycerol appears to play a major role in the basal production of arachidonic acid, but not in its production in response to Ca^{2+}. It is known that the activities of arachidonoyl-CoA synthetase and acyl-CoA: lysophosphatide acyltransferase (acyl-CoA: 1-acyl-*sn*-glycero-3-phosphocholine acyltransferase) in many cell types exceed the activity of phospholipase A_2 [36]. This has led to the suggestion that these enzymes, which act to re-incorporate arachidonic acid into phospholipids, may regulate the level of free arachidonic acid in the cell [30]. A recent paper by Fuse *et al.* [37] demonstrates that protein kinase C activation by PMA results in the inactivation of both arachidonoyl-CoA synthetase and arachidonoyl-CoA: lysophosphatide acyltransferase. They interpret this result as providing evidence that the PMA-induced increase in arachidonic acid release from platelets is due to a protein kinase C-induced inhibition of these enzymes, resulting in a decrease in arachidonic acid re-incorporation and hence a net increase in free arachidonic acid in the cell. Clearly the results of the present study, where Ca^{2+}-dependent arachidonic acid release appears to involve activation of neither phospholipase A_2 nor phospholipase C, but is completely dependent on protein kinase C activation, are consistent with this hypothesis. Thus, in the chromaffin cell, Ca^{2+} may act to increase the level of free arachidonic acid not by directly stimulating phospholipid hydrolysis, but (through protein kinase C) by inhibiting the basal re-incorporation of arachidonic acid into phospholipids. The physiological role of this increase in the cellular level of free arachidonic acid is unknown.

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