Insulin Stimulation of Na^+-K^+ Pump in Clonal Rat Osteosarcoma Cells

MARIO A. DE LUISE AND MICHAEL HARKER

Clonal osteoblast-like cells derived from a rat osteogenic sarcoma (UMR 106-06) were shown to possess specific, high-affinity binding sites for insulin, with a receptor density of 22,000/cell. The hormone, at physiologic concentrations (1–10 ng/ml), was found to stimulate active K^+ transport into these cells, the effect being mediated via the Na^+-K^+ pump. Alterations in insulin-receptor status by treatment of cells with glucocorticoids or exposure to subphysiologic pH was reflected in parallel changes in the sensitivity of the K^+-uptake process to the hormone. We conclude that insulin can directly affect the metabolism of bone cells and that the hormone's action on transmembrane ion transport may be linked to interaction with its cell surface receptors. *Diabetes* 37:33–37, 1988

Insulin deficiency has been associated with a reduction in bone mass in human type I (insulin-dependent) diabetes mellitus (1–3) and with experimentally induced diabetes in animals (4). The mechanism(s) involved has not been elucidated, but insulin is known to exert direct effects on bone metabolism. Thus, the hormone, at relatively low concentrations, can stimulate collagen synthesis in fetal rat bone (5). At much higher, nonphysiologic levels, insulin can also increase the rate of DNA synthesis in cultured rat calvaria (6). Other potential effects of the peptide on bone cell metabolism have not been investigated to any significant extent. The availability of the well-characterized clonal osteoblast-like cell UMR 106-06, which is responsive to other hormones that control bone turnover (7), has allowed study of direct interaction between insulin and bone cells. This study reports the existence of typical insulin receptors in these cells and the stimulatory effects of physiologic concentrations of the hormone on cation transport at the cell membrane level. Because alterations in Na^+ and K^+ fluxes and Na^+-K^+ pump activity have been implicated at an early phase in the stimulation of cellular proliferation by various growth factors (8,9), the effect of insulin on these ion-transport processes in bone cells may point to a potential mechanism whereby the hormone is involved in normal bone turnover.

From the University of Melbourne, Department of Medicine, Repatriation General Hospital, Heidelberg, Australia.
Address correspondence and reprint requests to Dr. M. De Luise, Department of Medicine, Repatriation General Hospital, Heidelberg 3081, Australia.
Received for publication 22 December 1986 and accepted in revised form 4 June 1987.

MATERIALS AND METHODS

A clonal line of cells originally derived from a ^{32}P-induced rat osteogenic sarcoma, the UMR 106, was kindly provided by T.J. Martin (Univ. of Melbourne, Melbourne, Australia). The UMR 106-06 subclone was used in these studies and maintained in Eagle's minimum essential medium (MEM) with nonessential amino acids, HEPES (20 mM), gentamycin (80 mg/L), and 10% fetal calf serum (FCS) (Flow, Sydney, Australia). The characteristics of these cells have been described previously (7). They are phenotypically osteoblasts and closely resemble normal osteoblasts with regard to their responses to agents such as parathyroid hormone, prostaglandin E_2, and epidermal growth factor, all of which modulate bone turnover (10,11). Cells initially grown in 75-cm^2 plastic culture flasks were subcultured (0.0125% trypsin/0.5 mM EDTA) into 6- or 12-well plates at an initial density of 10^5 cells/well and grown for 24 h in MEM supplemented with 5% FCS. After substitution of serum-free MEM for 24 h, the cell monolayers were used to measure insulin binding and cation transport.

^{125}I-labeled insulin binding. Porcine monocomponent insulin (Novo, Copenhagen) was iodinated to sp act 180 μCi/μg according to the method of Freychet et al. (12). Binding of the hormone to cell monolayers was measured by incubation with tracer amounts of iodinated peptide (0.1–0.2 ng) and increasing concentrations of unlabeled hormone (0.5–1000 ng/ml) at 22°C for 120 min. The incubation buffer consisted of 1 ml Dulbecco's phosphate-buffered saline (PBS) supplemented with $CaCl_2$ (1 mM), $MgCl_2$ (1 mM), dex-

