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Ca^{2+} and phorbol ester effect on the mast cell phosphoprotein induced by cromolyn

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Abstract

Several phosphoproteins are involved in stimulus-secretion coupling. The β and γ subunits of immunoglobulin E binding protein (FcεRI) and three other protein bands get phosphorylated during stimulation of mast cell secretion. These additional proteins of 42, 59 and 68 kDa are also phosphorylated when secretion is stimulated by compound 48/80 (C48/80). A 78 kDa band, however, is phosphorylated as secretion wanes after stimulation with C48/80 and by the anti-allergic drug disodium cromoglycate (cromolyn). Phosphorylation was blocked by protein kinase C inhibitors. We investigated the isozyme involved by first showing that a cation ionophore prevented the phosphorylation of the 78 kDa protein, while a Ca^{2+} chelator did not affect phosphorylation even though it enhanced the inhibitory effect of cromolyn. This protein was identified as moesin by immunoprecipitation. Protein kinase C activators had no effect on 78 kDa protein phosphorylation either in the presence or absence of Ca^{2+} ions, but prevented its phosphorylation by cromolyn. Protein phosphatase inhibitors prolonged the duration, but not the amount of phosphate incorporated in the 78 kDa protein band while cromolyn had no effect on protein phosphatase action in vitro. The insensitivity of the 78 kDa protein phosphorylation to calcium and protein kinase C activators suggests that an atypical protein kinase C isozyme may be involved. Western blot analysis identified the presence of isozymes α, β, δ and ζ, of which only the latter fits the profile suggested by the present findings.

Keywords: Exocytosis; Inhibition; Mast cell; Moesin; Protein kinase C; Phosphatase; Phosphorylation; Secretion

1. Introduction

Increasing evidence indicates that protein phosphorylation plays a key role in the control of cell function (Hunter, 1995). For instance, phosphoproteins such as synapsin I regulate neurosecretion through the fraction of the synaptic vesicles available for release (McCloskey and Cahalan, 1990; Tarelli et al., 1992; Greengard et al., 1993; Levitan, 1994). Immunologic stimulation of mast cells results in both tyrosine and serine/threonine phosphorylation of FcεRI (Beaven and Metzger, 1993; Scharenberg and Kinet, 1994). In particular, the *src*-related tyrosine kinases *lyn* and *syk* become sequentially activated following antigenic stimulation, leading to phosphorylation of the β and γ subunits of FcεRI and of the *syk* tyrosine kinase, itself (Scharenberg and Kinet, 1994). First, *lyn* seems to phosphorylate the β and γ subunits of FcεRI which cross-link allowing *syk* to bind and become phosphorylated by *lyn*. Phosphorylated *syk* then phosphorylates phospholipases which become translocated to the plasma membrane (Beaven and Metzger, 1993; Scharenberg and Kinet, 1994). Four other proteins with molecular mass of 42, 59, 68 and 78 kDa also become phosphorylated following stimulation with the mast cell secretagogue compound 48/80 (C48/80; Sieghart et al., 1978) or IgE and specific antigen (Wells and Mann, 1983). The first three proteins are phosphorylated within 10 s of challenge (Sieghart et al., 1978; Theoharides et al., 1981; Wells and Mann, 1983); of these, the 59 kDa phosphoprotein was identified as Vimentin, one of the intermediate filaments of cytoskeletal proteins (Izushi et al., 1992). The 78 kDa protein, however, is phosphorylated by 2 min when secretion is over and was postulated to possibly be involved in mast cell recovery (Sieghart et al., 1978). Phosphorylation of this protein is also induced by the clinically available 'antiallergic' drug cromolyn in a manner which paralleled the dose–response and time course of its inhibition of mast

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cell secretion (Theoharides et al., 1980). These findings led to the premise that this protein may be involved in inhibition of mast cell secretion (Theoharides et al., 1980). We identified the 78 kDa phosphoprotein as moesin (Correia et al., 1996) a protein thought to link the plasma membrane to the cytoskeletone (Furthmayr et al., 1992) and showed that its phosphorylation in mast cells was prevented by inhibitors of protein kinase C, but not protein kinase A and protein kinase G (Correia et al., 1996). A recent paper reported that protein kinase C activators *inhibited* cromolyn-induced phosphorylation of the 78 kDa protein (Cox et al., 1998). They concluded that phosphorylation of the 78 kDa protein is not mediated by a phorbol ester sensitive protein kinase C, but that such an enzyme may regulate its dephosphorylation (Cox et al., 1998). The phosphorylation of the 78 kDa protein is now shown to be regulated by a Ca^{2+}-independent and phorbol ester-insensitive protein kinase C.

2. Experimental procedures

2.1. Materials

Antisera to protein kinase C isozymes and phosphatases were purchased from Upstate Biotechnology (Lake Placid, NY). Cromolyn, cation ionophore A23187, C48/80, 1,2-bis (2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′,-tetraacetic acid (BAPTA), phorbol myristate acetate (PMA), phorbol 12,13 dibutyrate (DPB), okadaic acid, cyclosporine and FK506 were purchased from Sigma (St. Louis, MO).

2.2. Mast cell purification

Rat peritoneal mast cells were obtained from 300 g male Sprague–Dawley rats (Charles River Labs, NY) and were purified (> 90%) over 22.5% metrizamide (Accurate Scientific Westbury, NY) as described previously (Theoharides et al., 1980).

2.3. Phosphorylation in vivo

Purified mast cells were loaded with carrier-free [^{32}] ortho phosphate (Pi) and treated with cromolyn as previously described (Theoharides et al., 1980). The cells were lysed and analyzed on 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), except when noted that the gel composition was 10%. Autoradiography was carried out for 24 h at −70°C.

