An Insulin-sensitive Cation Channel Controls $[\text{Na}^+]_i$ via $[\text{Ca}^{2+}]_o$ -regulated Na^+ and Ca^{2+} Entry

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The insulin-stimulated cation channel previously identified in patch-clamped muscle preparations is here shown to be responsible for bulk Na^+ entry into the cell. The mainly Na^+ current of the channel was shown to be accompanied by an inhibitory Ca^{2+} component responsible for oscillations. Here, using quantitative fluorescence imaging of Fura-2- and SBFI-loaded soleus muscle, we measure changes in $[\text{Na}^+]_i$ and $[\text{Ca}^{2+}]_i$ related to channel function. Insulin increased $[\text{Na}^+]_i$ and $[\text{Ca}^{2+}]_i$ in a transient spike of <1-min duration. There was a momentary dip in $[\text{Na}^+]_i$ related to inhibition of the channel by the Ca^{2+} spike, and changes in external Ca^{2+} were shown to alter $[\text{Na}^+]_i$ via the cation channel, all effects being blocked by the specific channel inhibitor μ -conotoxin, but not by tetrodotoxin. The $[\text{Ca}^{2+}]_i$ spike could also be induced by 8-bromo cyclic-guanosine 5'-monophosphate, an analogue of the channel-activator cyclic-guanosine 5'-monophosphate (cGMP). In addition it was noted that insulin reduced the $[\text{Ca}^{2+}]_i$ rise upon subsequent muscle depolarization by a factor of 3.5. Insulin could be substituted with phorbol ester for the same effect and HA1004, a protein kinase inhibitor, blocked the reduction.

INTRODUCTION

In this paper we show that an insulin-stimulated cation channel, previously identified in skeletal muscle (McGeoch and Guidotti, 1992), is a major vehicle for the Na^+ entry that sustains the activity of the (Na^+ , K^+)ATPase (Na^+ pump). There are many possible ways in which Na^+ can enter cells: Na^+/H^+ exchangers (Aronson and Boron, 1986), $\text{Na}^+/\text{Ca}^{2+}$ exchangers (Allen *et al.*, 1989), $\text{Na}^+/\text{K}^+/\text{Cl}^-$ cotransporters (Haas, 1989), $\text{Na}^+/\text{solute}$ cotransporters (Schultz, 1986), voltage-gated tetrodotoxin (TTX)-sensitive Na^+ channels (Trimmer and Agnew, 1989), and nonspecific cation channels (Penner *et al.*, 1988). The $\text{Na}^+/\text{Ca}^{2+}$ exchanger would be limited in this role by the small amount of available internal Ca^{2+} . When insulin is employed to stimulate cation transport in skeletal muscle, there is a lack of involvement in most of these channels (Clausen and Flatman, 1987; Hsu and Guidotti, 1991), but patch-clamp experiments by McGeoch and Guidotti (1992) identified an insulin-sensitive cation channel. The realization that the channel played a major role in Na^+ entry and controlled baseline $[\text{Na}^+]_i$ came from 1) monitoring channel-dependent changes in $[\text{Na}^+]_i$ and $[\text{Ca}^{2+}]_i$

on insulin addition and 2) monitoring channel-dependent changes in $[\text{Na}^+]_i$ and $[\text{Ca}^{2+}]_i$ related to changes in $[\text{Ca}^{2+}]_i$ and $[\text{Ca}^{2+}]_o$. Ca^{2+} is the prime regulator of the channel and we show that the action of insulin, while providing a clearly defined channel response that indicates its role in ion balance, is via the less direct agency of a kinase pathway.

The insulin-stimulated cation channel has properties that make it an ideal candidate vehicle for sustained Na^+ delivery because it has a high conductance for Na^+ at physiological potential (153 pS in 150 mM Na^+ at -80 mV) and the Ca^{2+} that accompanies Na^+ entry through the channel is inhibiting on the cytoplasmic side, causing a controlled, intermittent conductance (McGeoch and Guidotti, 1992). This entity can therefore deliver Na^+ without depolarizing the cell, which otherwise would elicit a signal that could be confused with that of the acetylcholine receptor and cause action potentials or muscle contraction.

