0026-895X/94/061215-06$03.00/0

MOLECULAR PHARMACOLOGY, 45:1215–1220, 1994.

Insulin-like Growth Factor-I Induces a Rapid Increase in Calcium Currents and Spontaneous Membrane Activity in Clonal Pituitary Cells

RICHARD H. SELINFREUND and LESLIE A. C. BLAIR

Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510

Received November 23, 1993; Accepted March 4, 1994

SUMMARY

The role of growth factors in the adult brain is largely unknown, although receptors for factors such as insulin-like growth factor-I (IGF-I) have been localized on nondividing mature neurons. Because neurons use the frequency and pattern of action potentials to encode information, we assessed the ability of IGF-I to modulate rapidly the electrical properties of GH_4C_1 cells, a spontaneously active pituitary line with neuronal L- and T-type calcium currents. Electrical quiescence (the absence of spontaneous activity) was induced by culture in serum-depleted conditions. IGF-I, which is synthesized locally in mammalian brain, induced a rapid increase in electrical activity that was accompanied by increased activation of calcium channel currents. These effects were dose and time dependent. The spontaneous activity of cells exposed to 20 ng/ml IGF-I increased in ~10 sec and, after a brief exposure, continued increasing for at least 8 hr. Currents carried by calcium channels doubled within 10 sec. Both the increase in spontaneous activity and the increased activation of calcium channel currents were blocked by tyrosine kinase inhibitors. These results suggest that IGF-I can act as a rapid neuromodulator of calcium currents.

Growth factor receptors, including those for EGF, fibroblast growth factor, insulin, and the IGFs, have been identified on terminally differentiated neurons in the adult central nervous system (1–5). Their effects are not well understood. By contrast, in dividing cells, including neuronal precursors (6, 7), serum-derived growth factors are known to exert a variety of effects (8). Among the delayed responses are the induction of intermediate proto-oncogenes, commitment to cell cycle progression, and cellular division. Rapid responses include pH changes, transient membrane hyperpolarizations, increased intracellular calcium levels, and the activation of enzymes such as phospholipase C-γ and phosphatidylinositol 3-OH kinase (9–11). Which, if any, of these effects occur in nondividing neuronal cells and their importance in cellular functions have yet to be defined. However, it has been recently demonstrated that EGF can activate calcium and calcium-dependent ion channels in non-neuronal cell lines transfected with the EGF receptor (12, 13), suggesting that one possible function of growth factors is a rapid tyrosine kinase-dependent modulation of ion channel activity.

For mature mammalian neurons and endocrine cells, the IGFs are of particular interest. They are widely distributed in the adult brain, are present in high concentrations in the cerebrospinal fluid, and have a family of receptor tyrosine kinases, together implying important roles for IGFs in the central nervous system (1, 2, 14). GH_4C_1 cells, a subclone of GH_3 cells, are derived from an anterior pituitary tumor (15). They were chosen for this study because they express IGF and insulin receptors (16, 17), spontaneous action potential activity, and multiple ionic currents, including L- and T-type calcium currents indistinguishable from those found in sensory and hippocampal neurons (18, 19). Because currents carried by voltage-sensitive ion channels underlie the ability to produce action potentials, we focused on the rapid regulation of ion channel activity by IGF-I, assessing its ability to influence calcium currents and spontaneous electrical behavior.

Experimental Procedures

Materials. GH_4C_1 cells were a gift from Dr. Priscilla Dannies (Yale University). Human recombinant insulin, IGF-I, and IGF-II were from Collaborative Biomedical Products. αMEM (without ribo- and deoxyribonucleosides) and fetal calf serum were from GIBCO.

Cell culture. Control GH_4C_1 cells and stock cultures were maintained in αMEM with 10% fetal calf serum and 2 mM L-glutamine but without ribo- or deoxyribonucleosides. To reduce the high concentrations of growth factors found in standard, high serum media, experi-

This work was supported by National Institutes of Health Grant R01-CA51496 to L.A.C.B.; the salary for R.H.S. was from National Institutes of Health Training Grant T32-NS07136–14.

ABBREVIATIONS: EGF, epidermal growth factor; IGF, insulin-like growth factor; MEM, minimal essential medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

mental cells were synchronized in the G_o/G_1 phase of the cell cycle by mitotic shake and serum arrest (20, 21). Cells became electrically quiescent after two passages in αMEM with 0.1% serum; a single passage in αMEM failed to induce full quiescence. To ensure the absence of synaptic contacts, cells were plated at low density.

Electrophysiology. Electrophysiological properties were monitored by standard patch-clamp techniques (22). The whole-cell configuration in the current-clamp mode was used to record action potential activity, resting membrane potential, and fluctuations in membrane potential. The whole-cell configuration in the voltage-clamp mode was used to assess macroscopic currents. The patch-clamp amplifiers were a List EPC-5 and a List EPC-9, which was used in conjuction with MacIntosh-based data acquisition and analysis software (HEKA; Instrutech Corp.) [low pass filter, 3 kHz (−3 dB, digital Gaussian filter); sample interval, 50 μsec; *P*/4 leak subtraction]. For analysis of kinetic properties, barium currents were evoked by depolarizing voltage pulses (−60 to +40 mV) from a holding potential of −80 mV; for each test cell, currents were recorded immediately before and 10–300 sec after IGF-I addition. The rising phase was fit with a single exponential function. At the end of each test pulse, the membrane potential was returned to −80 mV, revealing tail currents that were fit with the sum of two exponential functions. Each dose-response curve was obtained by a nonlinear, least squares fit of a four-parameter function. This was done using the Levenberg-Marquardt nonlinear curve-fitting procedure in the Kaleidagraph program (Synergy Software), where $y = (I - F)/(1 + ([\text{concentration of factor}]/EC_{50})^{slope}) + F)$, with I being the initial level and F the final saturating level.