2.4. Immunoprecipitation

Mast cell extracts were immunoprecipitated using a polyclonal antiserum to recombinant moesin as described previously (Correia et al., 1996). Mast cells were loaded with [^{32}]Pi, were treated with cromolyn for 1 min and then boiled for 5 min in 1% SDS. Double-strength inhibitor solution consisted of phosphate buffered saline (PBS) containing 20 mM NaPP, 100 mM NaF, 2 mM EGTA, 5% NP40, and protease inhibitors at a final concentration of 10 μg/ml); it was added to the sample at 1:1 dilution. Proteins in the lysate were precleared by incubation for 20 min with 50 μl of Protein A linked to Sepharose. The clear supernatant was transferred to 10 μl of the rabbit anti-rat moesin polyclonal antibody and incubated for 30 more minutes. To the reaction mixture, 50 μl of Protein A linked to Sepharose was added and incubated for another 30 min and spun at $10{,}000 \times g$ for 1 min. The pellet was washed twice with 1 ml of SII (150 mM NaCl, 15 mM Hepes, 1 mM EGTA, 0.5% NP-40, pH 7.4). To the pellet, 100 μl of solubilizing SDS buffer was added, boiled for 5 min, and analyzed by SDS-PAGE gels (Correia et al., 1996).

2.5. The effects of BAPTA on mast cell secretion

Mast cells were purified, suspended (2×10^6/ml) in Locke's buffer (Theoharides et al., 1980), loaded with [^{3}H] serotonin (5 μCi/ml) at 37°C for 1 h and washed twice with Locke's buffer. The cells were resuspend in Locke's buffer at 2×10^6/ml. A 500 μl aliquot of cell suspension (about 10^6 cells) was placed into Eppendorf tubes and BAPTA was added for 10 min at 37°C. C48/80 was then added to a final concentration of 0.5 μg/ml and the cells were incubated at 37°C for another 10 min. The samples were then centrifuged at 4°C and scintillation fluid was added to both supernatants and pellets and radioactivity was determined in a beta-counter. In order to establish the time course of the effect of BAPTA, mast cells were incubated at 37°C for the times indicated.

2.6. Immunoblot analysis of protein kinase C isozymes

Immunoblot analysis was carried out on purified rat peritoneal mast cells and rat basophil leukemia (RBL) cells grown as previously described (Tamir et al., 1982). Mast cells or RBL cells (10^6 cells per lane) were lysed, boiled immediately for 5 min, loaded on 7.5% SDS gels and analyzed by SDS-PAGE. Rabbit polyclonal antibodies (Upstate Biotechnology) to six different protein kinase C isoforms (α, β, γ, δ, ε and ζ) were used at a concentration of 1:1000. The corresponding MW blots (78 kDa) were cut in single lanes and were incubated overnight with 5 μl of the primary antibody (in 5 ml total volume), followed by 20 μl of the secondary antibody, goat anti-rabbit-horseraddish peroxidase (1:250 in 5 ml). Detection was with diaminobenzidine.

2.7. Interaction of BAPTA, PMA and cromolyn

Mast cells were purified, loaded with [^{32}]Pi at 37°C for 1 h, washed three times and resuspended in Locke's buffer

(without any bovine serum albumin or Ca^{2+}) as 10^6 cell/ml. BAPTA (4 mM) and PMA (0.1 μM) were added at 37°C for 5 min, following which cromolyn (0.2 mM) was added for 30 more seconds. SDS sample buffer was