The channel has been identified thus far in skeletal muscle, heart, pyramidal neurones, neuroblastoma cells, adipocytes and white blood cells and is therefore likely to have a ubiquitous distribution in the body. It is identifiable by its cyclic-guanosine 5'-monophosphate

(cGMP) gating ($K_m = 0.3 \mu\text{M}$), Ca^{2+} inhibition ($K_i = <1 \mu\text{M}$), and μ -conotoxin inhibition ($K_i = 50 \text{ nM}$) (McGeoch and Guidotti, 1992). Also it has a strongly curved current-voltage (I-V) characteristic, only opening at cytoplasmic potentials more negative than -40 mV and it is stimulated by insulin ($K_m = 5 \text{ nM}$). Kinase pathways from the insulin receptor to the channel may be involved and are discussed below. The channel's properties are similar to those of the vertebrate cyclic-nucleotide-gated channels, particularly the voltage-sensitive cone channels (Yau and Baylor, 1989) and olfactory cGMP/cAMP-gated cation channel (Dhallen *et al.*, 1990), and thus it may be a member of the superfamily of voltage-gated cation channels (Jan and Jan, 1990). Many of this family of channels have regulation by Ca^{2+} , either directly (Stern *et al.*, 1986; Zufall *et al.*, 1991) or indirectly, via Ca^{2+} regulation of a phosphodiesterase that degrades the cyclic nucleotide necessary for channel gating (Kamamura and Murakami, 1991) or Ca^{2+} regulation of a cyclase that generates the gating cyclic nucleotide (Koch and Stryer, 1988). The Ca^{2+} regulation of the cation channel has been shown to involve a cooperative interaction of subunits (McGeoch and McGeoch, 1994) that is tissue specific, as indicated by the Fourier transform of the current fluctuations.

Continuing from prior work (McGeoch and Guidotti, 1992) in which cation currents were measured in patch-clamped reconstituted membrane and whole muscle fiber cells, we have now measured the cumulative effect of such currents via quantitative fluorescence imaging of the internal Na^+ and Ca^{2+} levels ($[\text{Na}^+]_i$ and $[\text{Ca}^{2+}]_i$) in response to (principally) insulin, μ -conotoxin, and external ion changes. We observed $[\text{Na}^+]_i$ to fluctuate in a manner consistent with periodically interrupted entry of that ion and to decrease over time in response to insulin, reflecting insulin stimulation of the $\alpha 2$ isoform of the Na^+ pump (Resh *et al.*, 1980) outstripping insulin stimulation of the channel conductance. Also insulin caused a transient rise in $[\text{Ca}^{2+}]_i$ that decayed to physiological baseline within tens of seconds, the decay being assisted by the action of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. It was apparent that the Ca^{2+} rise, or "spike," was sufficiently high to cause temporary channel inhibition, as reflected in a momentary dip in $[\text{Na}^+]_i$.

The work revealed that this cation channel in skeletal muscle is one of the main entry routes for Na^+ in the cell and that extracellular Ca^{2+} tightly regulates the supply of Na^+ to the cell. The oscillatory nature of the current seen in the voltage-clamped muscle (McGeoch and Guidotti, 1992) and in the $[\text{Na}^+]_i$ fluctuations in the present experiments is a manifestation of the fine control of Na^+ entry and $[\text{Na}^+]_i$ by the inhibitory Ca^{2+} component of the cation channel conductance. As the channel is present in many cell types, its function as the principal route of sodium entry is likely to be widespread.

Additionally we observed in the course of these measurements that the intracellular Ca^{2+} rise brought about by the depolarization of skeletal muscle with KCl was strongly decreased by prior exposure of the muscle to insulin. Insulin is known to activate several serine/threonine protein kinases (Boulton *et al.*, 1991; Lavionne *et al.*, 1991; Robbins and Cobb, 1992; Hei *et al.*, 1993), including protein kinase C (Cooper *et al.*, 1990) so an investigation was undertaken with the kinase stimulator phorbol 12-myristate 13-acetate (TPA) and the kinase inhibitor *N*-(2'-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride (HA1004), which revealed the potential involvement of protein kinase C and other kinases in the phenomenon.