Patch electrodes were fabricated from Corning 8161 glass. For current-clamp analysis of membrane fluctuations, the patch electrodes were filled with (in mM) 150 KCl, 10 glucose, 5 HEPES, pH 7.2 (electrode resistance, ~5 MΩ). The extracellular recording saline solution was (in mM) 150 NaCl, 5 KCl, 2 $CaCl_2$, 1.3 $MgCl_2$, 10 glucose, 5 HEPES, pH 7.2. For analysis of calcium/barium currents, the pipet solution was (in mM) 140 *N*-methyl-D-glucamine, 2 Mg-ATP, 10 EGTA, 10 HEPES, pH 7.2; the extracellular saline solution was (in mM) 105 NaCl, 10 $BaCl_2$ or 1.6 $CaCl_2$, 1.3 $MgCl_2$, 30 tetraethylammonium chloride, 5 4-aminopyridine, 10 HEPES, pH 7.2. Under these conditions, calcium channel currents could be recorded for 5 min without displaying significant 'rundown.'

Tyrosine kinase inhibitors. Genistein was dissolved in 20% dimethylsulfoxide at a concentration of 3.5 mM and then diluted into culture medium. With a final concentration of genistein of 7 μM, the final dimethylsulfoxide concentration was 0.04%; this was the highest concentration and was used for inhibitor vehicle controls.

Results

One of the most important neuronal functions is the ability to carry information encoded in the frequency and pattern of action potential firing. Factors that modulate the underlying ionic currents could regulate the activity of the cells directly exposed to the factor and, through this regulation, indirectly alter the activity of neural circuits or release patterns in endocrine cells. To assess the ability of growth factors to rapidly regulate ionic currents, isolated cells were maintained under minimal serum conditions (0.1%) before testing to avoid preexposure to the high and potentially saturating concentrations of factors found in serum (Fig. 1) (20, 21). We found that growth under these conditions both blocked spontaneous action potential activity (Fig. 1, A and B) and significantly reduced the voltage-sensitive currents that underlie the action potential. However, the resting electrophysiological properties (input resistance and resting potential), which are often considered indicators of cellular fitness, did not differ significantly from those of cells grown under high serum conditions. Values for input resistance were 1.24 ± 0.10 GΩ (mean ± standard error,

