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Pertussis toxin does not affect the time course of exocytosis in mast cells stimulated by intracellular application of GTP-γ-S

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Exocytosis was studied in single rat peritoneal mast cells. Granule fusion was monitored by time-resolved capacitance measurements using the patch-clamp technique. Intracellular stimulation of mast cells with 20 μM GTP-γ-S stimulates exocytosis with a calcium-dependent time course. Secretion in response to receptor-mediated stimulation with compound 48/80 was completely abolished by treatment with pertussis toxin (IAP) at 180 ng/ml for 4 h. The time course of exocytosis in response to GTP-γ-S remained unaffected in IAP-treated cells supporting the involvement of a second GTP-binding protein in stimulus-secretion coupling.

Mast cell; Exocytosis; Ca^{2+}; G-protein; Islet-activating protein; Patch-clamp; (Pertussis toxin)

1. INTRODUCTION

Mast cells secrete histamine and other mediators in response to various receptor-directed stimuli like antigen or polycations. Secretion has also been observed after elevating intracellular calcium by calcium ionophores [1] or permeabilisation with ATP^{4-} [2]. The receptors can also be bypassed by introducing non-hydrolysable GTP analogs into the cells, which stimulate exocytosis in a concentration-dependent manner [3–5]. An early event following receptor-directed stimulation is the activation of a polyphosphoinositide phospholipase C [6], which can also be stimulated by GTP-γ-S [7].

It has been reported that exposure of rat mast cells to IAP, pertussis toxin, simultaneously inhibits inositol phospholipid breakdown, arachidonic acid release and histamine secretion in mast cells stimulated by compound 48/80 [6]. We have used the patch-clamp technique to monitor exocytosis [8] in response to extracellular stimulation with the polyamine compound 48/80 and to intracellular stimulation with GTP-γ-S. The time course of exocytosis in response to GTP-γ-S depends linearly on the inverse GTP-γ-S concentration [7]. We now demonstrate that the time course is also affected by the intracellular calcium concentration. In contrast to previous results [9] we found that IAP-treated cells which lost their responsiveness to stimulation with compound 48/80 still respond normally to intracellular application of GTP-γ-S. This result supports the existence of a second GTP-binding protein G_E [10,11] which directly controls exocytosis in mast cells.

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Abbreviations: IAP, islet-activating protein; GTP, guanosine 5′-triphosphate; GTP-γ-S, guanosine 5′-*O*-(3-thiotriphosphate)

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2. MATERIALS AND METHODS

Rat peritoneal mast cells were obtained by peritoneal lavage of large male Wistar rats with buffer A (143 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 45 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 10 mM Hepes-NaOH, pH 7.2, 9 mM glucose). The resulting cell suspension was used without further purification. About 200 μl of the suspension was placed in petri dishes with a glass bottom and after settling of the cells 2–3 ml buffer A or buffer A containing 200 ng/ml IAP was added giving a final IAP concentration of about 180 ng/ml. The cells were then kept in an incubator under 7% CO_2, 93% air at 37°C in a humidified atmosphere.

Time-resolved patch-clamp capacitance measurements were made by analysis of the current relaxations in response to voltage pulses as described [8]. The response to stimulation with compound 48/80 was measured in the slow-whole-cell configuration [12]. In these experiments the pipette contained 125 mM K-glutamate, 30 mM NaCl, 500 μM EGTA, 10 mM Hepes-NaOH, pH 7.2, and 20 μM Na_2ATP. Intracellular stimulation with GTP-γ-S was performed in the fast-whole-cell configuration with a pipette solution containing 125 mM K-glutamate, 10 mM NaCl, 7 mM $MgCl_2$, 5 mM EGTA, 4.5 mM $CaCl_2$, 200 μM Na_2ATP, 20 μM GTP-γ-S and 10 mM Hepes-NaOH, pH 7.2, if not otherwise indicated. The bath solution was always buffer B (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes-NaOH, pH 7.2, 25 mM glucose). All experiments were carried out at room temperature.

IAP was from Sigma, GTP and GTP-γ-S from Boehringer Mannheim. In some experiments HPLC-purified GTP-γ-S (a gift from Dr Fritz Eckstein, Göttingen) was used. Only minor differences were observed between results obtained with purified GTP-γ-S and the particular batch of non-purified GTP-γ-S used in the present experiments.

3. RESULTS

Degranulation of rat peritoneal mast cells is associated with a pronounced morphological change which can be easily observed under a light microscope equipped with Nomarski optics [12]. Cells which were incubated in the presence of 180 ng/ml IAP for at least 4 h showed no signs of degranulation under the microscope after stimulation with compound 48/80 at 0.5 or 5 μg/ml. At this time all the control cells were fully responsive (5 experiments). The fusion of secretory granules with the plasma membrane leads to an increase in plasma membrane area which is associated with a proportional increase in membrane capacitance. This capacitance change can be measured in single mast cells using the patch-clamp technique (see [8] for details). Since mast cells do not respond to extracellular stimulation if dialysed with a patch-pipette for some minutes [12,13], we have used the slow-whole-cell method [12] to monitor exocytosis stimulated by the polycation compound 48/80. Stimulation of mast cells with compound 48/80 at 0.5 μg/ml leads to very fast exocytosis associated with an about 2-fold increase in membrane area as shown in fig.1a. Fig.1b shows that no capacitance