MATERIALS AND METHODS

Preparation of Soleus Muscle Loaded with Ion Probes

The experiments were performed on Fura-2- and SBFI-loaded neonatal rat soleus muscle of age 3–14 d. Measurements were taken from 6–20 fibers per muscle. In older rats (21–25 d) 5–10 fibers were dissected free with their intact tendons for ion measurements. The changes in $[\text{Na}^+]_i$ and $[\text{Ca}^{2+}]_i$ were the same for both intact neonatal muscle and dissected fiber bundles from the older rats. The neonatal soleus is $\sim 100 \mu\text{m}$ deep $\times 2\text{--}5 \text{ mm}$ wide $\times 7\text{--}10 \text{ mm}$ long from birth to 14 d of age and in physiological saline at 25°C can remain metabolically viable for 2–3 h because it is small enough for adequate substrate and ion exchange between the muscle and physiological saline. The relatively flat soleus muscle in the neonate is also shallow enough to allow accurate quantitative fluorescence imaging of dye-loaded fibers in the intact muscle. In older rats it is difficult to focus on the intact soleus, so dissection of the fibers was performed. Rats were killed by decapitation, and the whole soleus muscle was dissected out with tendons or individual fibers in older rats and placed in a temperature-controlled microincubation chamber (25°C) containing $8 \mu\text{M}$ Fura-2,AM (Gryniewicz *et al.*, 1985) for 1 h or $5 \mu\text{M}$ SBFI,AM (Minta and Tsien, 1987; Harootunian *et al.*, 1989) for half an hour in 3 ml of physiological saline (concentration in mM: 20 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4, 140 NaCl, 4 KCl, 1 CaCl_2 , 5 glucose, and 0.8 mM MgCl_2) for 1 h. The soleus muscle was loaded with Fura-2,AM/SBFI,AM and was washed free of the dye in physiological saline minus MgCl_2 ($\times 3$) or with 0.5 mM $[\text{Mg}^{2+}]_o$. The cation channel under study will transport all cations, and therefore inward Mg^{2+} could interfere with the potential inward Ca^{2+} that we wished to monitor. $[\text{Mg}^{2+}]_o$ was therefore reduced in most experiments. It was established that the reported changes in $[\text{Na}^+]_i$ and $[\text{Ca}^{2+}]_i$ were still present when 0.5 mM $[\text{Mg}^{2+}]_o$ was present. The muscle and fiber bundles were prevented from movement in the incubation chamber by attaching the tendons to the chamber base with microliter drops of cyanoacrylate glue.

Quantitative Fluorescence Imaging

The muscle and fiber bundles were illuminated via the epifluorescence port of the microscope and a 425-nm dichroic mirror, with alternating 340- and 380-nm bandpass filters. Fluorescence was collected (8 frames for 0.27 s at 340 nm, 0.25 s to switch to 380 nm, 8 frames over 0.27 s at 380 nm, 0.25 s to switch to 340 nm, and so on) via a Quantex ICCD camera and counted at $\sim 1 \text{ Hz}$. $[\text{Na}^+]_i$ and $[\text{Ca}^{2+}]_i$ were calculated from the ratio of the fluorescence at the two excitation wavelengths. Single wavelength fluorescence measurements were at 340 nm at the rate of 4–30/s. In each experiment with whole muscle, fluorescence was measured from 6 to 20 fibers of the muscle, collected from an area $25 \times 25 \mu\text{m}$ per fiber. Similar sized areas per fiber were measured

