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MASTOPARAN INDUCES OSCILLATIONS OF CYTOSOLIC Ca^{2+} IN RAT PANCREATIC ACINAR CELLS

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Microfluorimetry of fura-2 was used to monitor $[Ca^{2+}]_i$ in single cells stimulated with the G-protein activating agent mastoparan. Mastoparan induced the generation of $[Ca^{2+}]_i$ oscillations, which in contrast to oscillations induced by low concentrations of CCK were acutely dependent on the presence of extracellular Ca^{2+}. Oscillations were inhibited by phorbol ester. Sodium fluoride, a known activator of G-proteins, gave similar results. Both mastoparan and CCK induced turnover of inositol phosphates, at concentrations higher than necessary to induce oscillations.

Stimulation of many cell-types with phospholipase C linked agonists often results in a complex, oscillating $[Ca^{2+}]_i$ signal [1]. In pancreatic acinar cells stimulation by low concentrations of acetylcholine, cholecystokinin or bombesin will give rise to such an oscillating $[Ca^{2+}]_i$ signal [2,3,4]. However the mechanisms which underlie these oscillations are far from clear [1,5,6 for review], but are thought to at least initially involve the products of the receptor-linked, and G-protein coupled, breakdown of a membrane-bound inositol lipid, phosphatidylinositol 4,5 bisphosphate (PIP_2), [7].

Recently it has been found that mastoparan, a amphiphillic tetradecapeptide isolated from wasp-venom, has a wide variety of cellular actions; these include mast-cell degranulation [8], insulin secretion [9], stimulation of glycogenolysis [10], and secretion of catecholamines by adrenal chromaffin cells [11]. These effects of mastoparan have been ascribed to the activation of G-proteins on the basis of the ability of the peptide to stimulate GTPase activity of a number of purified G-proteins [12]. Stimulation of G-proteins is believed to be brought about by the peptide inserting into the plasma membrane and forming a highly structured α-helix, which appears similar in structure to the intracellular loops of G-protein coupled receptors. In analogy with receptor activation, it is believed mastoparan interacts with the c-terminal domain of the G-protein α-subunit, thus bringing about G-protein activation [12]. Sodium fluoride can also activate G-proteins, but by a different mechanism. It forms a

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complex with aluminium, which appears structurally analogous to the terminal phosphate of GTP [13]. In this study we have used mastoparan and NaF, two membrane-permeable agents, to directly activate G-proteins, and by bypassing the receptor determine if G-proteins are directly involved in stimulating oscillations of acinar cell $[Ca^{2+}]_i$.

METHODS AND MATERIALS

The methods are essentially similar to those previously published [2,3]. In brief pancreata were excised from adult male rats (200-250g) and acini prepared by enzymatic digestion with collagenase. Acini were resuspended in a physiological salt solution (PSS) containing 1% BSA, 0.1mg/ml soybean trypsin inhibitor (SBTI) and (in mM); 137 NaCl, 4.7 KCl, 0.56 $MgCl_2$, 1.28 $CaCl_2$, 1.0 Na_2HPO_4, 10 HEPES, 2 L-Glutamine, 5.5 D-glucose. The pH was adjusted to 7.4 and equilibrated with 100% O_2. For fluorescense measurements the PSS was identical, except no BSA or SBTI was added. In addition where Ca^{2+} free media is indicated, $CaCl_2$ was omitted from the PSS and 1mM EGTA was added.

Isolated acini were loaded with 1μM fura-2 AM at ambient temperature for 30 min. Loaded cells were then washed and resuspended in fresh PSS. For measurement of intracellular Ca^{2+}, loaded acini were transferred to a closed chamber mounted on the stage of a Nikon Diaphot, inverted microscope, and continuously superfused (1ml/min) at 37°C with PSS. Solution changes were rapidly accomplished by means of a valve attached to an 8 chambered superfusion reservoir. Individual cells protruding from an acinus were isolated optically by means of a pin-hole diaphragm. Dual excitation, alternating at 340 and 380nm was provided by a SPEX fluorolog system (SPEX Industries Inc, Edison, New Jersey.) and emission at 505nm associated with this excitation was stored and analysed by DM3000cm software (SPEX). Calibration of fluorescent ratio signals was accomplished as previously described according to the equation of Grienkiewicz *et al*, [14] in which $[Ca^{2+}]= k_d.\beta.\{R-Rmin\}/\{Rmax-R\}$. Rmax, Rmin and β = 11.4, 0.64 and 8.1 respectively.

