Vol. 97, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 28, 1980 Pages 716-724

STIMULATION OF MONOVALENT ION FLUXES AND DNA SYNTHESIS IN 3T3 CELLS BY MELITTIN AND VASOPRESSIN IS NOT MEDIATED BY PHOSPHOLIPID DEACYLATION

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Received October 21, 1980

SUMMARY

Melittin at subtoxic concentrations stimulates monovalent ion fluxes and, together with insulin, synergistically increases DNA synthesis, but has little effect on phospholipase activity. The Na^+ ionophore monensin increases Na-K pump activity without affecting phospholipase activity; whereas, the divalent cation ionophore A23187 has reciprocal effects. Finally, vasopressin and insulin potently stimulate ion fluxes and DNA synthesis but have no effect on phospholipase activity. Thus, the polypeptide mitogens melittin, vasopressin, and insulin alter membrane function and subsequently stimulate DNA synthesis in quiescent 3T3 cells by mechanisms independent of phospholipid deacylation.

Quiescent fibroblasts can be stimulated to divide by the addition of serum or combinations of pure growth factors (1,2). Neither the critical initial event nor the mechanism by which this signal is translated into the stimulation of DNA synthesis is yet known. Evidence from this laboratory supports the view that early changes in monovalent ion fluxes underlie the action of a variety of mitogens (2,3). Other laboratories have recently suggested that alterations in phospholipid methylation and/or deacylation may be the regulatory signals mediating concanavalin A-stimulated mitogenesis in murine T-lymphocytes (4) and serum-stimulated mitogenesis in murine fibroblasts (5).

* ABBREVIATIONS: PLase: phospholipase A_2. DMEM: Dulbecco's modified Eagle's medium.

0006-291X/80/220716-09$01.00/0

We have reported that melittin, the amphipathic polypeptide toxin from bee venom (6), stimulates an early influx of Na^+ and activation of Na-K pump activity, and subsequent initiation of DNA synthesis in quiescent 3T3 cells, without affecting the other biochemical events usually associated with mitogenesis (7). Because melittin is known to activate phospholipase-A_2 (PLase: phosphatide 2-acylhydrolase, E C 3.1.1.4)* and hence phospholipid deacylation (6,8-10), we have investigated the relationship of phospholipid deacylation, activation of ion fluxes, and DNA synthesis stimulated by melittin and other mitogens in quiescent 3T3 cells. We report here that phospholipid deacylation is not causally related either to alterations in ion fluxes or DNA synthesis in this experimental system.

MATERIALS AND METHODS

Cell Culture. Swiss 3T3 cells (11) were maintained as previously described (12). Cells were subcultured into 30 mm Nunc tissue culture dishes with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The medium was changed two days after plating and studies were performed on quiescent, confluent cultures 4 to 8 days later.

Phospholipase A_2 assays. The medium from quiescent cultures was aspirated and centrifuged to remove detached cells, and the monolayer cultures were washed once with serum-free medium. (^{3}H)-Arachidonic acid (0.5-1.0 µCi/ml) was added to the original conditioned medium, and the cells incubated for 18 to 24h in 1 ml of this medium. Under these conditions, 95% of the incorporated radioactivity is found in phospholipids in murine fibroblasts (10,13). The cells were then washed three times with serum-free medium. Cell-associated radioactivity was assayed by lysing replicate monolayers in 1 ml of serum-free DMEM containing 1% Triton X-100 and assaying radioactivity in a Triton-Toluene scintillation cocktail. Release of radioactivity was measured by incubating the cells in 1 ml of serum-free medium containing factors as indicated. The medium was aspirated and total radioactivity assayed as above. Under these conditions more than 80 to 90% of the released ^{3}H-radioactivity is free arachidonic acid; the remainder being found primarily in prostaglandins (5,10,13).

Na-K pump activity. The ouabain-sensitive uptake of ^{86}Rb, a K tracer, was measured as previously described (3,7). All measurements were made on duplicate dishes.

DNA synthesis. Incorporation of ^{3}H-thymidine (1 μCi/ml, 1μM) was measured over a 40 to 42 h period as previously described (12). Quiescent monolayer cultures were all washed three times with serum-free medium prior to addition of thymidine-containing medium.

