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MITOGENS AND MELITTIN STIMULATE AN INCREASE IN INTRACELLULAR FREE CALCIUM CONCENTRATION IN HUMAN FIBROBLASTS

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Intracellular free Ca^{+2} concentration was measured in cultured human fibroblasts (HSWP) utilizing the Ca^{+2}-sensitive fluorescent probe quin2. The addition of peptide growth factors to serum-deprived HSWP cells induced an immediate rise in intracellular Ca^{+2} concentration. This mitogen-induced rise in Ca^{+2} concentration could be blocked by the addition of the intracellular Ca^{+2} antagonist TMB-8. Addition of the phospholipase activator, melittin, to cells in the absence of growth factors also caused a dramatic rise in intracellular Ca^{+2} concentration which was blocked by TMB-8.

The addition of mitogens to serum-deprived fibroblasts stimulates a series of biochemical events which ultimately lead to DNA synthesis and cell division. In order to understand the process by which binding of mitogens to their membrane receptors leads to proliferation, it is important to characterize the initial events following mitogen binding. In recent years, it has become apparent that a number of the initial events stimulated by mitogens involve a movement of ions either across the plasma membrane or out of intracellular storage sites. For example, studies have shown that mitogens stimulate Na^+ influx (1), Ca^{+2} influx (2), Ca^{+2} efflux (3) and Na^+/K^+ pump flux (4). It has been postulated that one or more of these early ion movements may serve as a trigger mechanism for subsequent biochemical events. Thus, for the past several years our laboratory has been studying the activation of Na^+/H^+ exchange by mitogens to characterize the mechanism by which mitogens stimulate Na^+ influx via this transport pathway. Our evidence indicates that the activation of Na^+/H^+ exchange is the result of a rise in cytosolic free Ca^{+2} concentration whose action is mediated by the Ca^{+2} dependent regulatory protein calmodulin. This hypothesis is based on the observations that: 1) Na^+ influx can be

Abbreviation: TMB-8/ 8-(N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate.

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induced in a mitogen-free medium by the addition of the divalent cation ionophore A23187 (5); 2) the mitogen-induced Na^+ influx can be blocked by known inhibitors of calmodulin (6) and 3) the mitogen-induced Na^+ influx can be blocked by the proposed intracellular Ca^{+2} antagonist TMB-8 (7). Although there is substantial evidence that the mitogen stimulation of Na^+ influx is secondary to a rise in intracellular Ca^{+2} activity, to prove this hypothesis, it is necessary to show a rise in intracellular Ca^{+2} activity in response to mitogens. Recent work from our laboratory and by others have shown that mitogens stimulate Ca^{+2} efflux from fibroblasts in a manner that is consistent with a mobilization of intracellular Ca^{+2} from intracellular stores (3). However, to date, there have been no reports of a rise of intracellular Ca^{+2} activity in cultured fibroblasts in response to mitogen stimulation. With the recent introduction of quin2 as a suitable fluorescent probe for intracellular Ca^{+2} activity (8), it is now feasible to measure Ca^{+2} activity in cells as small as cultured fibroblasts. Our present results demonstrate that the use of quin2 is not limited to suspension cells, where all the previous studies have been performed (8,9), but its use can be extended to cultured fibroblasts, grown on a solid substratum, to show a mitogen-induced rise in intracellular Ca^{+2} activity.

MATERIALS AND METHODS

Human fibroblasts (HSWP), derived from human foreskin, were obtained from James Regan (Oak Ridge National Laboratory) and were cultured in Eagle's Minimal Essential Medium (EMEM) (GIBCO) containing 10% fetal bovine serum (FBS) (KC Biologicals). Cells were grown at 37°C in a 95% air, 5% CO_2 atmosphere and were used between the 10th and 25th passages. Cells were removed from stock flasks by trypsinization and were subcultured onto 40x40 mm glass coverslips for use in fluorescent experiments. The cells were used 3-5 days after subculture while in the logarithmic phase of growth.