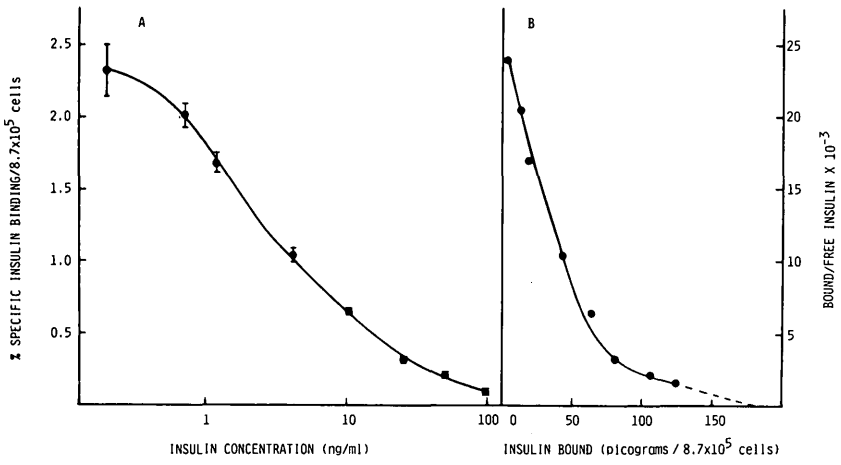

FIG. 1. *A*: specific ^{125}I-insulin binding as function of insulin concentration. Each *point* represents mean ± SE of 4 determinations. Nonspecific binding, 0.066%/well. Insulin concentration for half-maximal inhibition of tracer binding, 3.2 ng/ml. *B*: Scatchard analysis of binding data, with extrapolation to abscissa to give receptor density of 2.2 ± 10^4/cell.

trose (10 mM), and bovine serum albumin (0.3% wt/vol), pH 7.8. Preliminary experiments had indicated that these conditions resulted in an optimal degree of specific binding that reached equilibrium during the specified time. At the end of the incubation, monolayers were washed three times with ice-cold PBS, and cells were solubilized in 0.3 M NaOH for determination of cell-associated radioactivity.

Monovalent cation transport. Potassium uptake by cells was measured by a modification of the method of Rozengurt and Heppel (13), with ^{86}Rb used as a convenient analogue of K^+ (14). Cells growing in serum-free MEM in 12-well plates were exposed to insulin (0–10 ng/ml) for appropriate times, and ^{86}Rb uptake was determined over a 30-min period after addition of the isotope (250,000 dpm/well, sp act 37–296 M Bq/mg) to all wells and of the digitalis glycoside ouabain (final concn 0.5 mM) to half. After three washes of the monolayers with cold isotonic (112 mM) $MgCl_2$ solution, intracellular radioactivity was released into 1 ml of 5% (wt/vol) trichloroacetic acid, and an aliquot of the supernatant was transferred to a liquid scintillation cocktail for counting by a Beckman liquid scintillation spectrometer (L3801).

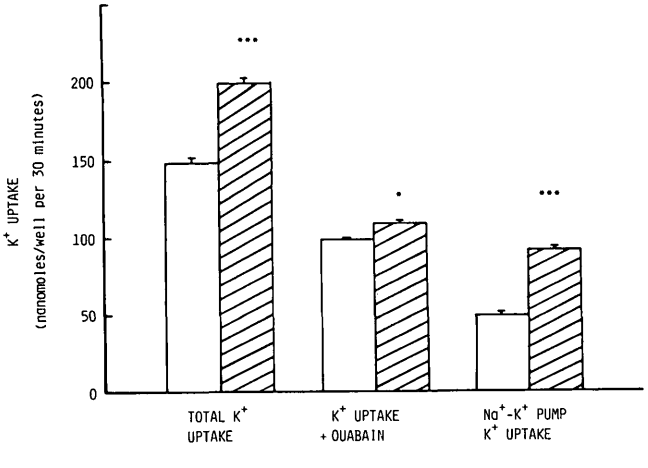

FIG. 2. Potassium uptake by UMR 106-06 cells in multiwell plates determined in presence and absence of ouabain (0.5 mM). *Open bars*, uptake in control cells; *hatched bars*, uptake by cells exposed to insulin (10 ng/ml) for 180 min. Results are means ± SE for 6 replicate wells, each well containing 5.2 × 10^5 cells. *$P < .05$, ***$P < .001$, control vs. insulin treatment.