For the measurement of total $[^3H]$-inositol phosphate production, acini were labeled with 20μCi/ml $[^3H]$-inositol for 90mins at 37°C. The labeled acini were washed twice with PSS containing 10mM $LiCl_2$ and resuspended in the same solution. Aliquots of acini (0.6ml) were incubated with agents for 90 secs and the incubation terminated by addition of an equal volume 20% ice-cold trichloroacetic acid. After centrifugation at 1000g for 15min, 0.9 ml of each supernatant was washed three times with water-saturated diethylether, neutralized with 1 M $KHCO_3$, and diluted with 2.5ml of water. Analysis of total $[^3H]$-inositol phosphates was carried out by methods as described by Berridge [15].

RESULTS

STIMULATION OF SINGLE CELLS WITH MASTOPARAN. The effect of mastoparan (0.5-2 μM) on the fura-2 fluorescent signal of superfused acini can be seen in figure 1 A. These applications were associated with an increase in $[Ca^{2+}]_i$, rising from a mean basal value of 89±7 nM (S.E.M.) (n=18) to a peak of 344±21 nM (n=10). The signals were characterized by a long latency, averaging 135± 31 secs, prior to the Ca^{2+} increase. In many cases a gradual increase in $[Ca^{2+}]_i$ was observed prior to the appearance of an oscillating $[Ca^{2+}]_i$ signal. The frequency of these oscillations averaged 1.07± 0.14 cycles per minute. The oscillations were characterized by pulsitile increases in the fluorescent signal,

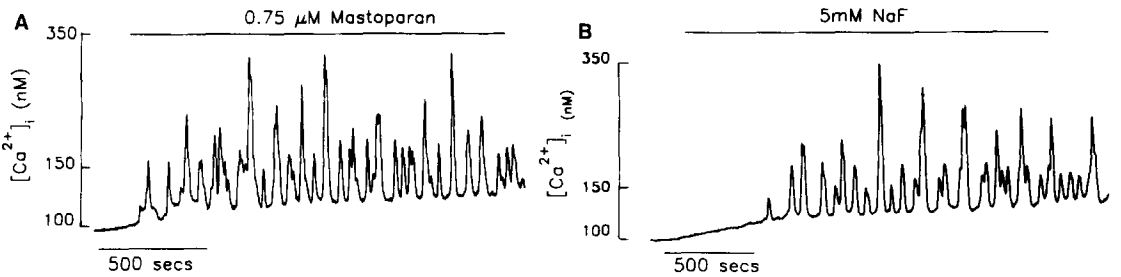

FIGURE 1 - Mastoparan stimulates oscillations in $[Ca^{2+}]_i$. Stimulation with 0.75μM mastoparan (n=4) (A) or 5mM sodium fluoride (n=5) (B) induces the appearance of an oscillating $[Ca^{2+}]_i$ signal.

originating from at the basal concentration and punctuated by more rapid spikes, superimposed on a plateau region. Removal of the agents did not markedly attenuate the rise in $[Ca^{2+}]_i$. Superfusion of higher concentrations of mastoparan (>2μM) was associated with the generation of a more sustained $[Ca^{2+}]_i$ signal (data not shown).

EFFECT Of EXTRACELLULAR Ca^{2+} AND THE INFLUENCE OF PROTEIN KINASE C ON $[Ca^{2+}]_i$ OSCILLATIONS. The dependence on extracellular Ca^{2+} of the mastoparan induced oscillations was tested by removal of Ca^{2+} from the bathing solution. Chelation of extracellular Ca^{2+} resulted in a rapid termination of the oscillatory Ca^{2+} signal and a return of the signal to basal values, indicating a marked dependence on extracellular Ca^{2+} (Figure 2A). Readdition of Ca^{2+} to the extracellular bathing solution always resulted in a further increase in $[Ca^{2+}]_i$. This marked dependence on extracellular Ca^{2+} is in contrast to oscillations generated by low concentrations of cholecystokinin (CCK) in this cell-type, which are able to continue to oscillate for some minutes in the absence of extracellular Ca^{2+} (Figure 2C). It has been previously reported that phorbol esters, known activators of protein kinase C (PKC), will inhibit oscillations induced by CCK in pancreatic acinar cells [3,16]. The effects of phorbol ester were therefore tested on oscillations induced by mastoparan. Application of 0.1 μM PMA caused an attenuation of the oscillations, which resulted in the signal returning to at or near basal levels within 5 mins. If subsequently the PKC inhibitor staurosporine (100-300nM) was added with phorbol ester, the signal was always characterized by the return of oscillations. This confirms that the site of phorbol ester action is most likely PKC and indicates that PKC may be involved in modulating mastoparan induced oscillations, possibly through a negative feed-back loop (Figure 3).