Prior to measurement of intracellular Ca^{+2} activity, cells were serum deprived overnight in EMEM containing 0.1% FBS. Previous studies have shown that this is sufficient time in a low serum medium to reduce the Na^+ influx to the level seen in quiescent cells. Cells were then incubated in Hepes-buffered, amino acid-free EMEM containing 50 μM quin2 acetoxymethyl ester (quin2/AM) for a period of 90 minutes. The cells were then washed free of the extracellular probe and incubated in Hepes-buffered Hank's salt solution for another 30-60 minutes. The coverslip was then mounted as the top of a flow through chamber which was maintained at 37°C. The fluorescence of a field of appoximately 5 cells was monitored in a microspectrofluorometer (10). The presence of intracellular quin2 was established by obtaining corrected fluorescence spectra of cells loaded with the probe. To determine the effects of mitogens, melittin and TMB-8 on cellular Ca^{+2}, these agents were injected

into the chamber through a side port while the fluorescence was continuously monitored.

The TMB-8 was obtained from Aldrich. Melittin, insulin and vasopressin were obtained from Sigma. Lys-bradykinin was obtained from Peninsula Laboratories, Inc., EGF from Collaborative Research and ionomycin was a gift from Hoffman LaRoche.

RESULTS AND DISCUSSION

In initial studies, the excitation and emission spectra of the cells was monitored at various times during the loading process to assess whether the quin2/AM was being taken up by the fibroblasts and whether the compound was being de-esterified once it entered the cytoplasm. The data in Figure 1 show the fluorescence spectrum of quin2/AM before it is loaded into fibroblasts and the spectrum of cells following a 90 minute loading period. These data clearly show that there is a substantial red shift in the emission spectrum and a blue shift in the excitation spectrum of the probe once it has loaded into the cells. These spectral shifts are consistent with those seen when quin2/AM is treated with esterase in a buffer solution (Fig. 1), suggesting that once loaded into fibroblasts, the probe is de-esterified to produce quin2 which then can be used to monitor changes in cytoplasmic Ca^{+2} activity.

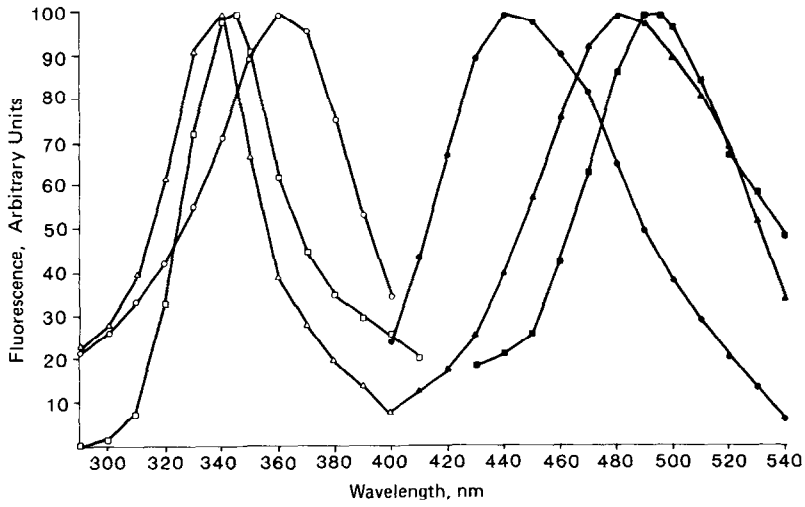

FIG. 1. Quin2 excitation and emission spectra. (○,●) The spectra of the quin2 acetoxymethyl ester, quin2/AM. (△,▲) Spectrum of quin2 after a two hour incubation with bovine esterase. Both spectra were measured in a Perkin-Elmer fluorescence spectrophotometer. (□,■) Serum deprived HSWP cells were loaded with quin2 as described in "Methods". The spectrum was determined with the microspectrofluorimeter, using background subtraction and computer smoothing. Open symbols are excitation and closed symbols are emission spectra.