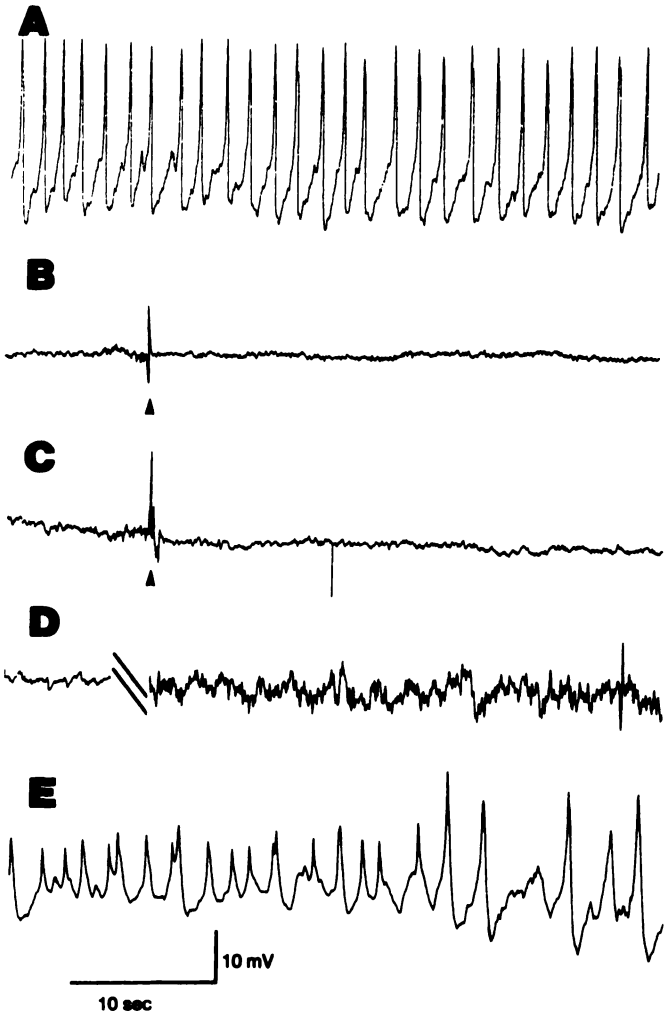

Fig. 1. IGF-I rapidly increases electrical activity in GH_4C_1 pituitary cells. A, GH_4C_1 cells maintained under high serum culture conditions fire spontaneous action potentials. Shown is the activity in a single cell; recordings were also made from an additional six cells, all of which displayed similar behavior. B-E, Before testing, GH_4C_1 cells were maintained under reduced growth factor (low serum) conditions. B, Cells plated in 0.1% serum are electrically silent. Addition of vehicle alone is without effect; *arrowhead* indicates the brief movement artifact associated with the sham addition (n = 13 cells, all electrically inactive and all failing to respond to vehicle). C, Low concentrations of IGF-I (0.2 ng/ml) fail to induce activity. Data show a continuous recording from one cell before, during (*arrowhead*), and after growth factor addition (n = 3 cells, with none responding). D, IGF-I at 20 ng/ml rapidly increases electrical activity. Shown are measurements from one cell immediately before and 20–55 sec after IGF-I addition (n = 8 cells, all responding). E, Electrical activity dramatically increases with increasing time after a brief exposure. Shown is the activity occurring 8 hr after a 10-min exposure to 20 ng/ml IGF-I (n = 6 cells, all responding). *Calibrations*: *vertical*, 10 mV; *horizontal*, 10 sec.

n = 6 cells grown under high serum conditions) and 1.35 ± 0.16 GΩ (n = 12 serum-depleted cells), and values for resting potential were −45 ± 4 mV (n = 7 cells grown under high serum conditions) and −45 ± 4 mV (n = 13 serum-depleted cells). Similar values have been reported for GH_4C_1 cells maintained under standard conditions (18).

It was found that physiological concentrations of IGF-I rapidly and dramatically increased spontaneous electrical fluctuations in isolated cells. The effects were dose responsive, with very low doses (0.2 ng/ml final concentration) being unable to

induce detectable activity (Fig. 1C) while higher concentrations (2–20 ng/ml) significantly increased both small fluctuations in membrane potential and small spontaneous spike-like activity (Figs. 1, D and E, and 2). Sham bath changes produced no effect (Fig. 1B).

The rapid increase in electrical activity was quantitated by two means. First, the ability of a factor to induce small spontaneous depolarizations within several minutes of IGF-I addition was measured (Fig. 2). For cells grown under high serum conditions, action potentials were small (~40-mV), long-duration (300–500-msec) events (Fig. 1A). To assess rapid IGF-I effects in cells grown under reduced serum conditions, small spike-like events were defined as spontaneous depolarizations of 10–30 mV, lasting 300–500 msec. No spikes occurred before IGF-I addition (Fig. 1B) or after addition of IGF-I at very low concentrations (0.2–2 ng/ml) (Fig. 2). However, using 20–200 ng/ml IGF-I, spontaneous depolarizations were induced within 2–4 min of exposure. During this time period, all depolarizing activity remained small, with few events of >20 mV and none approaching a full action potential. All responses appeared to be mediated by IGF-I receptors; the half-maximal dose for IGF-I was near 8 ng/ml (i.e., 1 nM). Moreover, insulin, which could also increase small spike-like activity, was 2 orders of magnitude less potent, suggesting that its effects were mediated through IGF-I receptors (23). IGF-II (20 ng/ml) was ineffective ($n = 7$ cells) (data not shown).

The response to growth factors was also evaluated by quantitating the rapid increase in small membrane potential fluctuations (see Fig. 1D). IGF-I (2–200 ng/ml) consistently produced this effect, which was evident within seconds of exposure. Before addition of 20 ng/ml IGF-I, rhythmic 5–10-mV fluctuations were not detectable; by 2 min after addition, fluctuations had risen to 30 ± 8/min (mean ± standard error; $n = 6$ cells). When lower (2 ng/ml) doses were used, smaller fluctuations, typically <5 mV, were induced during the first 2 min (2.4 ± 1.7/min; $n = 5$ cells). At very low concentrations (0.2 ng/ml), no fluctuations were observed during the recording period (3–7 min; $n = 3$ cells). In addition, it was also found that the time to onset of a response was dose dependent. With 2 ng/ml IGF-I, no spike-like activity occurred during the ~5-min recording periods and 49 ± 24 sec elapsed before the onset of small fluctuations ($n = 5$ cells). However, when a quiescent cell was exposed to 20 ng/ml IGF-I, small fluctuations increased in as little as 2 sec (12 ± 2 sec) and small spike-like activity was induced within 4 min (1.3 ± 0.5 min; $n = 6$ cells).