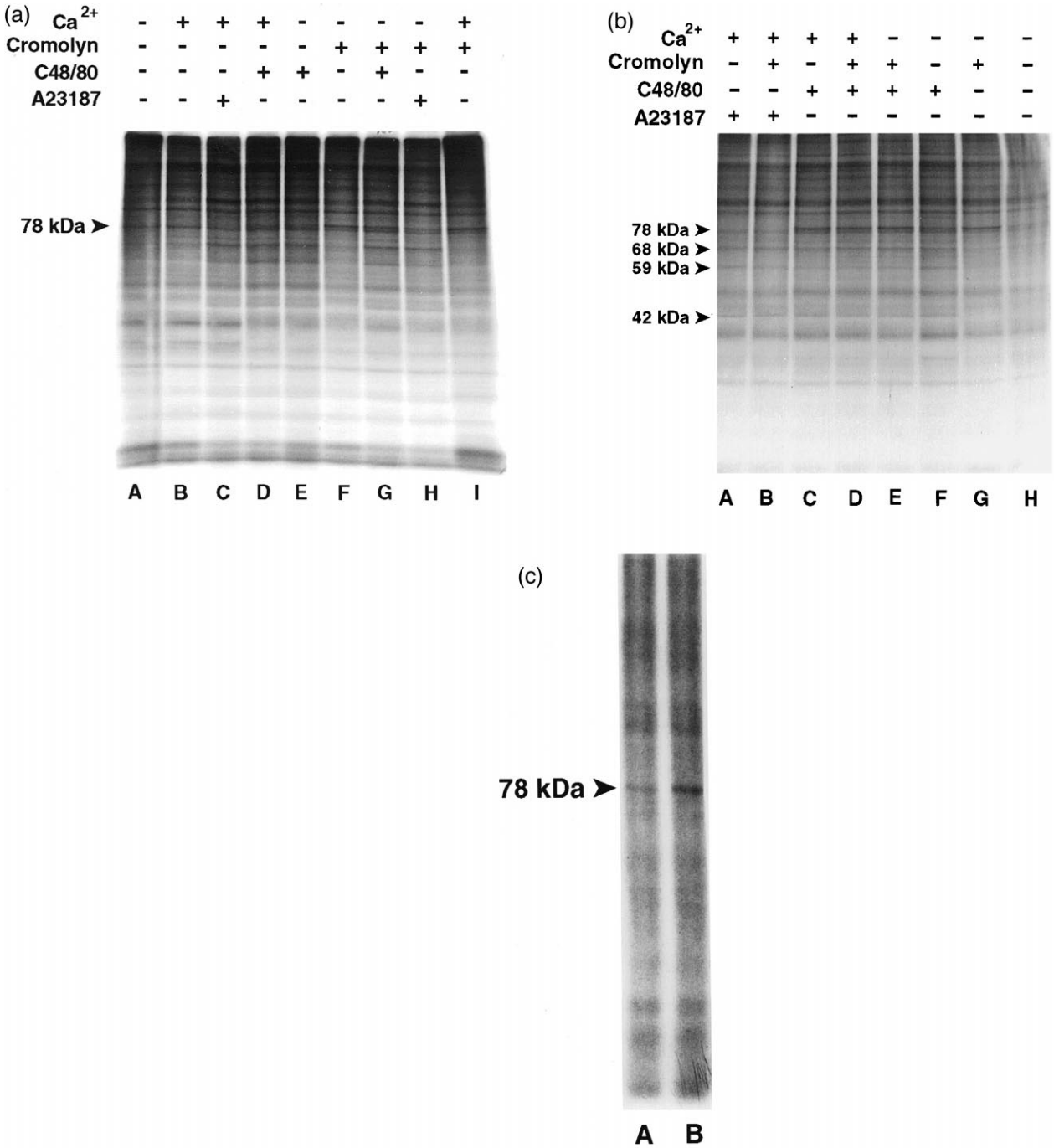

Fig. 1. (A) Effect of extracellular Ca^{2+} on phosphorylation of the 78 kDa protein by 0.2 mM cromolyn for 30 s ($n = 3$). Mast cells were prepared and treated as described in Methods except that the SDS-PAGE was 10%. A23187 was used at 0.1 μg/ml for 1 min and C48/80 at 0.5 μg/ml for 1 min. Cromolyn (0.2 mM) was used for 30 s followed by 0.1 μg/ml A23187 for one more minute or 0.5 μg/ml C48/80 for 1 min. No Ca^{2+} indicates that no extracellular Ca^{2+} was added and 0.1 mM EGTA was added. The description of the lanes is shown on the legend over the gel. Note there was no difference in the control with or without Ca^{2+} (lanes A and B) or in cromolyn treated cells with or without Ca^{2+} (lanes F and I) or the cromolyn treated cells. A23187 appears to substantially reduce phosphorylation of the 78 kDa protein (compare lanes F and H). (B) Effect of extracellular Ca^{2+} ions on phosphorylation of the 78 kDa protein ($n = 5$). The conditions of this figure are the same with those described for (A). Note that the presence or absence of Ca^{2+} has no effect on the phosphorylation induced either by C48/80 (compare lanes C and F) or by cromolyn (lane G). The presence of the cation ionophore A23187 reduced phosphorylation of the 78 kDa protein (compare lanes B and G). (C) Immunoprecipitation of the 78 kDa phosphoprotein with anti-moesin serum ($n = 3$). Mast cell extracts from [^{32}]Pi-labeled mast cells treated in situ without (A) or with (B) 0.2 mM cromolyn for 30 s and were processed for Western blot analysis. The arrow indicates the position of moesin.

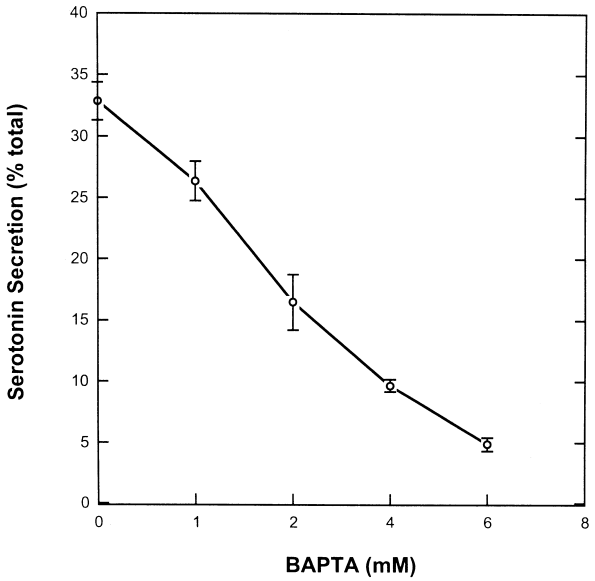

Fig. 2. Dose–response of the effects of BAPTA on mast cell secretion ($n = 5$). Mast cells were purified as before and were loaded (2×10^6/ml) with [^{3}H] serotonin (5 μCi/ml at 37°C for 1 h in Locke's buffer without Ca^{2+}. Cells were washed and incubated (10^6 cells/500 μl) at 37°C for 5 min with the designated concentrations of BAPTA. Secretion was then stimulated with C48/80 (0.5 μg/ml) at 37°C for an additional 1 min.

then added to the samples and they were boiled for 5 min for SDS-PAGE and autoradiography. In other experiments, PDBu or the inactive analog 4-α-PDBu (100 nM) were also added for 5 min at 37°C prior to the addition of cromolyn for 30 more seconds.