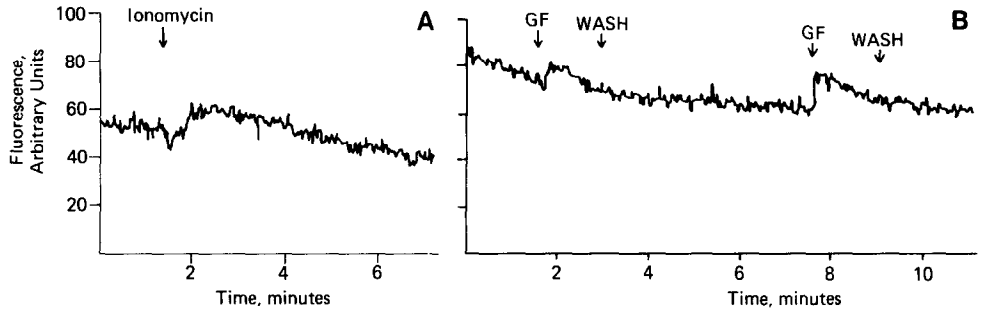

FIG. 2. Effect of ionomycin and growth factors on intracellular calcium activity. A. Cells were prepared as described in "Methods". At the time indicated by the arrow, ionomycin was injected into the flow-through chamber to a final concentration of 0.5 μM. Ionomycin was dissolved in dimethyl sulfoxide (DMSO). DMSO final concentration was 0.1%, and controls showed that it alone had no effect on fluorescence. B. Peptide growth factors were injected at the time indicated by the arrow. Final concentrations are insulin, 1 μg/ml, EGF, 50 nM, bradykinin, 0.1 μg/ml, vasopressin, 0.1 μg/ml. All were dissolved in normal saline.

A representative trace of the response of the quin2 signal to the addition of the Ca^{+2} ionophore ionomycin is shown in Figure 2. Following addition of ionomycin, the fluorescence signal rises to a peak and then decays, indicating that the signal is responsive to changes in cytoplasmic Ca^{+2} activity. Also shown in Figure 2 is the response of quin2 signal to the addition of a mixture of mitogens that we have previously shown will maximally stimulate both Na^{+} influx and DNA synthesis in HSWP cells (11). The addition of mitogens causes an immediate rise in fluorescence which peaks in less than 30 seconds and then gradually declines over the next minute. Thus, these data indicate that mitogens induce a rapid rise in intracellular Ca^{+2} activity which is of a transient nature. The observation that mitogens which stimulate Na^{+} influx also increase Ca^{+2} activity, further supports our hypothesis that the actions of these mitogens on Na^{+} influx are mediated by a Ca^{+2} dependent event. In addition, the demonstration of an early rise in intracellular Ca^{+2} activity in response to mitogens, strengthens the contention that Ca^{+2} plays an important role in the mitogenic process. In recent studies, it was demonstrated that melittin, a peptide component of bee venom which stimulates phospholipase activity, could activate Na^{+} influx in a manner similar to serum (12,13). This together with the observation that phospholipase inhibitors block the serum stimulation of Na^{+} influx (13) lead us to suggest that the

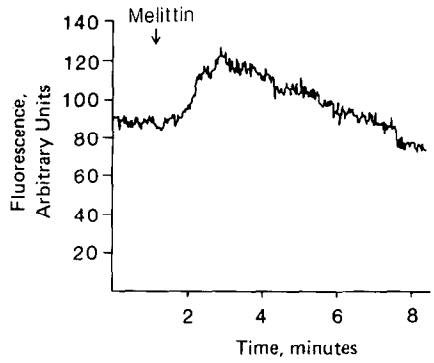

FIG. 3. Effect of melittin on intracellular calcium activity. Cells were prepared as described in "Methods". At the time indicated by the arrow, melittin was injected to a final concentration of 0.6 μg/ml.

mitogen-stimulation of phospholipase activity is an important step in the activation of Na^+ influx. Since recent studies in insect salivary glands have suggested that a product of phospholipase C activity may be involved in the mobilization of intracellular Ca^{+2} (14), we were interested in determining whether stimulation of phospholipase activity by melittin could mimick the mitogen-induced rise of intracellular Ca^{+2} activity. The data in Figure 3 show that the addition of melittin, at a dose that gives substantial stimulation of Na^+ influx in HSWP cells and of cell proliferation in mouse 3T3 cells (12), produces a dramatic rise in intracellular Ca^{+2} activity. This observation supports our previous contention that melittin stimulates Na^+ influx in a manner similar to that of mitogens and in addition suggests that the effects of melittin on cell proliferation may be mediated by a Ca^{+2} dependent process.