in the fiber bundles of older rats. Dyes were calibrated for ratio wavelength measurement by measuring fluorescence of buffered (pH 7.4) solutions of calcium and sodium (+2 M sucrose) with the free acids of Fura-2 and SBFI (Gryniewicz *et al.*, 1985). The dyes were not calibrated in dissected fibers or fiber bundles because the variation in size between different experiments would have necessitated calibration for every experiment. Muscle fibers also proved difficult to load with high concentrations of Na⁺ (100 mM) necessary for the calibration. The figures either have calibrated [Na⁺]_i values in millimolar [Na⁺]_i or the 340:380 nm ratio value with a scale for [Na⁺]_i. In these experiments it is the relative change in the [Na⁺]_i that is important rather than the absolute [Na⁺]_i. Ligand additions were made via pipette at a distance of 0.5 cm from the muscle in the 3-ml bath, in microliter volumes at 100 times the final concentration. It was calculated that for 1 s the muscle fiber surface would encounter up to a maximum of 10 times the final concentration and in 2 s it would see the desired concentration. (To eliminate the possibility that zinc in the insulin preparation entering the muscle would interact with Fura-2 to give artifactual "calcium" rises, soleus muscle preparations were treated with the addition of 100 μM ZnCl₂. No change in the basal calcium was noted.)

When monitoring [Na⁺]_i, it became apparent that the baseline level fluctuated. As we were potentially studying changes in [Na⁺]_i related to a cation channel that in patch-clamp experiments oscillated (McGeoch and Guidotti, 1992), these fluctuations were of interest. It was necessary, however, to be sure that the irregular fluctuations were not artifacts due to the imaging apparatus. Apparatus artifacts were ruled out, as on a given day's experiments with every condition constant, when one muscle preparation showed clear fluctuations, another did not display the phenomenon (Figure 1A). The fluctuations were also seen when [Na⁺]_i was monitored at a single wavelength (340 nm). The background noise can be high with the Na⁺ dye (SBFI), because the degree of loading that gives a sensitive [Na⁺]_i response (for instance, a change in [Na⁺]_i to insulin or to changing [Ca²⁺]_o) is lower than that for Fura-2 when [Ca²⁺]_i is being monitored. High levels of SBFI loading with a strong fluorescence signal give high resting [Na⁺]_i levels indicative of toxic action in the muscle. The background noise from SBFI monitored with 6 mM NaCl did not show the fluctuations seen in living tissue.

RESULTS

Baseline [Na⁺]_i Oscillates and Is Controlled by [Ca²⁺]_o

The fluctuating characteristics of the baseline [Na⁺]_i and the [Ca²⁺]_o-dependent changes in [Ca²⁺]_i and [Na⁺]_i were monitored in Fura-2- and SBFI-loaded whole neonatal soleus muscles and in fiber bundles from 21- to 25-d-old rats. There were no differences in the effects between whole muscle or fiber bundles. Baseline [Ca²⁺]_i was 117 ± 30 nM (n = 43) and that of [Na⁺]_i 3.4 ± 1.5 mM (n = 19).

In many preparations baseline [Na⁺]_i constantly fluctuated by ~1 mM when monitored by taking ratio measurement of the fluorescence (340:380 nm) (Figure 1A) or by measurement at a single wavelength (340 nm). In the 12-min trace, irregular [Na⁺]_i oscillations are approximately two to three per minute. The baseline [Na⁺]_i here is 6 mM and drops with insulin addition, which will be discussed later. Although the fluctuations in baseline [Ca²⁺]_i were above those of apparatus noise, they were less pronounced than the fluctuations in [Na⁺]_i.