2.8. Treatment with phosphatase inhibitors

Purified mast cells were loaded with [^{32}]Pi as described before and were preincubated with 1 or 10 μM of okadaic acid for 1, 10 or 30 min prior to addition of cromolyn (0.2 mM) for 30 s at 37°C. In the case of the specific protein phosphatase-2B inhibitor, FK506 (1 μM) was added for 60 min and the status of phosphorylation of moesin was then assessed by autoradiography at 30 s and 5 min following the addition of cromolyn (0.2 mM).

2.9. Effect of cromolyn on phosphatase activity in vitro

Protein phosphatase-1, protein phosphatase-2A and protein phosphatase-2B activity was assayed by the addition of the respective enzymes (Upstate) and substrate in a final volume of 100 μl. Inorganic phosphate released from paranitrophenyl phosphate over 30 min at 37°C was colorimetrically assayed using an enzyme-linked immunosorbent assay (ELISA) reader at 410 nM.

2.10. Presentation of results

The number of experiments performed is indicated in the respective legend and results of serotonin release are shown as mean ± S.D.

3. Results

3.1. Effect of calcium ions on phosphorylation of the 78 kDa protein in rat mast cells

Phosphorylation of the 78 kDa protein was induced in purified mast cells with 0.2 mM cromolyn for 1 min in the presence or absence of Ca^{2+} (Fig. 1A). C48/80 also induced phosphorylation of the 78 kDa protein band, along with protein bands of 42, 59 and 68 kDa, both in the presence and absence of extracellular Ca^{2+} (Fig. 1B, compare lanes C and F); in this series of experiments, cromolyn again induced phosphorylation in the absence of extracellular Ca^{2+} (Fig. 1, compare lanes G and H). Simultaneous addition of cromolyn (Fig. 1, lanes 4 and 5) and C48/80 could not increase the phosphorylation of the 78 kDa protein over that seen with C48/80 alone (Fig. 1, compare lanes C, D, E and G). The cation ionophore A23187 (0.1 μg/ml for 1 min) which is a powerful stimulus of mast cell secretion, induced phosphorylation of the 42, 59 and 68 kDa proteins associated with secretion, but *not* of the 78 kDa protein (Fig. 1, lane A). Moreover, 0.2 mM cromolyn for 1 min could not induce phosphorylation of the 78 kDa protein in the presence of A23187 (compare Fig. 1A (lanes F and H) and B (lanes B and G)), suggesting that excess of Ca^{2+} ions may prevent phosphorylation of the 78 kDa protein. In fact, A23187 appeared to decrease base line phosphorylation of the 78 kDa protein occasionaly seen in control (Fig. 1B, compare lanes A and H). Immunoprecipitation showed that the band phosphorylated by cromolyn was moesin (Fig. 1C).

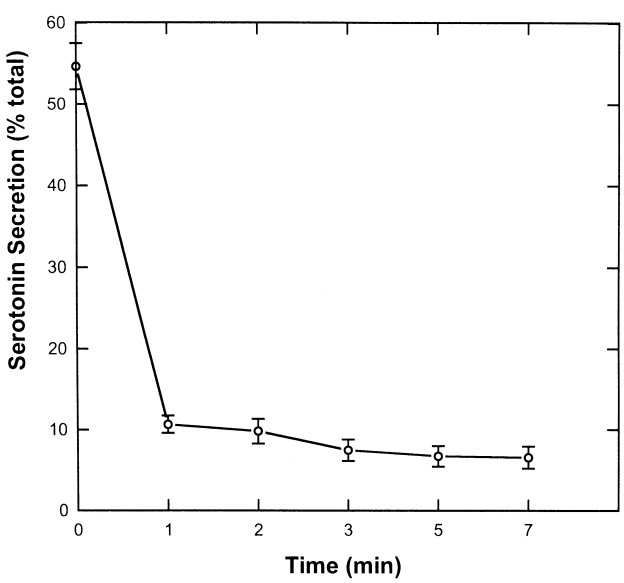

Fig. 3. Time course of the effect of BAPTA on mast cell secretion ($n = 5$). Mast cells were prepared as in Fig. 2 above except that they were incubated at 37°C for the times indicated with BAPTA at a final concentration of 4 mM. They were then stimulated by C48/80 (0.5 μg/ml) for 1 min at 37°C.

In order to further investigate the requirement for Ca^{2+} ions, we used BAPTA which is a specific Ca^{2+} ion chelator (Tsien, 1980). Incubation of mast cells with different concentrations of BAPTA for 5 min (Fig. 2) inhibited mast cell secretion in response to 48/80, a process known to require intracellular Ca^{2+} ions. This inhibition was complete when 4 mM BAPTA was used for more than 2 min (Fig. 3). Phosphorylation of the 78 kDa protein was then examined in mast cells pretreated with 4 mM BAPTA for 5 min prior to addition of cromolyn (0.2 mM). The phosphorylation was unaffected under conditions during which mast cell secretion was effectively blocked (Fig. 4). In other words, the 78 kDa protein must have been phosphorylated by a Ca^{2+}-independent protein kinase. In fact, BAPTA pretreatment potentiated the inhibitory effects of cromolyn on mast cell secretion (Fig. 5) and on phosphorylation of the 78 kDa protein (Fig. 6). These results support our findings with A23187 that a Ca^{2+}-dependent process may be either antagonizing the ability of cromolyn to phosphorylate the 78 kDa protein, or inactivating it by promoting its dephosphorylation.