The rate of uptake of ^{86}Rb in the presence and absence of ouabain has been shown to be linear with time for at least 60 min under the conditions described above. Data are expressed as nanomoles of K^+ taken up per well per 30 min calculated from the known K^+ concentration of the medium (5.4 mM) and the fractional uptake of ^{86}Rb. The activity of the Na^+-K^+ pump is given by the difference in uptake of ^{86}Rb by cells incubated with and without the pump inhibitor ouabain. Cell counts, when required, were performed by treating replicate monolayers with 0.0125% trypsin/0.5 mM EDTA to obtain a single cell suspension and determining cell density by Coulter counter. Cellular protein per well was measured in parallel after suspension of cells in 0.01 M Tris HCl, pH 7.4, and sonication (15). Data are expressed as means ± SE. Statistical analysis employed Student's *t* test for unpaired samples.

RESULTS

Insulin was found to bind specifically and with high affinity to monolayers of UMR 106-06 osteogenic sarcoma cells (Fig. 1). Nonspecific binding was low (<5% of ^{125}I-insulin tracer binding). Half-maximal inhibition of tracer binding was noted at an unlabeled hormone concentration of 3.2 ng/ml. Unrelated peptides (i.e., parathyroid hormone, calcitonin, and ACTH) were found not to compete with ^{125}I-insulin binding to UMR 106-06 cells (data not shown).

Binding of insulin was saturable, and Scatchard analysis of binding data resulted in a curvilinear plot typical of other classic insulin receptors (16; Fig. 1). Extrapolation of the Scatchard curve to the abscissa, assuming a single class of binding sites exhibiting negative cooperativity (17), indicated a receptor density of 2.2 × 10^4/cell in the preparation used in this experiment. In four other experiments, receptor numbers ranged between 1.1 × 10^4 and 1.94 × 10^4/cell.

The insulin receptors on UMR 106-06 cells could also be downregulated. Exposure of cells to insulin (8 ng/ml for 24 h) resulted in a significant ($P < .05$) reduction in specific ^{125}I-insulin binding from 3.5 ± 0.08 to 2.93 ± 0.07%/mg protein at a tracer concentration of 0.13 ng/ml.

The existence of specific insulin receptors in these cells would suggest that the hormone might exert one or more biologic effects on them. We have previously shown that the active transport of monovalent cations in UMR 106-06 cells

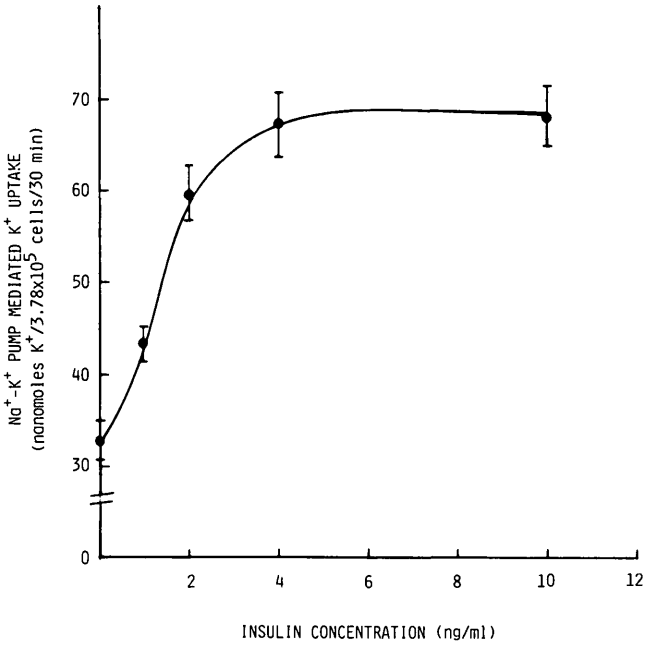

FIG. 3. Dose-response curve for insulin stimulation of Na^+-K^+ pump in UMR 106-06 cells. Duration of insulin exposure was 180 min. Data shown represent difference in K^+ uptake by cells in absence and presence of ouabain. Each *point* is mean ± SE of 5 determinations. Half-maximal stimulation observed at insulin concentration of 1.5 ng/ml.