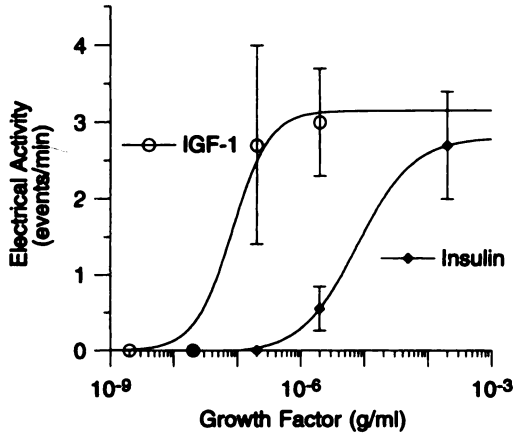

Fig. 2. Dose dependence of the rapid IGF-I- and insulin-induced rise in membrane fluctuations. The activity of GH_4C_1 cells plated in low serum was monitored continuously before, during, and after the bath addition of either IGF-I or insulin. The rate of small spontaneous spike-like events was averaged over the 2 min preceding growth factor addition and 2–4 min after addition. Acceptable activity was based on the small (40-mV), long duration (300–500-msec) action potentials observed in cells cultured under high serum conditions. For the test cells, an event was scored when a 300–500-msec depolarization exceeded 10 mV. Before factor addition, no activity occurred. However, within 2–4 min of IGF-I (○) or insulin (◆) addition, small spike-like activity was initiated. Typically, these small depolarizations were one fourth to one half the magnitude of a full action potential. Data for each point are from three to eight cells and represent the mean ± standard error. *Curves* were obtained by nonlinear, least squares fits of a four-parameter function, giving EC_{50} values of 8 ± 4 nM for IGF-I and 780 ± 100 nM for insulin.

The activity induced by IGF-I began within several minutes of growth factor addition, increased with time, and was sustained for hours. In all cells, small fluctuations preceded the induction of small spike-like activity and the spike-like activity increased with increasing time after factor addition. When cells incubated for 1 hr with 20 ng/ml IGF-I were compared with those incubated for 3 min with the factor, the magnitude and frequency of small spike-like activity increased 1.4- and 4-fold, respectively ($n = 6$ cells at 3 min in IGF-I; $n = 4$ cells at 1 hr). This increased activity occurred in the absence of detectable effects on either resting potential or input resistance. Before IGF-I addition, these were −45 ± 4 mV ($n = 13$ cells) and 1.40 ± 0.21 GΩ ($n = 6$ cells), respectively. Indistinguishable values of −42 ± 5 mV and 1.37 ± 0.20 GΩ, respectively, were obtained when the same cells were examined after addition of 20 ng/ml IGF-I. Membrane fluctuations not only increased during extended exposure to IGF-I but also increased after a brief exposure. When cells were tested 6–8 hr after a 10-min exposure to 20 ng/ml IGF-I, spontaneous depolarizing activity was high, with all cells producing numerous small spikes as well as occasional action potential-like events ($n = 6$ cells) (Fig. 1E).

In GH_4C_1 cells, unlike the closely related GH_3 cells, the rising (depolarizing) phase of the action potential results from calcium influx through voltage-sensitive calcium channels (18). We found that IGF-I induced a rapid and substantial increase in calcium channel currents (Fig. 3). Experiments were performed using either calcium or barium as the charge carrier. Currents were elicited by depolarizing voltage-clamp steps from a holding membrane potential of −80 mV. It was found that within 10 sec of exposure to IGF-I (20 ng/ml) the magnitude of the currents increased dramatically (Fig. 3, A and B) and, additionally, that this increase occurred consistently over a wide range of membrane potentials (−60 to +40 mV) (Fig. 3C). The increases occurred without perceptible changes in kinetic properties, including the voltage dependence of activation (Fig. 3C), the rates of current rise or tail current decay (Table 1), and the peak current potential (before IGF-I, −5.0 ± 3.4 mV; after IGF-I, −5.8 ± 3.3 mV; $n = 6$ cells).

The rapid increase in electrical activity in response to IGF-I suggests a relatively direct mechanism of controlling ion channel behavior. The absence of synaptic interactions eliminates the possibility that IGF-I secondarily modulated calcium currents through modification of synaptic behavior. However, both insulin and IGF-I receptors possess intrinsic tyrosine kinase activity, as well as the ability to activate other intracellular tyrosine kinases. To assess whether transduction of the rapid IGF-I effects involves tyrosine phosphorylation, we used genistein, a membrane-permeant tyrosine kinase inhibitor. To allow