EFFECT OF SODIUM FLUORIDE ON $[Ca^{2+}]_i$. Application of low concentrations of sodium fluoride (1-5mM) gave effects very similar to mastoparan. Fluoride induced the appearance of $[Ca^{2+}]_i$ oscillations, similar in appearance to those induced by mastoparan (Figure 1B) characterized by a long latency and often

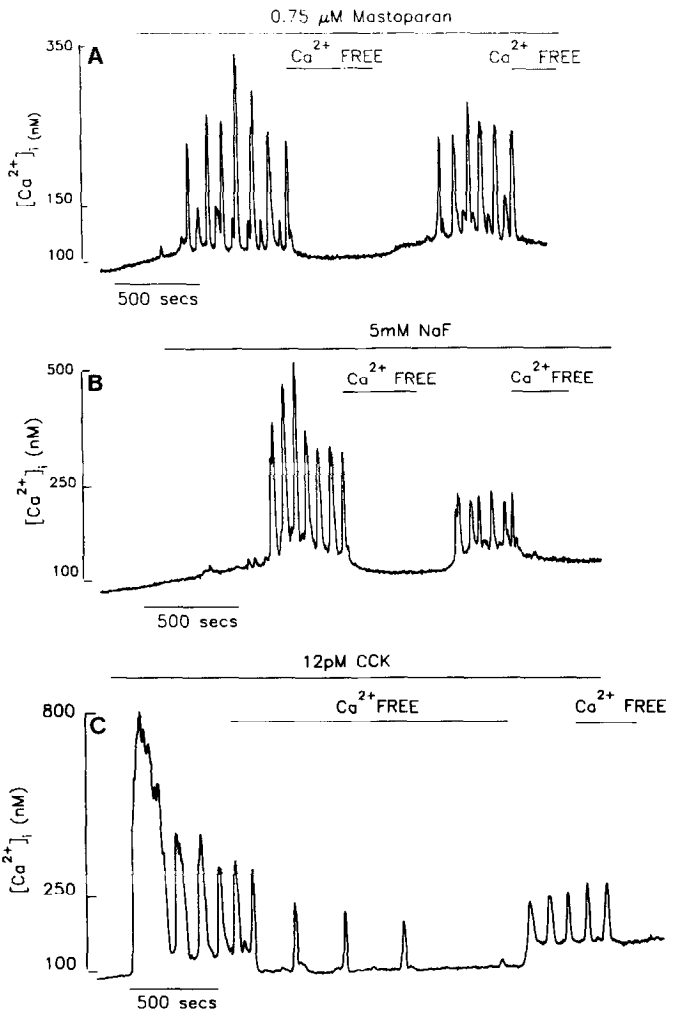

FIGURE 2- Extracellular Ca^{2+} dependancy of oscillations.
After stimulation with either 0.75μM mastoparan (n=4) or 5mM sodium fluoride (n=4), removal of Ca^{2+} from the extracellular bathing solution rapidly attenuates the oscillations.In C oscillations induced by CCK (12pM), in contrast are not abolished immediately, but continue at a reduced frequency for several minutes. Readmission of external Ca^{2+} results in a return of the oscillating signal in each case.

accompanied by a slow increase in the basal signal. The average amplitude of these oscillations was 431± 25 nM (n=8). The oscillations were also extremly sensitive to removal of external Ca^{2+}, being rapidly abolished when external Ca^{2+} was removed (Figure 2B). Application of phorbol ester also caused an attenuation of NaF induced oscillations, which was reversed by staurosporine in 4 experiments (data not shown).

STIMULATION OF INOSITOL PHOSPHATE TURNOVER BY MASTOPARAN. It has been previously reported that sodium fluoride will cause PI turnover in a number of cell-types including the rat exocrine pancreas [17], an effect which has