Materials. Melittin, lot no. 128C-0170, was purchased from Sigma; this melittin is reported to be possibly contaminated with phospholipase A_2 up to 50 units per mg. A preparation of purified melittin free from phospholipase A_2 contamination (8) was kindly provided by Drs. C. Mollay and G. Kreil (Salzburg). Both preparations gave similar results with respect to arachidonic acid release, Na-K pump activity, and DNA synthesis, except that the PLase-free melittin showed less toxicity at 600-1000 ng/ml during long-term (20-40 h) incubation. Insulin, (arg)-vasopressin, and ouabain were all purchased from Sigma. (5,6,8,9,11,12,14,15-^{3}H) arachidonic acid (120 Ci/mmol, 4.4 TBq/mmol), (methyl-^{3}H)-thymidine (20 Ci/mmol), and ^{86}Rb (13.1 mCi/mg, 486 MBq/mg) were obtained from the Radiochemical Centre, Amersham. Monensin and A23187 were obtained from the Eli Lilly Company.

RESULTS AND DISCUSSION

In order to assess whether the effects of melittin on phospholipid deacylation and Na-K pump activity are causally related, we have examined the concentration - dependence for both phenomena in the same quiescent cells. As shown in figure 1A, the dose-reponse relationships for the two effects of melittin are clearly dissociated. Over a melittin concentration range of 0 to 400 ng/ml, 50% of the maximal stimulation of ^{86}Rb uptake is achieved, but only 5% of the maximal stimulation of arachidonic acid release. The maximal stimulation of ^{86}Rb uptake in these experiments (30 min incubation) was seen at 1 μg/ml melittin, a concentration which was apparently toxic with respect to Rb transport using other melittin preparations (7). Results identical to the ones shown were obtained using a purified preparation of melittin free of phospholipase A_2 contamination.

As shown in figure 1B and reported previously (7), melittin in combination with insulin causes a synergistic enhancement of DNA synthesis. Maximal stimulation of thymidine incorporation

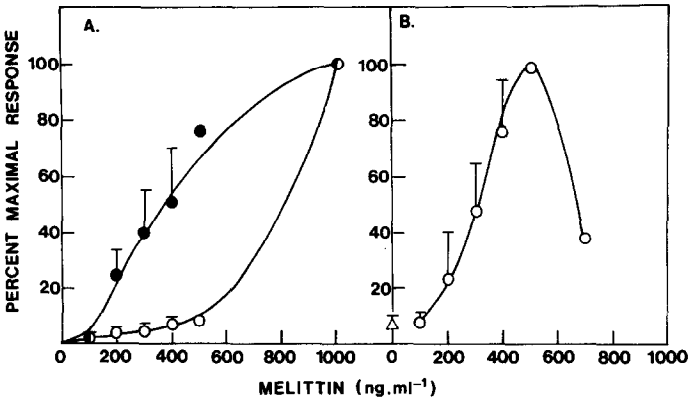

Figure 1 Concentration dependence of the melittin stimulation of (^{3}H)-arachidonic acid release, ^{86}Rb uptake, and DNA synthesis in quiescent Swiss 3T3 cells. A. Cellular phospholipids were metabolically labeled with [^{3}H]-arachidonic acid as described in Materials and Methods. Release of radioactivity (O) was then measured over a 30 min period in quadruplicate cultures. After aspiration of the medium for assay of released radioactivity, ^{86}Rb uptake (●) was measured for 10 to 12 min, in the presence or absence of ouabain, in the same cultures incubated with fresh serum-free medium containing the original concentration of melittin. The results of five independent experiments have been pooled and the data expressed as percentage of the maximal response obtained with melittin. The error bars represent SEM; data for melittin at 500 ng/ml represent the mean of two experiments. Cell-associated arachidonic acid radioactivity at time 0 averaged 2.7 x 10^5 cpm/dish. The S.D. for quadruplicate dishes in a single experiment was <7%. In the absence of any added factors, an average of 1.3±0.2% (mean ± SEM) of cell-associated radioactivity was released in 30 min. The average maximal response achieved with 1 µg/ml melittin in these experiments represented the release of 15±3% of cell-associated radioactivity in 30 min. 10µg/ml insulin caused the release of 1.4±0.2% of the radioactivity; and 10% fetal bovine serum, 4.2±0.9%. The maximal response elicited by 1 µg/ml melittin with respect to ouabain-sensitive ^{86}Rb influx was a 129±17% increase in rate of influx. Insulin stimulated Rb influx 65±5%; serum, 95±19%. B. In three of these five experiments, DNA synthesis was measured in parallel cultures as described in "Materials and Methods". Melittin alone does not stimulate DNA synthesis; therefore, 10 µg/ml insulin was present at all melittin concentrations tested. Insulin alone (△) stimulated approximately 5% as much thymidine incorporation as insulin plus 500 ng/ml melittin. The data are expressed as the percentage of maximal response achieved with melittin; the error bars represent SEM.