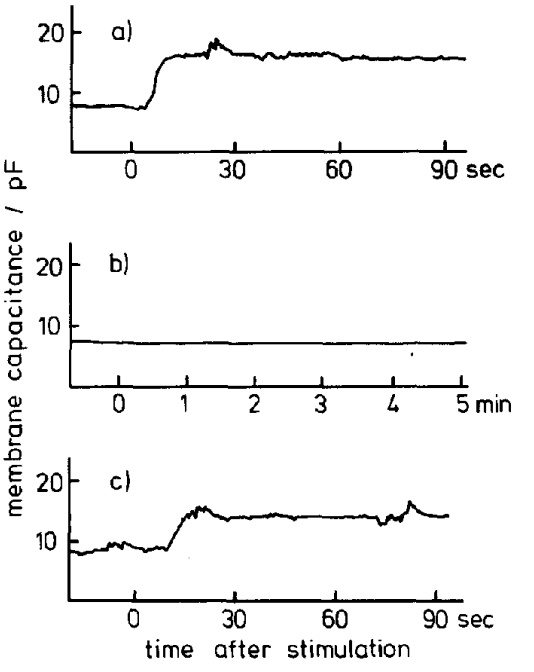

Fig.1. Changes of membrane area in response to stimulation with compound 48/80 recorded in the slow-whole-cell configuration. (a) Typical time course of exocytosis after stimulation with 48/80 at 0.5 μg/ml. (b) No granule fusion is detectable in cells which were incubated with IAP (180 ng/ml, 5 h) before stimulation. (c) Exocytosis recorded from a control cell incubated for 10 h in the absence of IAP.

change is observed in a cell incubated with IAP for 5 h. The control cells incubated in the absence of IAP, however, were still responsive even after 10 h, giving a very similar response at this time (fig.1c). These results clearly demonstrate that the loss of responsiveness was due to the action of IAP and was not an effect of the incubation time of several hours.

In the fast-whole-cell configuration the pipette solution rapidly exchanges with the cytosol [14]. This technique can thus be used to introduce certain substances into the cells and simultaneously measure exocytosis as a capacitance increase [4,5,8]. Intracellular application of 20 μM GTP-γ-S in the presence of about 1 μM free intracellular Ca^{2+} leads to exocytosis with a slower time course and higher amplitude than with compound 48/80 as shown in fig.2a. Exocytosis is not observed when the pipette contains high Ca^{2+} in the absence of GTP-γ-S (fig.2b). If the free Ca^{2+} is reduced to about 10^{-8} M, GTP-γ-S still causes exocytosis, with a similar amplitude, but with a markedly delayed time course. To investigate the effect of IAP on GTP-γ-S-induced degranulation we have thus used the most effective procedure employing 20 μM GTP-γ-S in the presence of about 1 μM free Ca^{2+}. GTP itself did not reproducibly stimulate exocytosis at concentrations up to 1 mM.

When IAP-treated cells are stimulated by intracellular application of GTP-γ-S exocytosis occurs with a similar time course and amplitude as shown in fig.3a. When compound 48/80 was added to the other cells in the same dish none of the observed cells showed visible signs of degranulation. This result was confirmed by slow-whole-cell capacitance measurements and the trace of fig.1b was actually obtained from a cell in this dish. After about 30 min the dish was washed free of compound 48/80 using the perfusion system of the patch-clamp set-up. Intracellular application of GTP-γ-S to another one of these cells still caused normal secretion as shown in fig.3b. It should be noted that the lag period between application of the GTP analog and onset of degranulation is not

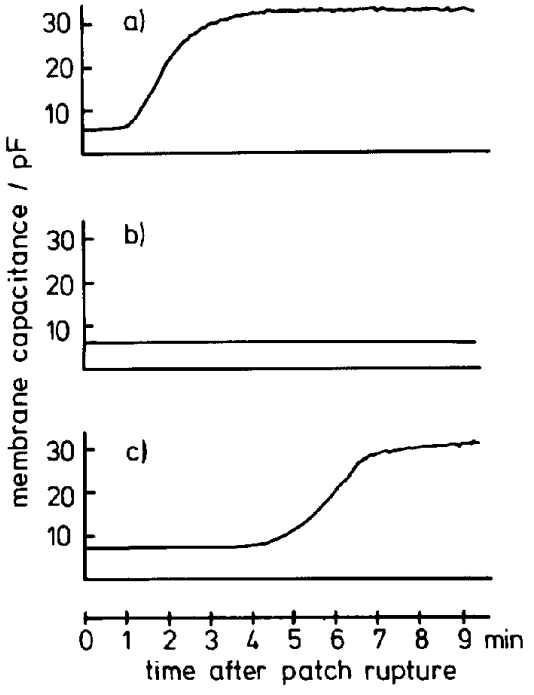

Fig.2. Time course of membrane area recorded in mast cells dialysed with patch pipettes containing: (a) 20 μM GTP-γ-S, 5 mM EGTA, 4.5 mM $CaCl_2$ (p$Ca \approx 6$), (b) no GTP-γ-S, p$Ca \approx 6$ as in (a), (c) 20 μM GTP-γ-S, 0.5 mM EGTA, no $CaCl_2$ (p$Ca \approx 8$). All pipette solutions at pH 7.2. The calculated pCa values were confirmed by measurements with a calcium electrode.