is under hormonal control, with stimulation of the Na^+-K^+ pump by parathyroid hormone (14). We therefore chose to examine the possibility that insulin may influence ion transport, in addition to the reported stimulation of glucose uptake (18), an effect confirmed in our laboratory (data not shown). Figure 2 shows that the hormone stimulates the active uptake of K^+ against a concentration gradient. Use of ouabain indicates that the effect of insulin is exerted mainly on the Na^+-K^+ pump, which in these cells accounts for 30–40% of total K^+ uptake.

The dose dependence of the insulin-stimulatory effect on the Na^+-K^+ pump activity of UMR 106-06 cells is shown in Fig. 3. A significant ($P < .01$) increase in pump activity (131% of basal) is noted at the lowest concentration used (1 ng/ml), i.e., well within the physiologic range of insulin levels. A maximal stimulatory effect (doubling of basal activity) is seen in the 4–10 ng/ml hormone concentration range.

The data shown in Figs. 2 and 3 were obtained with cells exposed to insulin for 180 min before measurement of K^+ uptake. A smaller but still significant effect of the hormone is measurable at earlier time points (Table 1), i.e., within the first 30 min of the simultaneous addition of ^{86}Rb and insulin. Stimulation persists, and its magnitude is enhanced with more prolonged incubations (up to 24 h).

We then explored the potential involvement of insulin receptors in the hormonal stimulation of Na^+-K^+ pump activity. The dose-response relationship between insulin concentration and either specific binding (Fig. 1) or stimulation (Fig. 3) of Na^+-K^+ pump would suggest that maximal stimulation of K^+ uptake via the pump occurs when only 24% of the receptors are occupied. Such analysis clearly does not prove that the hormone effect is indeed mediated through its receptors. To obtain more direct evidence for such a relationship, two approaches were used to alter receptor status and subsequently determine the effect of such alterations on the insulin sensitivity of the K^+-uptake process.

First, the cells were treated with dexamethasone, a glucocorticoid that has been shown to alter insulin binding in cultured adipocytes (19). Exposure of UMR 106-06 cells to the steroid was found to induce a significant increase in insulin binding (Fig. 4), with no change in cell numbers. The steroid effect on binding was delayed and measurable only after a minimum of 8 h of exposure of cells to dexamethasone. It was also found to depend on the dose of steroid, with a minimum concentration of 10 nM required to produce a significant increase in insulin binding (data not shown). The increase in binding was apparently the result of an increase in receptor numbers with no change in receptor affinity, as indicated by the data shown in Fig. 4 and the parameters derived from Scatchard analysis of the same data (control vs. dexamethasone-treated cells: B_{max} 10.6 vs. 25.9 pg/10^5 cells; K_d, 1.8 vs. 1.9 ml/ng).

The stimulatory effect of insulin on K^+ uptake was found to be enhanced in cells that had previously been exposed to dexamethasone (100 nM for 24 h) and therefore had greater ability to bind ^{125}I-insulin at tracer (0.1 ng/ml) concentrations (Fig. 5, *upper panel*). Such a significant ($P < .01$) enhancement of cation transport was clearly evident at both low and high concentrations of the peptide.

The second approach used to determine the relationship between insulin binding and K^+ transport made use of the observed dependence of binding on pH. When insulin binding and stimulation of K^+ uptake were compared in cells exposed to media of differing pH (7.0 and 7.4), the expected decrease in binding at the lower pH was accompanied by a reduced sensitivity of the transport process to the stimulatory effects of the hormone (Fig. 5, *lower panel*). A significant ($P < .05$) stimulation of uptake was seen at each concentration of insulin in control (pH 7.4) cells, whereas stimulation was only significant at 10 ng/ml insulin in cells exposed to pH 7.0.