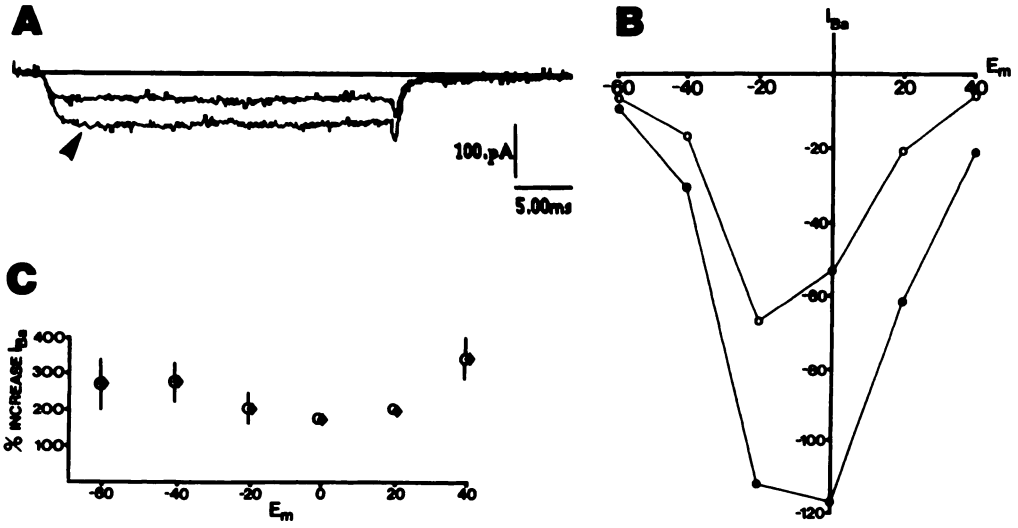

Fig. 3. IGF-I induces a rapid increase in voltage-activated barium currents. A, Ten seconds after addition of 20 ng/ml IGF-I, barium currents carried through calcium channels are 2-fold larger than before addition (before addition, −53 pA; after addition, −117 pA, *arrowhead*). Currents were evoked by 30-msec depolarizing voltage steps from −80 to 0 mV. B, Membrane current-voltage relation for the same cell before (○) and 10 sec after (●) IGF-I addition. C, Both peak (○) and plateau (◆) currents significantly and similarly increase within 10 sec of IGF-I addition ($n = 6$ cells analyzed over the full voltage range, both before and after IGF-I addition). Values are expressed as the percentage increase in current (mean ± standard error). At all potentials, errors for peak and plateau measurements overlapped; for test potentials of 0 and +20 mV, errors were within the size of the symbols denoting the means. The variability between the percentage increases at different membrane potentials was insignificant ($p > 0.1$).

TABLE 1

IGF-I does not alter kinetics of barium currents

Barium currents were evoked by depolarizing voltage pulses from a holding potential of −80 mV, immediately before and 10 sec after IGF-I (20 ng/ml) addition. The rising phase was fit with a single exponential function and, at any given test potential, there was no statistical difference between the time constants measured before and after exposure to IGF-I ($p > 0.6$). At the end of each test pulse, the membrane potential was returned to −80 mV, revealing tail currents that were fit with the sum of two exponential functions. Again, differences were insignificant ($p > 0.5$) ($n = 4$ cells analyzed over the full voltage range, both before and after IGF-I addition).

V_{test}	τ_{rise}		$\tau_{tail_{fast}}$		$\tau_{tail_{slow}}$	
	Before IGF-I	After IGF-I	Before IGF-I	After IGF-I	Before IGF-I	After IGF-I
mV	*msec*		*msec*		*msec*	
−20	2.0 ± 0.8	2.1 ± 0.8	0.8 ± 0.1	0.9 ± 0.2	1.9 ± 0.1	2.3 ± 0.7
0	1.3 ± 0.6	1.3 ± 0.6	0.8 ± 0.2	0.9 ± 0.3	1.8 ± 0.2	2.1 ± 0.5
20	0.6 ± 0.1	0.6 ± 0.04	0.7 ± 0.04	0.7 ± 0.03	2.0 ± 0.1	1.9 ± 0.3
40			0.8 ± 0.1	0.7 ± 0.1	1.8 ± 0.4	1.8 ± 0.4

access of the inhibitor to its potential intracellular targets, electrically quiescent cells were preincubated for 2 hr in 0.4 or 7 μM genistein or in the vehicle control, and then assessed before, during, and after addition of IGF-I (20 ng/ml). It was found that exposure to low concentrations of genistein (0.4 μM) partially inhibited the IGF-I response ($n = 2$ cells) (Fig. 4A). However, in all cells tested, normal doses (7 μM) fully blocked the IGF-I-dependent increase in spontaneous activity (electrical activity measured as in Fig. 2 was 0.0 both before and after IGF-I treatment; $n = 4$ cells) (Fig. 4B). Similarly, 7 μM genistein also prevented the IGF-I-induced increase in barium currents in all pretreated cells (before IGF-I, −74 ± 12 pA; 10 sec and 2 min after IGF-I, −71 ± 13 pA and −71 ± 11 pA, respectively; $n = 7$ cells) (Fig. 4C).

Several potential nonspecific effects of genistein and its vehicle were eliminated. The possibility of direct inhibitor-ion channel interactions was evaluated by examining whether the effective genistein concentration (7 μM) exerted a rapid block of IGF-I responses inconsistent with the time necessary for the inhibitor to enter the cells and complex with its targets. When genistein was added to quiescent cells 1 min before IGF-I, no effect on the subsequent IGF-I-dependent increase in membrane activity was seen in any cell ($n = 5$ cells). Furthermore, genistein caused no alteration in spike activity in control GH_4C_1 cells grown under high serum conditions, where spike height, duration, and firing frequency were examined ($n = 2$ pretreated cells and 7 untreated cells). Finally, a 2-hr exposure to vehicle alone exerted no effect on IGF responsiveness ($n = 5$ cells, all cells responding). Together, these results suggest that the inhibitor effects were specific and that the rapid IGF-I-induced increase in activity requires at least one tyrosine phosphorylation step.

Discussion

The role of growth factors in non-neuronal cells is well established. Typically, the rapid alkalinization of the cells, activation of nuclear binding proteins, and induction of cell cycle progression are followed by cellular division (8). It is also known that many of these factors are present in the mature central nervous system and that nondividing, differentiated neurons have membrane-associated growth factor receptors that bind factors such as IGF-I and EGF (1, 2, 3). Although the role or roles that these factors play in the function of