3.2. Effect of phorbol esters on in vivo phosphorylation of the 78 kDa protein

To further investigate the possible involvement of PKC in the phosphorylation of the 78 kDa protein, PMA was used. PMA itself could not induce the phosphorylation of moesin either in the presence, or absence of Ca^{2+} ions

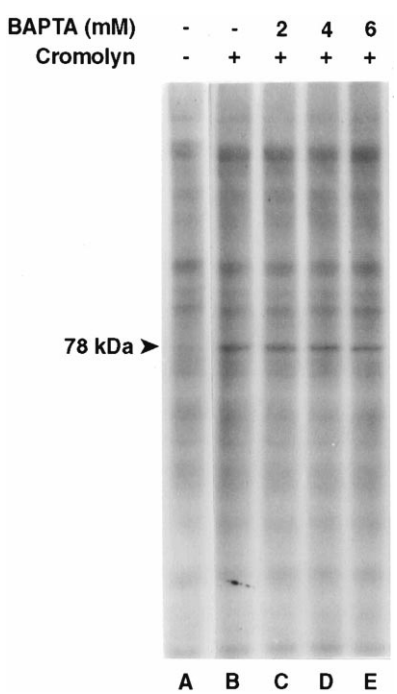

Fig. 4. Effect of BAPTA on phosphorylation of the 78 kDa protein ($n = 5$). Mast cells were prepared as in Fig. 3 above except that they were incubated at 37°C for 5 min with 2, 4 or 6 mM BAPTA prior to addition of cromolyn (0.2 mM) at 37°C for 30 s.

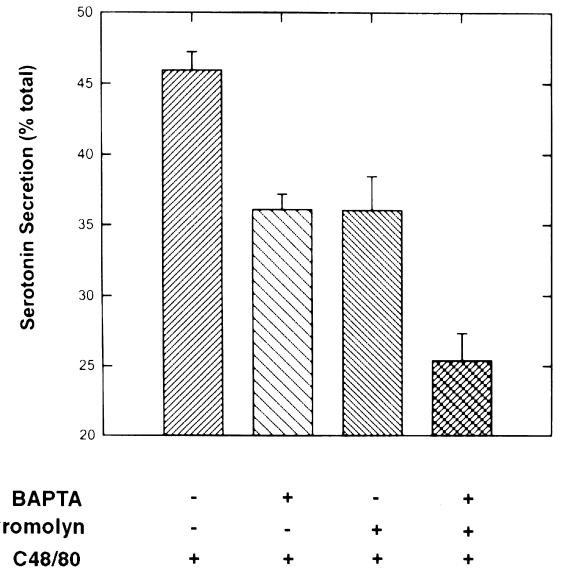

Fig. 5. The effect of combining BAPTA and cromolyn on mast cell secretion ($n = 5$). Mast cells were prepared as in Fig. 3 above, except that they were first treated with BAPTA (4 mM) for 5 min at 37°C or cromolyn (0.2 mM) at 37°C for 30 s or both. They were then stimulated in each case by C48/80 (0.5 μg/ml) for 1 min at 37°C. Note that increasing concentrations of BAPTA did not affect phosphorylation of the 78 kDa protein (compare lanes B–E).

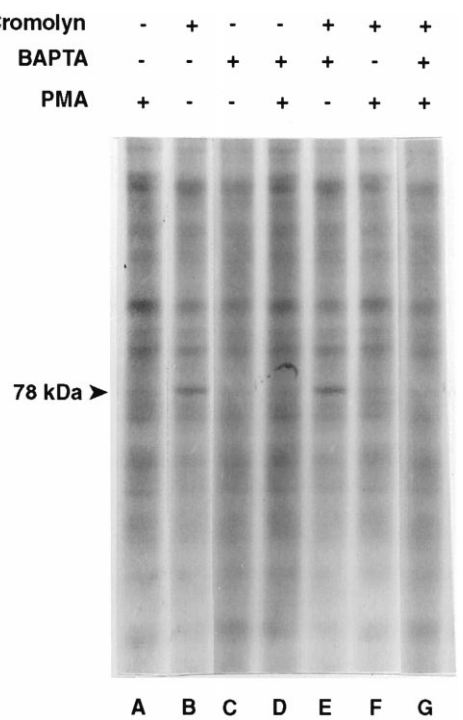

Fig. 6. The effect of PMA, with or without BAPTA, on the phosphorylation of the 78 kDa protein ($n = 5$). Mast cells were prepared as in Fig. 5 above, but were treated with or without BAPTA (4 mM) or PMA (0.1 FM) for 5 min at 37°C before addition of cromolyn (0.2 mM) at 37°C for 30 s. Note that PMA or BAPTA alone had no effect on the phosphorylation of the 78 kDa protein. The addition of PMA prevented phosphorylation of the 78 kDa protein in response to cromolyn (compare lanes B and F or lanes E and G).