was obtained at approximately 500 ng/ml melittin. At higher concentrations, toxicity manifested by detachment of cells was noted after 18 to 20 h incubation. The concentration-dependence of the melittin stimulation of DNA synthesis parallels that of ^{86}Rb uptake (half-maximal stimulation at approximately 300 to 400

ng/ml for both), and clearly differs from that of arachidonic acid release. Thus melittin stimulates Na-K pump activity and DNA synthesis in quiescent 3T3 cells at concentrations which stimulate only minimal increases in phospholipid deacylation. In contrast, there is an association between the toxic effects of melittin and its stimulation of PLase activity, as reported by Shier (10).

Phospholipase A_2 activity is stimulated by increasing intracellular Ca^{2+} concentration (4,5,10). Na-K pump activity, on the other hand, is stimulated by increasing intracellular Na^+ concentration, and this is thought to be the mechanism by which various mitogens enhance ^{86}Rb uptake (2,3,7,14). Hence, the mono- and divalent cation ionophores, monensin and A23187, provide a useful tool to investigate whether stimulation of phospholipid deacylation and of Na-K pump activity are causally related. A23187, at 1 μM causes a greater than three-fold increase in PLase activity but has no effect on ^{86}Rb influx. In contrast, monensin, at 2 μg/ml, causes the expected increase in ^{86}Rb influx, but has no effect on phospholipid deacylation (figure 2). These data further support the dissociation of regulation of phospholipid deacylation and of Na-K pump activity.

Vasopressin, at nanomolar concentrations, is a potent stimulator of Na^+ flux and Na-K pump activity (15), and acts synergistically with insulin to stimulate DNA synthesis (12). Therefore, we have compared the effect of this mitogen with that of melittin on the time course of phospholipid deacylation and on DNA synthesis in parallel cultures of quiescent 3T3 cells. As shown in figure 3A, 350 ng/ml melittin, in the absence or presence of 10 μg/ml insulin, causes only a minimal increase in (^3H)-arachidonic acid release over control, and this release

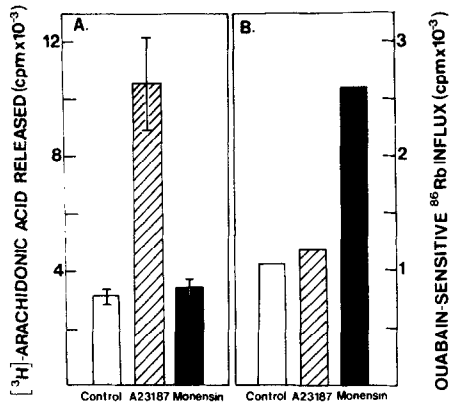

Figure 2. Effects of monensin and A23187 on arachidonic acid release and ^{86}Rb influx. A. Quiescent cultures of 3T3 cells were incubated with (^{3}H)-arachidonic acid (0.75 µCi/ml) for 20 h and washed as described in "Materials and Methods". Triplicate cultures were incubated for 25 min with 1 ml serum-free medium containing 1 µM A23187 (in 0.1% dimethyl sulfoxide) or 2 µg/ml monensin (in 0.02% ethanol) or no additions. Cell-associated radioactivity at time 0 was 318,200±11,700cpm/dish (mean ± S.D.). Error bars represent S.D. B. Parallel cultures were washed three times with serum-free medium, and incubated for 5 min with 1 ml of fresh serum-free medium containing A23187, monensin, or no additions. 50 µl ^{86}Rb/dish (10^{6}cpm) was then added in the presence or absence of 1 mM ouabain, and the incubation continued for 15 min. One µM A23187 does not interfere with the effect of monensin on ^{86}Rb uptake.