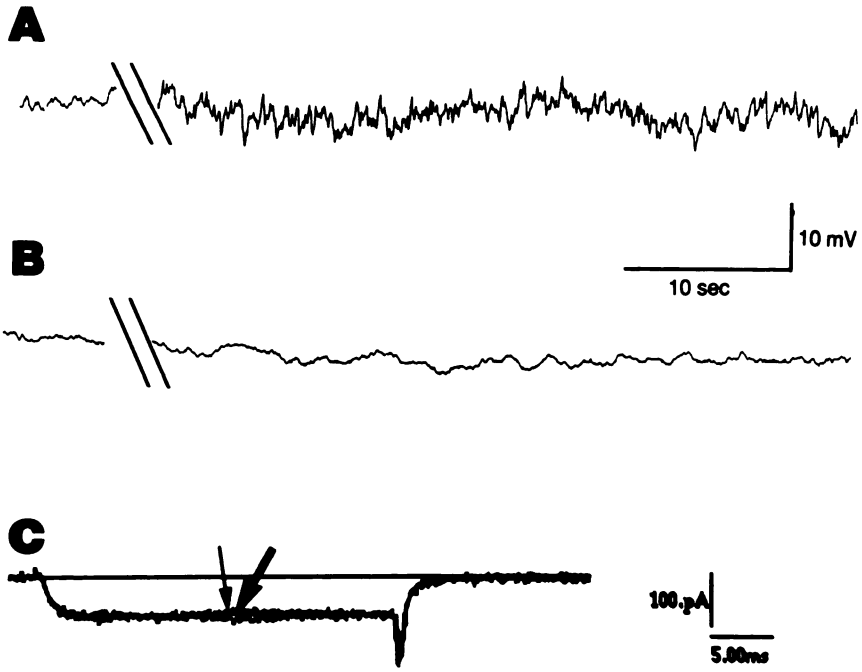

Fig. 4. Genistein, a membrane-permeant tyrosine kinase inhibitor, blocks the IGF-I response. Cells were preincubated with genistein for 2 hr at 37°. Activity was then continuously monitored before, during, and for up to 15 min after bath addition of IGF-I (20 ng/ml). A, Low concentrations of genistein (0.4 μM) reduce but fail to prevent an IGF-I response. Shown here is the activity of a cell immediately before and 3 min after IGF-I addition (n = 2 cells). B, Genistein (7 μM) entirely blocks the IGF-I-induced increase in membrane fluctuations. Again, the recording shows activity before and 3 min after IGF-I (n = 4 cells, with none responding to IGF-I). The resting membrane potential in the absence of genistein addition was −45 ± 7 mV and 2 hr after genistein addition was −37 ± 6 mV (mean ± standard error). *Calibrations*: *vertical*, 10 mV; *horizontal*, 10 sec. C, Genistein (7 μM) also eliminates the IGF-I-induced increase in barium currents. As in Fig. 3A, currents were evoked by 30-msec depolarizing voltage steps from −80 to 0 mV, and were measured before (−88 pA), and 10 sec (−88 pA) (*large arrow*) and 2 min (−83 pA) (*small arrow*) after IGF-I addition (n = 7 cells, none responding). *Calibrations*: *vertical*, 100 pA; *horizontal*, 5 msec.

differentiated neurons are largely undetermined, studies using embryonic neurons have suggested potential roles for the insulin family of factors: Insulin increases fetal retinal transmission (7), IGF-I stimulates RNA synthesis (24), and IGF-II stimulates neurite outgrowth (25).

Surprisingly, the IGF-I effects observed here were very rapid. In contrast to its well described effects on RNA and DNA synthesis, the actions of IGF-I on neuronal calcium currents and membrane electrical activity could be detected within seconds of addition and imply that IGF-I may act as a neuromodulator. Moreover, activity was elevated for at least 8 hr after a brief exposure to IGF-I, suggesting a potential role in the long term regulation of electrical excitability and an effect parallel to the sustained modification of neuronal activity observed during long term potentiation. Although a number of G protein-coupled receptors, such as muscarinic acetylcholine, α-adrenergic, and bradykinin receptors, have been shown to modulate neuronal calcium currents, they most commonly cause rapid and rapidly reversible decreases in current levels (26, 27). In contrast, β-adrenergic stimulation has been reported to enhance calcium currents in hippocampal neurons and cardiac myocytes, although again the effect is rapidly reversible (28–31). Here, the IGF-I effect appears to be rapid, sustained, and specific; IGF-I increased the activity of calcium channels without detectably altering resting membrane properties. Morever, the kinetic properties of the calcium/barium currents appeared unchanged, suggesting that a primary effect of IGF-I may be to increase the number of available channels, possibly by converting 'silent' channels to an active state (32). Additionally, because cells were grown at low density and only isolated cells were tested, the effects could not have arisen from transsynaptic regulation. Finally, the effects appear to be mediated through IGF-I receptors: Low nanomolar concentrations of IGF-I were effective, whereas physiological concentrations of IGF-II were ineffective and insulin was effective only at the very high concentrations that activate IGF-I receptors (23).

Our results also indicate that, similar to the known effects of IGF-I on cellular division, the rapid influence on electrical activity observed here is mediated through tyrosine kinase activity. Candidate molecules for tyrosine phosphorylation include the intracellular intermediates known to be phosphorylated during mitogenic responses as well as the IGF-I receptor itself, which autophosphorylates on specific residues (33–35). A further potential target for tyrosine phosphorylation is the neuronal calcium channel; the α_1 subunit of the L-type channel contains tyrosine residues that may serve as phosphorylation sites. Alternatively, or in addition, serine/threonine phosphorylation events may be involved; using GH_3 cells, Armstrong and Eckert (36) demonstrated a requirement for cAMP-dependent phosphorylation in the maintenance of calcium channel activity.