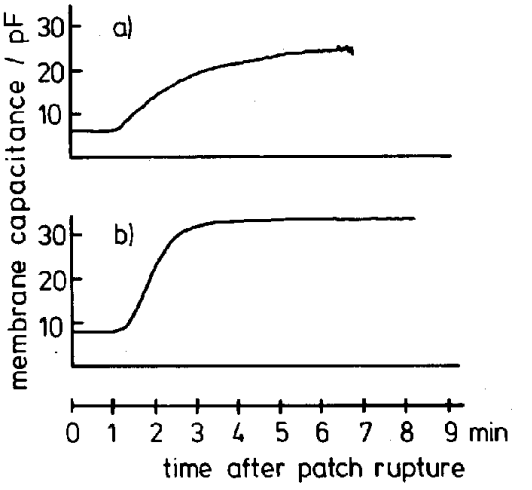

Fig.3. Changes of membrane area in response to intracellular application of GTP-γ-S (p$Ca \approx 6$) to: (a) a cell incubated with IAP for 9 h, (b) a cell incubated with IAP as in (a) and subsequently stimulated with compound 48/80. No degranulation occurred in response to the 48/80 stimulation. The initial capacitance is thus in the range usually measured in non-stimulated cells.

prolonged in IAP-treated cells. These results clearly show that cells which are rendered refractory to receptor-mediated stimulation with IAP respond normally to intracellular stimulation with GTP-γ-S.

4. DISCUSSION

Exocytosis in response to receptor-directed stimulation of mast cells is inhibited by IAP as shown by Nakamura and Ui [15]. This result was confirmed by the present investigation. After IAP treatment phosphoinositol breakdown and arachidonic acid release are also inhibited, whereas cyclic AMP levels are not significantly affected [6]. Since IAP ADP-ribosylates at least 3 different GTP-binding proteins [16,17] it was suggested that a G-protein might be the substrate of IAP in mast cells [6]. Calcium-dependent release of histamine and arachidonic acid from GPP(NH)P-loaded mast cells has also been reported to be partially inhibited by IAP [9] and by neomycin which is an inhibitor of the polyphosphoinositide phospholipase C [7]. These results were in agreement with the involvement of a single G-protein (G_p) regulating stimulus-secretion coupling in mast cells. The synergistic action of calcium and GTP-γ-S shown in fig.2 would be consistent with this view suggesting the activation of protein kinase C. In intact cells phospholipase C generates two second messengers. Inositol trisphosphate is generally believed to release calcium from internal stores. The other product diacylglycerol activates protein kinase C in a calcium-dependent manner [18]. In the fast-whole-cell configuration the internal calcium is buffered and the different time courses of exocytosis observed at different calcium concentrations would fit the above mechanism.

GTP-binding proteins can be persistently activated by non-hydrolysable GTP analogs. Activation of G_i with GTP-γ-S occurs after a lag phase which is proportional to the inverse GTP-γ-S concentration [19]. Exocytosis in mast cells in response to intracellular application of GTP-γ-S is also characterised by a lag period which depends linearly on 1/[GTP-γ-S] [5]. ADP-ribosylation of G_i by IAP does not prevent activation with GTP-γ-S, but the delay is significantly increased by about 5-fold compared to control membranes [20]. In contrast, intracellular application of GTP-γ-S stimulates exocytosis in mast cells with the same time course in IAP-treated and control cells (see figs 2a and 3). In particular, the lag phase between introduction of the GTP analog and onset of degranulation is always about 70 s under these conditions and is not significantly prolonged in IAP-treated cells.

In neutrophils receptor-mediated exocytosis is inhibited by IAP [21,22] whereas exocytosis stimulated with either non-hydrolysable GTP analogs or Ca^{2+} in Sendai virus-permeabilised cells is not [10,22]. Together with the observation that phorbol ester and GTP-γ-S differ in that secretion due to Ca^{2+} is inhibited by phorbol ester but amplified by GTP-γ-S, this has led to the suggestion of a second G-protein called G_E in stimulus-secretion coupling [11,22]. The present results strongly support the existence of an IAP-insensitive G_E and extend it to a second cell type. If GTP-γ-S were to activate the same G-protein which is ADP-ribosylated by IAP, then the turn-on reaction with the GTP analog would have to be completely unaffected, contrary to the results reported for the properties of G_i [20]. Recent experiments on streptolysin-O-permeabilised mast cells have shown that GTP-γ-S stimulates secretion under conditions where the phospholipase C is inhibited by neomycin [23] and this result also supports the existence of G_E. The molecular identification of this second GTP-binding protein has to await further studies.

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