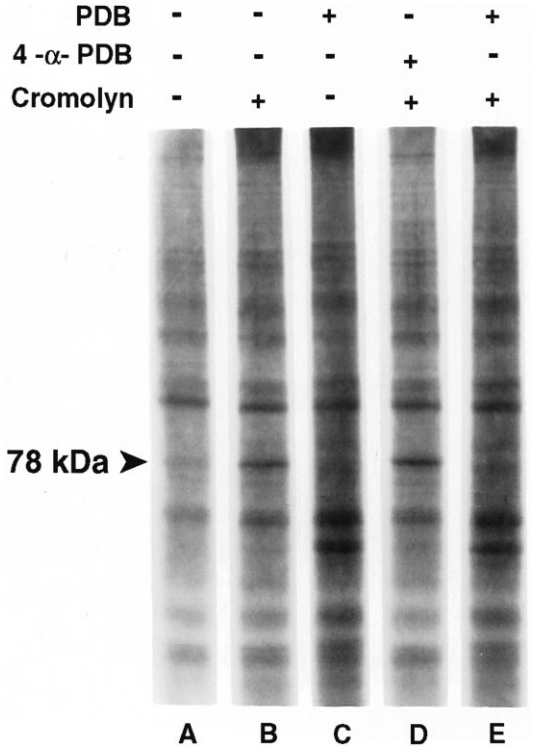

Fig. 7. The effect of phorbol esters on the phosphorylation of the 78 kDa protein ($n = 5$). Radiolabeled mast cells were pretreated with PDB (100 nM) or the negative control 4-α-PDB (100 mM) for 5 min and were then treated with cromolyn (0.2 mM) for 30 s at 37°C. Note that the presence of PDB prevented phosphorylation of the 78 kDa protein in response to cromolyn (compare lanes B and E), while this inhibition was not present in the case of the inactive 4-α-PDB (compare lanes B and D).

using BAPTA as before (Fig. 6). On the contrary, pretreatment of mast cells with PMA prevented cromolyn from phosphorylating the 78 kDa protein whether in the presence or absence of Ca^{2+} ions (Fig. 6). In order to determine whether the prevention of phosphorylation was a specific effect, the phorbol ester PDB with its appropriate negative control (4-α-PDB) was also used. Just like PMA, PDB alone did not induce phosphorylation of the 78 kDa protein. Pretreatment of mast cells with PDB for 5 min, followed by cromolyn, however, showed a rapid down-regulation of the subsequent phosphorylation of the 78 kDa protein (see Fig. 6), as described for PMA above. The inactive PDB analogue had no effect, demonstrating that the down-regulation of the phosphorylation of the 78 kDa protein was a specific effect.

We then investigated whether the effect of PMA observed was possibly due to activation of a Ca^{2+}-dependent protein kinase C which, in turn, activated the secretory pathway to override the phosphorylation of the 78 kDa protein. Mast cells were first treated with the specific intracellular calcium chelator BAPTA to prevent a Ca^{2+}-dependent protein kinase from being activated and were then stimulated with PMA. However, PMA still prevented the phosphorylation by cromolyn even in the presence of BAPTA (Fig. 7), suggesting that PMA's inhibitory action was not due to early activation of secretion or activation of Ca^{2+}-dependent kinase or phosphatase. Another possibility could be that PMA stimulates a Ca^{2+}-independent protein kinase C isozyme which activates some phosphatase rapidly leading to dephosphorylation of the 78 kDa protein. This possibility was investigated by pretreatment of mast cells with phosphatase inhibitors (see Section 3.3).

3.3. Effect of phosphatase inhibitors on the phosphorylation of the 78 kDa protein in vivo

Radiolabeled mast cells were pretreated with okadaic acid (10 μM) in the absence of extracellular Ca^{2+} for 1 and 10 min at 37°C. The cells were lysed with the sample buffer and analyzed by SDS-PAGE. A number of substrates, including the 78 kDa protein, incorporated more radiolabeled phosphate with increasing dose and time of preincubation with okadaic acid (Fig. 8). There was rapid incorporation of phosphate in a 55 kDa protein band, seen as early as 1 min with maximal incorporation by 10 min. The role of calcineurin (protein phosphatase-2B) was also assessed with the use of FK506, an immunosuppressant which is a potent inhibitor of this phosphatase. Mast cells

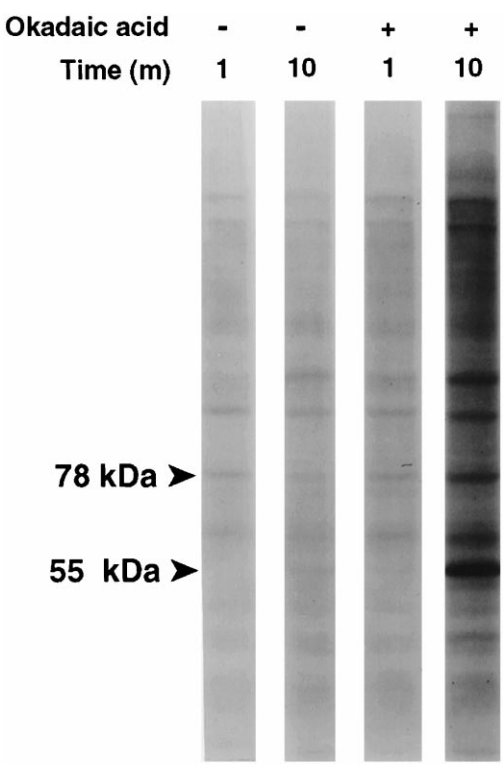

Fig. 8. Effect of okadaic acid on phosphorylation of the 78 kDa protein ($n = 5$). Radiolabeled mast cells were treated with 10 FM okadaic acid for 1 or 10 min prior to addition of cromolyn (0.2 mM) for 30 s at 37°C. Note that the addition of ocadaic acid greatly increased the incorporation of radioactive phosphate in a number of protein bands including the 78 kDa one.