Although multiple protein tyrosine kinase genes are expressed in the adult nervous system (37), their functions remain largely unknown. Tyrosine phosphorylation has been associated with long term potentiation (38), as well as with synaptic vesicles and neurotransmitter release (39). Recently, Peppelenbosch *et al.* (12, 13) demonstrated that tyrosine kinase activity is necessary for rapid EGF-induced activation of voltage-insensitive calcium channels in A431 fibroblast cells and in P19 undifferentiated embryonal cells transfected with the EGF receptor. Additionally, Wijetunge *et al.* (40) have reported a very rapid block of smooth muscle calcium currents by tyrosine kinase inhibitors. Our results using GH_4C_1 endocrine cells indicate that IGF-I-dependent tyrosine phosphorylation may mediate a potentially similar modulation of apparently neuronal types of voltage-sensitive calcium channels, and suggest that tyrosine kinase-associated growth factor receptors may utilize overlapping mechanisms to regulate ion channel behavior.

Acknowledgments

We thank Dr. Len Kaczmarek for providing space and equipment for the initial experiments, Dr. Priscilla Dannies for the gift of the GH_4C_1 cells, Dr. Murdock Ritchie for the loan of an EPC-5 amplifier, Drs. Fred Sigworth and Nathan Schoppa for the loan of an EPC-9 amplifier, and Drs. Mark Leibowitz, James Howe, and Jack Pledger for helpful comments on the manuscript.