reaches a plateau within 60 minutes. In contrast, this concentration of melittin, together with insulin, causes a very striking stimulation of DNA synthesis (figure 3B). At 1 µg/ml, melittin causes a large enhancement of PLase activity; but after 2 h, considerable cell toxicity (detachment from monolayer) occurs whether or not insulin is present. Fetal bovine serum causes a moderate stimulation of PLase activity and the expected large increase in DNA synthesis. Most striking is the observation that vasopressin (30 ng/ml) and insulin (10 µg/ml) added in combination cause no stimulation of (^{3}H)-arachidonic acid release over a four hour incubation period (figure 3A); whereas the two hormones synergistically stimulate DNA synthesis to almost the level of whole serum (figure 3B). These results clearly dissociate the effects of pure peptide mitogens in serum-

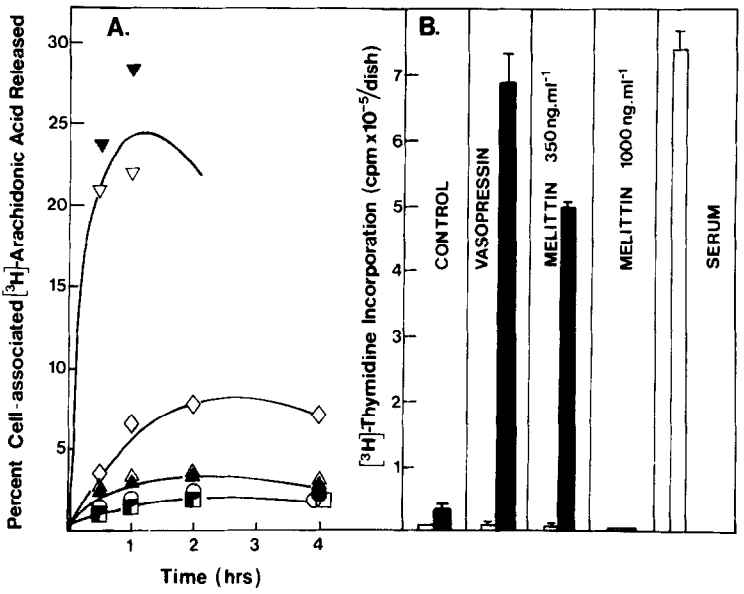

Figure 3. Effects of vasopressin, insulin, and melittin on arachidonic acid release and DNA synthesis. A. Quiescent cultures of 3T3 cells were incubated for 17 h with 0.5 µCi/ml (^{3}H)-arachidonic acid and washed as described. Cultures were then incubated in fresh serum-free medium without additions (○, ●), or containing 30 ng/ml (arginine)-vasopressin (□, ■) or 350 (△, ▲) or 1000 ng/ml (▽, ▼) melittin, in the presence (closed symbols) or absence (open symbols) of 10 µg/ml insulin, or with 10% fetal bovine serum (◇). At the times indicated, medium was aspirated from duplicate dishes for assay of released radioactivity. Cell-associated radioactivity at time 0 was 203,800±4000 (mean ± S.D., n = 4). B. Parallel cultures were washed three times with serum-free medium, and DNA synthesis assayed over 42 h in triplicate cultures incubated with the same factors. Open bars represent no insulin; closed bars, 10 µg/ml insulin. Error bars represent S.D. Cultures incubated with 1 µg/ml melittin in the presence or absence of insulin began to detach from the dish after 2 h incubation; no cells remained attached after 19 h.

free medium on DNA synthesis from their effects on phospholipid deacylation.

Alteration of membrane phospholipids by phospholipase A_2 might be expected to alter membrane fluidity and other properties (4,8) and thus membrane function. In addition, phospholipid deacylation is the first and probably rate-limiting step in prostaglandin synthesis (13). Indeed, it has been reported that phospholipid deacylation may mediate concanavalin A-stimulated

mitogenesis in lymphocytes (4) and serum-stimulated DNA synthesis in murine fibroblasts (5,13). Our findings, however, indicate that the effects of melittin, vasopressin, and insulin on monovalent ion fluxes and DNA synthesis can be clearly dissociated from activation of cellular PLase. Thus, phospholipid deacylation appears not to be required for the activation of early (ion fluxes) or late (DNA synthesis) biochemical events associated with the proliferative response of quiescent fibroblasts.

ACKNOWLEDGMENTS

We thank Drs. C. Mollay and G. Kreil for their gift of purified, PLase-free melittin. T.D. Gelehrter (present address: Dept of Human Genetics, Box 15, University of Michigan Medical School, Ann Arbor, MI, 48109) was a Faculty Scholar, Josiah Macy, Jr., Foundation, and was supported in part by grants CA 22729 and AM 22028 from the National Institutes of Health and PCM 78-07438 from the National Science Foundation.

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