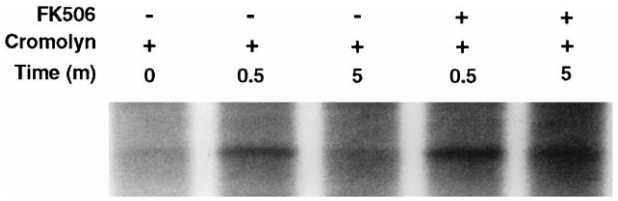

Fig. 9. The effect of FK506 on the phosphorylation of 78 kDa protein. Radiolabeled mast cells were pretreated with FK506 (1 μM) for 60 min and were then treated with cromolyn (0.2 mM) for 0.5 and 5 min. Note that the phosphorylation of the 78 kDa protein is still evident at 5 min in the presence of FK506.

were pretreated with FK506 (1 μM) for 1 h and then treated with cromolyn for 0.5 and 5 min. FK506 apparently decreased dephosphorylation since phosphorylation of the 78 kDa protein was still apparent at 5 min (Fig. 9). Both Ca^{2+}-dependent and -independent phosphatases may, therefore, be involved in the dephosphorylation of the 78 kDa protein.

3.4. Effect of cromolyn on protein phosphatase activity in vitro

A serine/threonine assay kit was used to investigate the effect of cromolyn on phosphatase activity directly in microtiter plates using *p*-nitrophenyl phosphate as substrate. Known inhibitors were used as positive controls and included okadaic acid for protein phosphatase-1 and protein phosphatase-2A, as well as cyclosporine for protein phosphatase-2B. Cromolyn even at 100 μM failed to inhibit any of these enzymes (results not shown) suggesting that cromolyn is not a direct phosphatase inhibitor, at least in vitro.

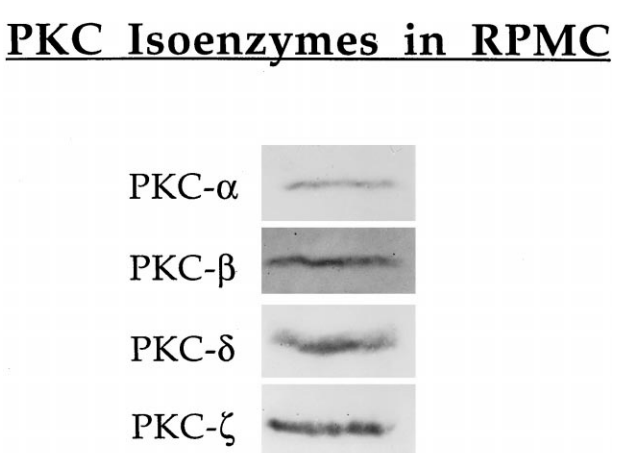

Fig. 10. Immunoblot analysis of protein kinase C isozymes in rat peritoneal mast cells ($n = 6$). Purified mast cells (10^6 cells per lane) were lysed and immediately boiled for 5 min; they were then loaded on 10% SDS gels and were analyzed as described in Section 2. The separated proteins were then blotted on nitrocellulose for 20 min at 15 V. The blots were cut in single lanes and were incubated overnight with the respective antisera.

3.5. Presence of PKC isozymes in rat mast cells

The presence of protein kinase C isozymes was investigated using Western blot analysis in purified rat peritoneal mast cells. They were shown to contain the α, β, δ and ζ isozymes (Fig. 10). The γ and ϵ isozymes could not be identified in rat peritoneal mast cells even though the ϵ isozyme was present in RBL cells (results not shown).

4. Discussion

We previously suggested that phosphorylation of moesin by cromolyn may be possibly mediated by protein kinase C (Correia et al., 1996) because sphingosine (100 μM), staurosporine (0.1 μM) and chelerythrine (100 μM) inhibited the phosphorylation of the 78 kDa protein (Correia et al., 1996). The concentrations of these inhibitors used were high and implied the possible involvement of a rather insensitive isozyme (Zhou et al., 1994). Phosphorylation of the 78 kDa protein by cromolyn is now shown to be independent of extracellular Ca^{2+} ions. Depletion of intracellular Ca^{2+} ions was assured by the use of the effective Ca^{2+} chelator BAPTA (Tsien, 1980) and was documented by showing that mast cell secretion was totally inhibited at the time cromolyn could still induce the phosphorylation of the 78 kDa protein. Our results (a summary of which is shown in Table 1) demonstrated that treatment of mast cells with BAPTA *enhanced* cromolyn's ability to induce the phosphorylation of the 78 kDa protein. It was also shown that an overabundance of intracellular Ca^{2+} ions by

Table 1
Summary of results

Drug	Conditions		Results	
	Cromolyn	Calcium	Effect on secretion	Effect on 78 kDa phosphorylation
None	+	+	none	↑
None	+	−	none	↑
C48/80	−	+	↑	↑
C48/80	−	−	↑	↑
C48/80	+	+	↓	↑
C48/80	+	−	↓	↑
A23187	−	+	↑	none
A23187	−	−	none	none
A23187	+	+	↑	↓
PMA	−	−	± ↑	none
PMA	+	−	?	↓
OA	−	−	none	none
OA	+	−	none	↑
OA + C48/80	−	+	none	none
OA + C48/80	−	−	none	none
OA + PMA	−	−	↑	none
OA + PMA	+	−	?	?
FK506	+	−	↓	↑ duration

+ = presence.
− = absence; in the case of Ca^{2+}, it means 0.1 mM EGTA was added to the medium.

the use of the cation ionophore A23187 *prevented* cromolyn from phosphorylating the 78 kDa protein. These results suggest that a Ca^{2+}-dependent process may be counteracting the Ca^{2+}-independent phosphorylation of the 78 kDa protein. Several isozymes of protein kinase C have been identified in RBL cells, a cell line resembling mucosal mast cells (Seldin et al., 1985); these include the Ca^{2+}-dependent α, β, and the calcium-independent δ, ϵ, and ζ isozymes (Ozawa et al., 1993a). Isozymes β and δ appear to facilitate secretion, while α and ϵ are associated with inhibition secretion (Ozawa et al., 1993b). Since the 78 kDa protein is phosphorylated by cromolyn which inhibits secretion, and since isozyme ϵ could not be detected in mast cells, it is likely that isozyme ζ may be involved in the phosphorylation of the 78 kDa protein by cromolyn. This possibility is strengthened by the fact that phorbol esters did not induce the phosphorylation of the 78 kDa protein, implying that phosphorylation of the 78 kDa protein is mediated by an isozyme that is insensitive to stimulation by PMA, such as ζ (Zhou et al., 1994).