DISCUSSION

Our studies demonstrate some novel aspects of insulin interaction with bone cells. First, high-affinity, specific receptors for the hormone have been detected in osteoblast-like

TABLE 1
Time course for insulin stimulation of Na^+-K^+ pump in UMR 106-06 cells

Duration of insulin treatment (h)	Na^+-K^+ pump-mediated K^+ uptake (nmol · well^{-1} · 30 min^{-1})		
	Control	Insulin	Change (%)
0.5	30.88 ± 0.82	42.77 ± 0.93	+38.5
3.5	33.56 ± 1.20	70.88 ± 1.31	+111.2
5.5	36.25 ± 1.81	69.68 ± 1.65	+92.2
24.5	34.42 ± 1.54	97.06 ± 1.59	+182.0

Cell monolayers were exposed to insulin (10 ng/ml) for the stated time intervals, and ^{86}Rb uptake ± ouabain was measured for the final 30 min of incubation. Results are means ± SE of 6 replicate determinations.

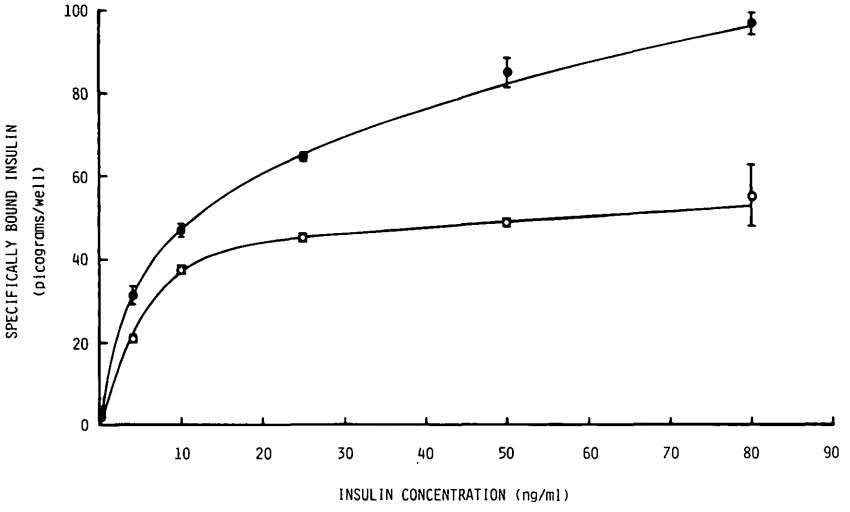

FIG. 4. Effect of dexamethasone on insulin binding to UMR 106-06 cells. Monolayers were treated with dexamethasone (100 nM) for 24 h. Total insulin binding over range of insulin concentrations was calculated from specific ^{125}I-insulin binding. Cell concentration was 6.3 × 10^5/well in control and 6.2 × 10^5/well in dexamethasone-treated plates. Each *point* represents mean ± SE of 4 replicate wells. ○, Control cells (B_{max} 60.5 pg/well, K_d 1.8 ml/ng); ●, dexamethasone-treated cells (B_{max} 140.0 pg/well, K_d 1.9 ml/ng).

cells. In all respects examined (pH dependence, affinity, specificity, and ability to downregulate), such receptors are similar to those of classic insulin target cells such as adipocytes (16). The possibility that their existence in UMR 106-06 cells merely represents an expression of the malignant phenotype cannot be excluded. However, these cells, although clearly neoplastic and derived from an osteogenic sarcoma, have been shown to be a useful and reliable model for normal osteoblasts with regard to the effects of other hormones known to influence bone metabolism (10,11). More important, in preliminary experiments we have demonstrated insulin binding on nonmalignant osteoblasts isolated by sequential collagenase digestion of neonatal mouse calvaria, although receptor density in these cells is somewhat lower (6 × 10^3/cell). Thus, results seen in the much more easily obtainable neoplastic cells can probably be extrapolated to normal osteoblasts.