[Ca²⁺]_o affected the [Na⁺]_i and [Ca²⁺]_i levels consistent with the characteristics of the inward Ca²⁺ component of the current being inhibitory. When the inhibition was removed by lowering [Ca²⁺]_o, more Na⁺ and Ca²⁺ could potentially enter. A drop in [Ca²⁺]_o caused a rise in [Na⁺]_i (Figure 1B) and a drop in [Ca²⁺]_i (Figure 1C). In the latter case raising [Ca²⁺]_o again caused a rise in [Ca²⁺]_i. A "history" of a drop in [Ca²⁺]_o was necessary to obtain this rise in [Ca²⁺]_i, and it did not occur if the [Ca²⁺]_o had not previously been reduced to 100 nM (Figure 1C, insert). The "history" is equivalent to removal of the inhibitory Ca²⁺ control of the channel. Many of the Ca²⁺ binding sites on the channel become unoccupied when [Ca²⁺]_o is lowered to <100 nM, and their repressive effect on channel conductance is relieved. Figure 1D shows that [Na⁺]_i drops when [Ca²⁺]_o is raised after it was previously reduced to <100 nM. Figure 1D (insert) shows that even a rise in [Ca²⁺]_o to 10 mM does not lower [Na⁺]_i in the absence of a prior reduction in [Ca²⁺]_o. Millimolar [Ca²⁺]_o reduces and regulates the channel conductance to Na⁺ and Ca²⁺ and features an oscillating [Na⁺]_i (Figure 1A). Decreasing [Ca²⁺]_o before raising it, increases channel conductance via removal of the Ca²⁺ inhibition. The changes in [Na⁺]_i and [Ca²⁺]_i related to [Ca²⁺]_o occurred whether [Mg²⁺]_o was micromolar or 0.5 mM.

Insulin-dependent Changes in [Na⁺]_i Monitored for 1 h

Insulin induces changes in [Na⁺]_i and [Ca²⁺]_i on the time scale of minutes (transient effects) and on the time scale of tens of minutes (longer term effects). The longer term insulin-dependent changes in [Na⁺]_i reveal the interaction of the channel and the Na⁺ pump on [Na⁺]_i regulation. Insulin effects changes in [Na⁺]_i that are equivalent to lowering [Ca²⁺]_o and can be regarded as acting to effect an allosteric reduction of the Ca²⁺ binding energy at one or some of the Ca²⁺ binding sites of the channel. The [Na⁺]_i changes to insulin result from increased channel delivery of Na⁺ and increased removal of the same by the insulin-stimulated Na⁺ pump. To observe the long-term rise in [Na⁺]_i to insulin, ouabain inhibition of the pump must be employed (Figure 2A; [Na⁺]_i rise = 350 μM/min). (Ouabain [2 mM] addition by itself causes a slow rise in [Na⁺]_i [140 μM/min] because of inhibition of the Na⁺ pump. This phenomenon has been previously documented in other SBFI-loaded tissue treated with Na⁺ pump inhibitors [ouabain: Negulescu *et al.*, 1990; strophanthidin: Satoh *et al.*, 1991].) To block both increased entry via the channel and increased exit via the pump, μ-conotoxin (the specific inhibitor of the channel [McGeoch and Guidotti, 1992] plus ouabain must be employed (Figure 2B). There is an initial reduction in the [Na⁺]_i in the experiment with μ-conotoxin and ouabain because μ-conotoxin inhibited

Figure 1. Baseline $[Na^+]_i$ fluctuates and is controlled by $[Ca^{2+}]_o$. The neonatal soleus muscles were loaded with the ion probes as outlined in MATERIALS AND METHODS. (A) (top trace) An example of baseline $[Na^+]_i$ in SBFI-loaded soleus muscle displaying irregular $[Na^+]_i$ fluctuations (2–3 per minute). The resting $[Na^+]_i$ was 6 mM and the trace, which is the average of 10 fibers, shows a decrease to insulin. (Bottom trace) Monitoring of baseline $[Na^+]_i$ in another set of 10 fibers where the fluctuations are much less marked. The decrease in $[Na^+]_i$ is due to the addition of 10 nM insulin. (B) The effect of changing $[Ca^{2+}]_o$ on $[Na^+]_i$ in SBFI-loaded muscle (average of 6 fibers). $[Na^+]_i$ rose by 2.5 mM when 1 mM $[Ca^{2+}]_o$ is reduced to <100 nM with BAPTA. (C) The effect of changing $[Ca^{2+}]_o$ on $[Ca^{2+}]_i$ in Fura-2-loaded muscles. Raising $[Ca^{2+}]_o$ after an initial reduction from 1 mM to <100 nM causes a rise in $[Ca^{2+}]_i$ (In 2 experiments the average $[Ca^{2+}]_i$ rise was 93 and 40 nM, respectively). In the graph insert at bottom right, $[Ca^{2+}]_i$ rose very little when $[Ca^{2+}]_o$ was raised (to 3 and 10 mM, respectively, depicted by Ca) without a prior decrease. (D) The effect of changing $[Ca^{2+}]_o$ on $[Na^+]_i$ in SBFI-loaded muscles. Raising $[Ca^{2+}]_o$ after an initial reduction from 1 mM to <100 nM causes a drop in $[Na^+]_i$ (In 10 fibers the average $[Na^+]_i$ drop = 1.8 mM). In the graph insert at bottom left, $[Na^+]_i$ remained steady when $[Ca^{2+}]_o$ was raised (to 3 and 10 mM, respectively, depicted by Ca) without a prior decrease. Values are the average $[Ca^{2+}]_i$ and $[Na^+]_i$ of ≥ 6 fibers per experiment. The decrease in $[Ca^{2+}]_o$ from 1 mM to <100 nM was achieved by the addition of buffered BAPTA to the physiological saline.