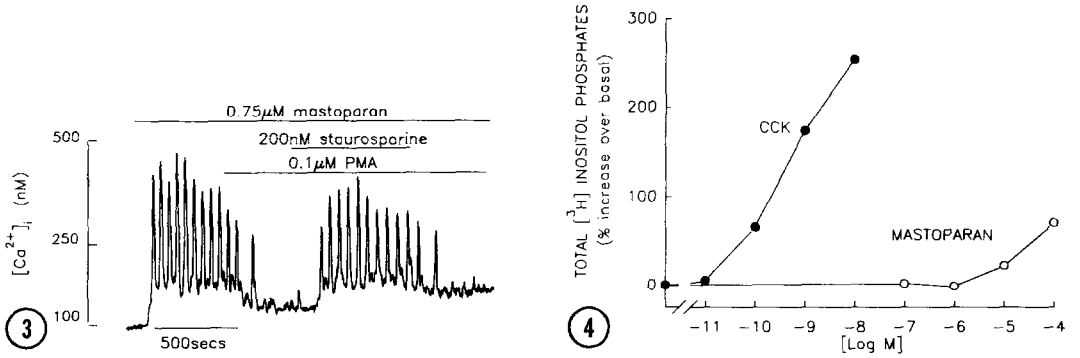

FIGURE 3- Phorbol ester inhibits $[Ca^{2+}]_i$ oscillations.
After stimulation with 0.75μM mastoparan, oscillations are attenuated by the phorbol ester PMA (0.1μM n=4). Subsequent concurrent application of the protein kinase C-inhibitor staurosporine (200nM) restores the oscillations.

FIGURE 4 - Mastoparan and CCK induce PI turnover.
Acini pre-labelled with $[^3H]$-inositol were incubated for 90 secs with either mastoparan or CCK in duplicate. The inositol phosphates extracted and assayed as detailed in "Materials and Methods." Data is presented as % increase above basal.

been ascribed to the activation of GTP-binding proteins. Figure 4 shows one experiment typical of two others, in which PI turnover induced by CCK and mastoparan is compared. Mastoparan induced a small PI turnover, compared to CCK. Moreover the majority of the increase above basal was seen at doses above 1μM. No increase in PI turnover was detected at concentrations of mastoparan which induced $[Ca^{2+}]_i$ oscillations. Sodium fluoride also induced PI turnover of magnitude intermediate between mastoparan and CCK (data not shown).

DISCUSSION

In this present study we have shown the ability of low concentrations of mastoparan and sodium fluoride to induce oscillations of cytosolic Ca^{2+} in rat pancreatic acinar cells. This is a characteristic response similar to that exhibited by low concentrations of agonists [2,3,4], and may indicate a common mechanism for generation. This finding is also consistant with that of Osipchuk *et al.*, [18], who showed that application of GTP-γ-S, through a patch electrode to mouse pancreatic acinar cells could induce $[Ca^{2+}]_i$ oscillations.

Mastoparan is a potent stimulator of GTP-binding proteins in a number of systems [8,9,10,11]. These include mast cells, where stimulation results in an increase in GTPase activity, an associated increase in Ins 1,4,5 P_3 levels, leading to release of intracellular Ca^{2+} and histamine secretion [8]. It is likely that since an increase in PI turnover was detected for both CCK and mastoparan, that an initial event in the generation of oscillations is an increase in Ins 1,4,5 P_3 and

the release of intracellular stored Ca^{2+}. However no increase in PI turnover was detected at concentrations of mastoparan or CCK, that induce $[Ca^{2+}]_i$ oscillations. This is a similar finding to Matozaki *et al.*, [19] who found, that an analogue of CCK which induces oscillations over all doses tested, caused no detectable PI turnover. The most accepted explanation for this finding is that small, localised changes in inositol phosphates may have occured, not detected by the assay system. However, the possibility of an alternative G-protein contributing to the induction of the oscillatory signal cannot be ruled out.

The subsequent cellular events which lead to the maintenance of the oscillations are far from clear, and are currently the subject of much debate [1,5,6]. The oscillations induced by both mastoparan and sodium fluoride were acutely dependent on the presence of Ca^{2+} in the extracellular bathing solution, and this would suggest that Ca^{2+} influx plays an important role in the maintenance of the oscillations. This is in some contrast to oscillations induced by CCK, which continue, at a somewhat reduced frequency in the absence of external Ca^{2+} for some minutes. However it has been reported that CCK stimulation can exert complex effects on both Ca^{2+} influx and efflux [20]. Whether all these effects are mediated by G-proteins activation, or by the same subset of G-proteins, activated by mastoparan, remains to be determined.

The observation that phorbol esters can inhibit oscillations induced by both GTP-binding protein activators and agonists, points to a possible role for protein kinase C in the mechanism which underlies these oscillations. Protein kinase C activation has been shown to influence many key cellular events, which could potentially influence the generation of oscillations [7]. It is, however, not as yet clear at what point PKC activation acts to inhibit oscillations. The fact that oscillations induced by G-protein activation are markedly influenced by phorbol esters points to a site of action distal to the occupation of receptors.

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