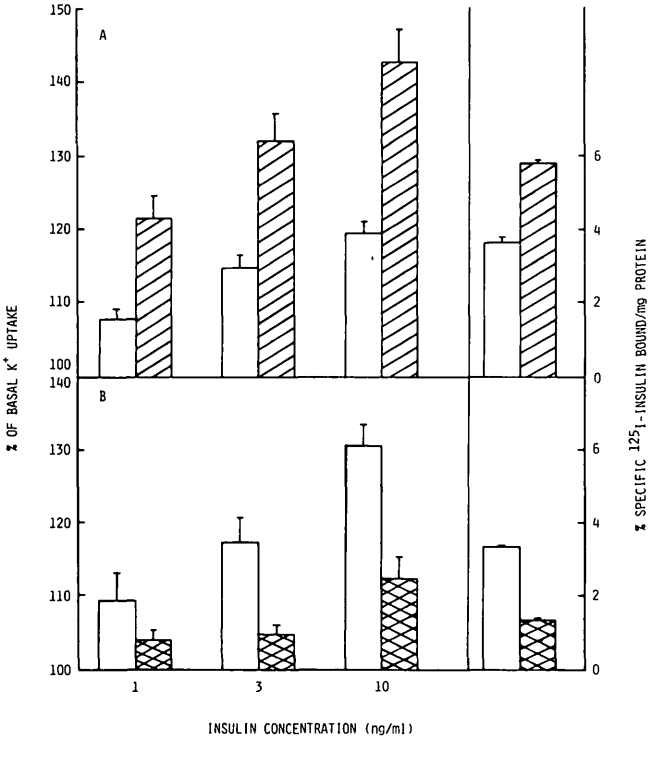

FIG. 5. Correlation between insulin binding and insulin stimulation of total K^+ uptake by UMR 106-06 cells. Specific ^{125}I-insulin binding per mg protein at tracer concentrations (0.1 ng/ml) of ligand is shown at *right* to demonstrate stimulatory effect of glucocorticoids (*A*) and inhibitory effect of low pH (*B*) on binding. In each *panel*, control values are shown by *open bars* and dexamethasone or pH 7.0 values by *hatched bars*. *Left* of each *panel*, percentage increase above basal in K^+ uptake by control (*open bars*) and dexamethasone (*A*)- or pH 7.0 (*B*)-treated (*hatched bars*) cells at different insulin concentrations (1, 3, and 10 ng/ml). Each *bar* represents mean ± SE of 9 replicate wells. Cell densities were 4.9 × 10^5/well in both control and dexamethasone-treated plates (*A*) and 3 × 10^5/well in all other plates (*B*).

In addition to binding to UMR 106-06 cells, insulin has been shown to stimulate active K^+ uptake by the Na^+-K^+ pump, with significant effects observed at hormone concentrations well within the normal physiologic range. The stimulatory effect is observable immediately on exposure of cells to the hormone, a result comparable to the rapid stimulatory action of insulin on glucose transport in its classic target cells. This observation suggests that the mechanism(s) involved in the hormonal stimulation of both transport processes may be similar in some respects. The observations linking insulin-receptor status and the insulin sensitivity of the K^+-uptake process support this contention. An inhibition of hormone binding at subphysiological pH is associated with a marked decrease in its ability to stimulate K^+ uptake, although the basal rate of transport of this ion is not significantly altered at the lower pH.

Treatment of UMR 106-06 cells with glucocorticoids was used initially to possibly reduce insulin binding, as has been observed in other tissues (19). Surprisingly, it was found that dexamethasone induced a time- and dose-dependent increase in insulin-receptor numbers in these cells. The significance of such an effect is not clear at this stage; it has recently been shown, however, that glucocorticoids can exert some anabolic effects on bone (20), and it may be speculated that the increase in insulin binding in steroid-treated osteoblast-like cells may represent such an anabolic action. Whatever the significance, a glucocorticoid-induced increase in insulin binding to UMR 106-06 cells is clearly reflected in a significant increase in the response of the cells to the peptide, at least in terms of K^+ uptake. Taken together, the data shown in Fig. 5 strongly suggest but do not establish

a close relationship between occupancy of receptors by insulin and stimulation of cation transport in osteoblast-like cells.

The significance of an insulin effect on cation transport in bone cells has yet to be determined. Much evidence links increases in Na^+ influx and stimulation of the Na^+-K^+ pump with initiation of DNA synthesis and cellular proliferation (8,9). The observed stimulatory effect of insulin on Na^+-K^+ pump activity in osteoblast-like cells may represent a potential pathway whereby the hormone exerts its anabolic effects on bone (5,6). In a state of insulin deficiency such as type I diabetes, the absence of such a direct effect of the hormone on bone cell metabolism may be manifested in the observed reduction in bone mass.