References

1. Quirion, R., D. Araujo, N. P. V. Nair, and J. Chabot. Visualization of growth factor receptor sites in rat forebrain. *Synapse* **2**:212–218 (1988).
2. Kar, S., J.-G. Chabot, and R. Quirion. Quantitative autoradiographic localization of [^{125}I]insulin-like growth factor I, [^{125}I]insulin-like growth factor II, and [^{125}I]insulin receptor binding sites in developing and adult rat brain. *J. Comp. Neurol.* **333**:375–397 (1993).
3. Fallon, J. H., K. B. Seroogy, S. E. Loughlin, R. S. Morrison, R. A. Bradshaw, D. J. Knauer, and D. D. Cuningham. Epidermal growth factor immunoreactive material in the central nervous system: location and development. *Science (Washington D. C.)* **224**:1107–1109 (1984).
4. Gospodarowicz, D., J. Cheng, G. Liu, A. Baird, and P. Bohlen. Isolation of brain fibroblast growth factor by heparin-Sepharose affinity chromatography: identity with pituitary fibroblast growth factor. *Proc. Natl. Acad. Sci. USA* **91**:6963–6967 (1984).
5. Go'mez-Pinilla, F., J. W. Lee, and C. W. Cotman. Basic FGF in adult brain: cellular distribution and response to entorhinal lesion and fimbria-fornix transection. *J. Neurosci.* **12**:345–355 (1992).
6. Barde, Y. A., D. Edgar, and H. Thoenen. Sensory neurons in culture: changing requirements for survival factors during embryonic development. *Proc. Natl. Acad. Sci. USA* **77**:1199–1203 (1980).
7. Puro, D. G., and E. Agardh. Insulin-mediated regulation of neuronal maturation. *Science (Washington D. C.)* **225**:1170–1172 (1984).
8. Baserga, R. *The Biology of Cell Reproduction*. Harvard University Press, Cambridge, MA (1985).
9. Ruderman, N. B., R. Kapeller, M. F. White, and L. C. Cantley. Activation of phosphatidylinositol 3-kinase by insulin. *Proc. Natl. Acad. Sci. USA* **87**:1411–1415 (1990).
10. Kapeller, R., K. S. Chen, M. Yoakim, B. S. Schaffhausen, J. Backer, M. F. White, L. C. Cantley, and N. B. Ruderman. Mutations in the juxtamembrane region of the insulin receptor impair activation of phosphatidylinositol 3-kinase by insulin. *Mol. Endocrinol.* **5**:769–777 (1991).
11. Jackson, T. R., L. R. Stephens, and P. T. Hawkins. Receptor specificity of growth factor-stimulated synthesis of 3-phosphorylated inositol lipids in Swiss 3T3 cells. *J. Biol. Chem.* **267**:16627–16636 (1992).
12. Peppelenbosch, M. P., L. G. J. Tertoolen, and S. W. deLaat. Epidermal growth factor-activated calcium and potassium channels. *J. Biol. Chem.* **266**:19938–19944 (1991).
13. Peppelenbosch, M. P., L. G. J. Terloolen, J. den Hertog, and S. W. De Laat. Epidermal growth factor activates calcium channels by phospholipase A_2/5-lipoxygenase-mediated leukotriene C_4 production. *Cell* **69**:295–303 (1992).
14. LeRoith, D., C. T. Roberts, H. Werner, C. Bondy, M. Raizada, and M. L. Adamo. Insulin-like growth factors in the brain, in *Neurotrophic Factors* (S. E. Loughlin and J. H. Fallon, eds.). Academic Press, San Diego, 391–414 (1993).
15. Dannies, P. S., and A. H. Tashjian, Jr. Effects of thyrotropin-releasing hormone and hydrocortisone on synthesis and degradation of prolactin in a rat pituitary cell strain. *J. Biol. Chem.* **248**:6174–6179 (1973).
16. Kiino, D. R., and P. S. Dannies. Insulin and 17β-estradiol increase the intracellular prolactin content of GH_4C_1 cells. *Endocrinology* **109**:1264–1269 (1981).
17. Yamasaki, H., D. Prager, S. Gebremedhin, L. Moise, and S. Melmed. Binding and action of insulin-like growth factor I in pituitary tumor cells. *Endocrinology* **128**:857–862 (1991).
18. Dubinsky, J. M., and G. S Oxford. Ionic currents in two strains of rat anterior pituitary tumor cells. *J. Gen. Physiol.* **83**:309–339 (1984).
19. Cohen, C. J., and R. T. McCarthy. Nimodipine block of calcium channels in rat anterior pituitary cells. *J. Physiol. (Lond.)* **387**:195–225 (1987).
20. Ashihara, T., and R. Baserga. Cell synchronization. *Methods Enzymol.* **58**:248–262 (1979).
21. Terasima, T., and L. J. Tolmach. Growth and nucleic acid synthesis in synchronously dividing populations of HeLa cells. *Exp. Cell Res.* **30**:344–362 (1963).
22. Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391**:85–100 (1981).
23. Cohick, W. S., and D. R. Clemmons. The insulin-like growth factors. *Annu. Rev. Physiol.* **55**:131–153 (1993).
24. Burgess, S. K., S. Jacobs, P. Cuatrecasas, and N. Sahyoun. Characterization of a neuronal subtype of insulin-like growth factor I receptor. *J. Biol. Chem.* **262**:1618–1622 (1987).
25. Recio-Pinto, E., M. M. Rechier, and D. N. Ishii. Effects of insulin, insulin-like growth factor-II, and nerve growth factor on neurite formation and survival in cultured sympathetic and sensory neurons. *J. Neurosci.* **6**:1211–1219 (1986).
26. Bernheim, L., D. J. Beech, and B. Hille. A diffusible second messenger mediates one of the pathways coupling receptors to calcium channels in rat sympathetic neurons. *Neuron* **6**:859–867 (1991).
27. Ewald, D. A., I.-H. Pang, P. C. Sternweis, and R. J. Miller. Differential G protein-mediated coupling of neurotransmitter receptors to Ca^{2+} channels in rat dorsal root ganglion neurons *in vitro*. *Neuron* **2**:1185–1193 (1989).
28. Gray, R., and D. Johnston. Noradrenaline and β-adrenoceptor agonists increase activity of voltage-dependent calcium channels in hippocampal neurons. *Nature (Lond.)* **327**:620–622 (1987).
29. Reuter, H. Calcium channel modulation by neurotransmitters, enzymes and drugs. *Nature (Lond.)* **301**:569–574 (1983).
30. Yatani, A., and A. M. Brown. Rapid β-adrenergic modulation of cardiac calcium channel currents by a fast G protein pathway. *Science (Washington D. C.)* **245**:71–74 (1989).
31. Hill-Smith, I., and R. D. Purves. Synaptic delay in the heart: an ionophoretic study. *J. Physiol. (Lond.)* **279**:31–54 (1978).
32. Strong, J. A., A. P. Fox, R. W. Tsien, and L. K. Kaczmarek. Stimulation of protein kinase C recruits covert calcium channels in *Aplysia* bag cell neurons. *Nature (Lond.)* **325**:714–717 (1987).
33. Fantl, W. J., J. A. Escobedo, G. A. Martin, C. W. Turck, M. del Rosario, F. McCormick, and L. T. Williams. Distinct phosphotyrosines on a growth factor receptor bind to specific molecules that mediate different signalling pathways. *Cell* **69**:413–423 (1992).
34. Cantley, L. C., K. R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, and S. Soltoff. Oncogenes and signal transduction. *Cell* **64**:281–302 (1991).
35. Pawson, T. Non-catalytic domains of the cytoplasmic protein-tyrosine kinases: regulatory elements in signal transduction. *Oncogene* **3**:491–495 (1988).
36. Armstrong, D., and R. Eckert. Voltage-activated calcium channels that must be phosphorylated to respond to membrane depolarization. *Proc. Natl. Acad. Sci. USA* **84**:2518–2522 (1987).
37. Lai, C., and G. Lemke. An extended family of protein-tyrosine kinase genes differentially expressed in the vertebrate nervous system. *Neuron* **6**:691–704 (1991).
38. O'Dell, T. J., E. R. Kandel, and S. G. N. Grant. Long-term potentiation in the hippocampus is blocked by tyrosine kinase inhibitors. *Nature (Lond.)* **353**:558–560 (1991).
39. Pang, D. T., J. K. Wang, F. Valtorta, F. Benfenati, and P. Greengard. Protein tyrosine phosphorylation in synaptic vesicles. *Proc. Natl. Acad. Sci. USA* **85**:762–766 (1988).
40. Wijetunge, S., C. Aalkjaer, M. Schachter, and A. D. Hughes. Tyrosine kinase inhibitors block calcium channel currents in vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **189**:1620–1623 (1992).

Send reprint requests to: L. A. C. Blair, Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06520–8066.