In recent studies, we have utilized the proposed intracellular Ca^{+2} antagonist TMB-8 (15) to block mitogen-stimulated Na^+ influx in HSWP cells (7). Although this agent blocked mitogen-stimulated flux with a K_i of 15 μM, it had no effect on A23187-stimulated Na^+ influx, indicating that TMB-8 is interfering with the regulation of Na^+/H^+ exchange rather than blocking the transport pathway itself. This together with the observation that TMB-8 blocks the stimulation of Ca^{+2} efflux from HSWP cells (3), supports the involvement of Ca^{+2} in the activation of Na^+ influx. However, although the blockade of the mitogen stimulated Ca^{+2} efflux is consistent with TMB-8 acting to reduce a mitogen-induced rise in intracellular Ca^{+2} concentration,

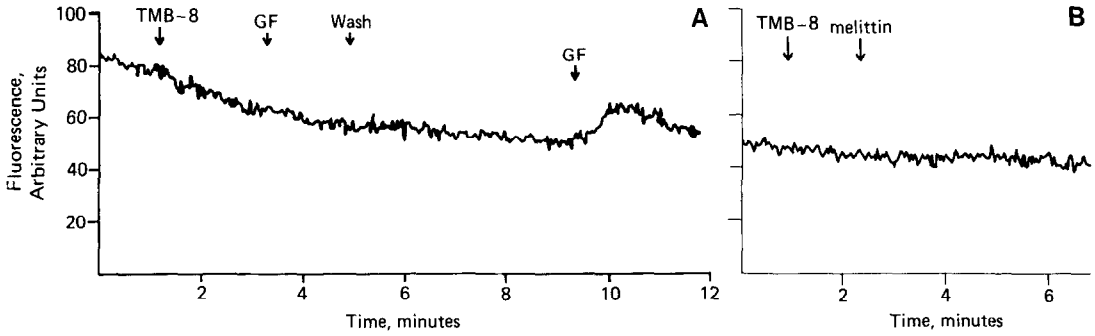

FIG. 4. Effect of TMB-8 on growth factor or melittin induced increase in intracellular calcium. A. At the first arrow, TMB-8 was injected into the flow-through chamber to a final concentration of 50 μM. Growth factors were injected at the second arrow and had no effect. The cells were then washed free of drug, and re-administration of growth factors caused an immediate increase in the calcium signal. B. TMB-8 was injected to a final concentration of 50 μM, then melittin was added to a concentration of 0.6 μg/ml. TMB-8 inhibits the melittin induced increase in calcium.

a more definitive test would be to determine its effect on intracellular Ca^{+2} activity in mitogen stimulated cells. The data in Figure 4 show the effect of TMB-8 on the quin2 response of cells stimulated by either a mixture of peptide growth factors or by melittin. The normal rise in cellular Ca^{+2} activity induced by these agents is dramatically inhibited by 50 μM TMB-8. This observation strongly supports the hypothesis that TMB-8 produces its effects on cellular processes by blocking the rise in intracellular Ca^{+2} activity in response to various hormones or growth factors. In addition, it provides further support for our hypothesis that the melittin stimulation and the mitogen stimulation of Na^{+} flux in HSWP cells are mediated by a similar mechanism which has as a critical step, a rise in intracellular Ca^{+2} activity. Studies are currently in progress to determine the mechanism by which peptide mitogens and melittin stimulate a rise in intracellular Ca^{+2} activity.

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