ACKNOWLEDGMENTS

This study was supported by a grant from the University of Melbourne.

REFERENCES

1. Hui SL, Epstein S, Johnston CC Jr: A prospective study of bone mass in patients with type I diabetes. *J Clin Endocrinol Metab* 60:74–80, 1985
2. Levin ME, Boisseau VC, Avioli LV: Effects of diabetes mellitus on bone mass in juvenile and adult-onset diabetes. *N Engl J Med* 294:241–45, 1976
3. McNair P, Christiansen C, Christensen MS, Madsbad S, Faber OK, Binder C, Transbol I: Development of bone mineral loss in insulin-treated diabetes: a one-and-a-half year follow-up study in sixty patients. *Eur J Clin Invest* 11:55–59, 1981
4. Goodman WG, Hori MT: Diminished bone formation in experimental diabetes: relationship to osteoid maturation and mineralization. *Diabetes* 33:825–31, 1984
5. Kream BE, Smith MD, Canalis E, Raisz LG: Characterization of the effect of insulin on collagen synthesis in fetal rat bone. *Endocrinology* 115:296–302, 1985
6. Canalis E: Effect of insulin-like growth factor I on DNA and protein synthesis in cultured rat calvaria. *J Clin Invest* 66:709–19, 1980
7. Partridge NC, Alcorn D, Michelangeli VP, Ryan GB, Martin TJ: Morphological and biochemical characterization of four clonal osteogenic sarcoma cell lines of rat origin. *Cancer Res* 43:4308–14, 1983
8. Kaplan JG: Membrane cation transport and the control of proliferation of mammalian cells. *Annu Rev Physiol* 40:19–41, 1978
9. Koch KS, Leffert HL: Ionic landmarks along the mitogenic route. *Nature* (Lond) 279:104–105, 1979
10. Forrest SM, Ng KW, Findlay DM, Michelangeli VP, Livesey SA, Partridge NC, Zajac JD, Martin TJ: Characterization of an osteoblast-like clonal cell line which responds to both parathyroid hormone and calcitonin. *Calcif Tissue Int* 37:51–56, 1985
11. Ng KW, Partridge NC, Niall M, Martin TJ: Stimulation of DNA synthesis by epidermal growth factor in osteoblast-like cells. *Calcif Tissue Int* 35:624–28, 1983
12. Freychet P, Roth J, Neville DM Jr: Monoiodo insulin: demonstration of its biological activity and binding to fat cells and liver membranes. *Biochem Biophys Res Commun* 43:400–408, 1971
13. Rozengurt E, Heppel LA: Serum rapidly stimulates ouabain sensitive ^{86}Rb uptake in quiescent 3T3 cells. *Proc Natl Acad Sci USA* 72:4492–95, 1975
14. De Luise M, Harker M: Parathyroid hormone stimulation of the Na^+-K^+ pump in clonal osteosarcoma cells. *J Endocrinol* 111:61–66, 1986
15. Lowry OH, Rosebrough JN, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–75, 1951
16. Olefsky JM, Jen P, Reaven GM: Insulin binding to isolated human adipocytes. *Diabetes* 23:565–71, 1974
17. De Meyts P, Bianco RA, Roth J: Site-site interactions among insulin receptors, characterization of the negative cooperativity. *J Biol Chem* 251:1877–88, 1976
18. Ituarte EA, Ituarte HG, Iida A, Hahn TJ: Insulin binding and stimulation of glucose analog transport in the UMR 106-06 osteoblastic cell line: regulation by insulin (Abstract). In *Proc Am Soc Bone Miner Res, Anaheim, California, 1986*, no. 39
19. Olefsky JM, Johnson J, Liu F, Jen P, Reaven GM: The effects of acute and chronic dexamethasone administration on insulin binding to isolated rat hepatocytes and adipocytes. *Metabolism* 24:517–27, 1975
20. Aubin JE, Bellows CG, Heershe JNM: Dexamethasone increases formation of bone nodules from rat calvaria cells in vitro (Abstract). In *Proc Am Soc Bone Miner Res, Anaheim, California, 1986*, no. 44