Figure 2. Insulin-dependent changes in [Na⁺]_i monitored over 1 h in fiber bundles of 21- to 25-d-old rats. The fiber bundles were loaded with SBFI as outlined in MATERIALS AND METHODS. Long monitoring of insulin-dependent changes in [Na⁺]_i of neonatal whole soleus

Na⁺ entry before the pump-dependent exit of the ion was inhibited by ouabain. However, insulin was added when [Na⁺]_i had plateaued, and no change in [Na⁺]_i occurred when the channel and the pump were inhibited. Figure 2C displays 100 min of data for fibers with uninhibited channels and pumps. Insulin lowers the [Na⁺]_i because stimulation of the Na⁺ pump outstrips that of the channel, and it reaches a new steady state that lasts 35 min before it starts to rise again.

Initial Insulin-dependent Transients in [Na⁺]_i and [Ca²⁺]_i

Focusing on the initial changes in [Na⁺]_i and [Ca²⁺]_i upon insulin addition to neonatal muscle reveals for [Na⁺]_i a fast spike (not always seen), a drop (0.99 ± 0.53 mM, $n = 6$), and a rise to baseline (frequently to a level just above the original baseline) before the steady drop to a new baseline, that lasts tens of minutes. Figure 3A1 shows a typical decrease in [Na⁺]_i to insulin. The fast spike is not visible here but is shown in a second trace (Figure 3A2). That the effects are blocked by the specific channel inhibitor, μ -conotoxin (Cruz *et al.*, 1985), is depicted in Figure 3A3.

The change in [Ca²⁺]_i is a transient spike (38 ± 14 nM, $n = 18$) of <1-min duration (Figure 3B). The first [Na⁺]_i drop lasts for precisely the duration of the [Ca²⁺]_i spike (Figure 3C). An explanation for this synchrony is that the inrush of increased Ca²⁺ upon insulin stimulation of the channel conductance closes the channel transiently (K_i for Ca²⁺ inhibition of the channel = $<1 \mu\text{M}$ [McGeoch and Guidotti, 1992]) until [Ca²⁺]_i decreases due to removal by diffusion, Ca²⁺ pumps, and the Ca²⁺/Na⁺ exchanger. The transient closure of channels lowers the [Na⁺]_i seen as the first drop in this ion concentration, and its rise represents the reopening of channels as the Ca²⁺ inhibition diminishes. For the next 5–10 min the [Na⁺]_i frequently has a slightly raised level relative to the initial baseline (Figure 3C, bottom trace monitoring [Na⁺]_i) before it