In fact, phorbol esters prevented or downregulated phosphorylation of the 78 kDa protein. Phorbol ester inhibition of the cromolyn-induced phosphorylation of 78 kDa protein was also observed independently (Cox et al., 1998). These authors concluded that a phorbol ester sensitive protein kinase C isozyme may be involved in the regulation of the dephosphorylation of the 78 kDa protein. One possibility could be that PMA stimulates the secretory mechanism which overrides the phosphorylation or makes the 78 kDa protein inaccessible for phosphorylation. However, this possibility seems unlikely because phorbol esters prevented the phosphorylation of moesin even when BAPTA had inhibited mast cell secretion. Moreover, PMA is not very effective in inducing mast cell secretion anyway (Sagi-Eisenberg, 1985). A more likely possibility is that phorbol esters stimulate a Ca^{2+}-independent protein kinase C isozyme (other than ζ), such as δ which then activates some phosphatase. This possibility is supported by our findings that pretreatment with phosphatase inhibitors prolonged the duration of phosphorylation of the 78 kDa protein by cromolyn, which does not inhibit phosphatase activity in vitro. Besides, it is known that phosphatase inhibitors do not inhibit kinase activity (Haystead et al., 1989). Taken together, our results indicate that both Ca^{2+}-dependent and -independent phosphatases may be involved in the dephosphorylation of the 78 kDa protein. Alternatively, another protein kinase may be involved in spite of the fact that this 78 kDa phosphoprotein has protein kinase C active sites (Correia et al., 1996).

Two previous reports suggested that okadaic acid inhibits immunologic but not non-immunologic mediated secretion from rat mast cells (Estévez et al., 1994) and human lung mast cells (Peirce et al., 1997). Our results indicate that okadaic acid *does not* inhibit rat mast cell secretion induced by C48/80 (results not shown). Similar findings had been reported previously (Botana et al., 1992). A phosphatase critical for the induction of secretion has been proposed by Lillie and Gomperts (1993) and activation of a phosphatase by a heterometric G protein downstream from protein kinase C was shown by Aridor et al. (1993) to activate exocytosis. Our results, however, do not support their suggestion that the action of cromolyn may be mediated by a direct inhibition of a calcium/phospholipid-dependent protein kinase C involved in stimulation of mast cell secretion (Sagi-Eisenberg, 1985). Our hypothesis is that cromolyn possibly activates a Ca^{2+}-independent and phorbol ester insensitive protein kinase isozyme such as ζ which then triggers phosphorylation of the 78 kDa protein directly or together with concurrent inhibition of some phosphatase. This premise could also explain the ability of C48/80 to both stimulate mast cell secretion *and* phosphorylation of the 78 kDa protein. In other words, C48/80 may act by first rapidly activating a Ca^{2+}-independent phosphatase (Botana et al., 1992), thus preventing or down-regulating phosphorylation of the 78 kDa protein, while also stimulating protein kinase C isozyme ζ to later phosphorylate the 78 kDa protein, an action that may be associated with termination of secretion. This possibility is supported by the fact that mast cell secretion in response to IgE and Ag was also associated with phosphorylation of the 78 kDa protein band (Wells and Mann, 1983).

Here, immunoprecipitation showed that the 78 kDa protein phosphorylated in the absence of extracellular Ca^{2+} by cromolyn was in fact moesin. The 78 kDa mast cell phosphoprotein had been purified (Correia et al., 1996) and the peptide sequence of two fragments was shown to have 100% homology to mouse and 97% to human moesin, respectively (Lankes and Furthmayr, 1991). Rat moesin was subsequently cloned and shown to have 90% sequence similarity to human moesin (Wang et al., 1995). Moesin was named for its involvement in the extension of filopodia (membrane-organizing extension spike protein, moesin; Furthmayr et al., 1992) and has high similarity with ezrin, radixin and merlin which belong to the erythrocyte band 4.1 superfamily, considered to link the plasma membrane to cytoskeletal components (Furthmayr et al., 1992). The fact that inhibition of mast cell secretion involved phosphorylation of moesin's Ser^{56}, Ser^{74} and Ser^{374} (Correia et al., 1996), while stimulation of platelet secretion resulted in moesin's phosphorylation only of Th^{558} (Nakamura et al., 1995) indicates that different phosphorylation sites may be associated with different functions (Pietromonaco et al., 1998). A phosphoprotein of 73 kDa and its kinase were recently isolated from acute myelogenous leukemia cells. This protein was identified as moesin and phosphorylation was mapped to a single Th^{558} of the actin binding domain of moesin (Pietromonaco et al., 1998). The kinase with a Mn^{2+} dependence and phosphatidylglycerol preference, was identified by immunodepletion to be protein kinase C-θ. Preliminary results indicate that this isozyme is absent in rat mast cells (results not shown).

The present results suggest that phosphorylation of the 78 kDa protein, possibly on serine residues, may lead to inhibition of secretion, while phosphorylation of other sites such as Th^{558} may be associated with stimulation of secretion. Conclusive evidence will have to await results using point mutations and knock-outs for moesin.

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