gave similar results. (A) There is a significant rise in [Na⁺]_i to insulin with an inhibited Na⁺ pump. Fiber bundles were pretreated with ouabain to display the insulin-dependent rise in [Na⁺]_i ($66 \mu\text{M Na}^+/\text{min}$). (B) There is no significant change in [Na⁺]_i to insulin with an inhibited Na⁺ pump and an inhibited channel. Pretreatment of the muscle with μ -conotoxin (inhibitor of the channel) + ouabain (inhibitor of the Na⁺ pump) blocked the channel-related rise in [Na⁺]_i seen in A. In B there was a small initial decrease in [Na⁺]_i due to the fact that ouabain takes some minutes to completely inhibit the Na⁺ pump. (C) There is a significant decrease in [Na⁺]_i to insulin with a functional Na⁺ pump and a functional channel. [Na⁺]_i drops because the stimulation of the Na⁺ pump by insulin outstrips that of the channel. The initial fast rate of decrease = $542 \mu\text{M Na}^+/\text{min}$ and the average rate from 18 to 60 min = $139 \mu\text{M Na}^+/\text{min}$. A ratio change of $0.14 = 1 \text{ mM [Na}^+]$ in the fiber bundles. The results are the average [Na⁺]_i in 4–10 fibers per experiment.

Figure 3. Initial insulin-dependent transients in $[Na^+]_i$ and $[Ca^{2+}]_i$. Neonatal soleus muscles were loaded with the ion probes as outlined in MATERIALS AND METHODS. (A) Characteristics of insulin-dependent $[Na^+]_i$ decrease in SBFI-loaded soleus muscle. (A1) A representative trace to show the decrease in $[Na^+]_i$ to insulin; average of 6 fibers. In 6 experiments (6–10 fibers/experiment) the average decrease in $[Na^+]_i$ to insulin = 0.99 ± 0.53 mM. (A2) An example of the initial Na^+ spike that is frequently seen and which represents an inrush of Na^+ (+ Ca^{2+} , which is only seen with Fura-2) before the rise in $[Ca^{2+}]_i$ closes the channels. The decrease in $[Na^+]_i$ as the channels close is followed by a steady rise as they reopen. This trace is the average of 2 fibers. (A3) Inhibition of the insulin-dependent $[Na^+]_i$ decrease by 175 nM μ -conotoxin. Twelve fibers in 2 experiments gave no $[Na^+]_i$ transient to insulin in the presence of μ -conotoxin. (B) Characteristics of the insulin-dependent $[Ca^{2+}]_i$ transient in Fura-2-loaded soleus muscles. A representative insulin-dependent $[Ca^{2+}]_i$ transient (left peak), followed by a $[Ca^{2+}]_i$ rise elicited by depolarizing doses of KCl (right peaks). The average insulin-dependent $[Ca^{2+}]_i$ transient = 38 ± 14 nM (18 separate experiments with ≥ 6 fibers per experiment). (C) A comparison of the transient $[Ca^{2+}]_i$ rise and $[Na^+]_i$ decrease to insulin. It is seen that during the time that $[Ca^{2+}]_i$ rises, $[Na^+]_i$ falls, and by the time that $[Ca^{2+}]_i$ has returned to baseline the $[Na^+]_i$ is starting to rise again. The $[Ca^{2+}]_i$ rise is the average of the 5 traces used in the decay constant calculation of Figure 4C, and the $[Na^+]_i$ decrease trace is from 1 experiment averaging 6 fibers.

slowly descends to a new lower steady-state baseline (Figure 2C). The rise in the 5- to 10-min period is variable (0.2–2 mM).

Monitoring of the $[Na^+]_i$ and $[Ca^{2+}]_i$ transients in the presence of 0.5 mM $[Mg^{2+}]_o$ rather than micromolar $[Mg^{2+}]_o$ gave similar results. Insulin caused the same transient drop in $[Na^+]_i$ (1.25 ± 0.25 mM in 10 fibers,

$n = 2$) and rise in $[Ca^{2+}]_i$ (78 ± 38 nM in 6 fibers, $n = 2$) with 0.5 mM $[Mg^{2+}]_o$.

Insulin-dependent Changes in $[Na^+]_i$ and $[Ca^{2+}]_i$ Are Inhibited by μ -Conotoxin

The short-term insulin-dependent changes in $[Na^+]_i$ and $[Ca^{2+}]_i$ are blocked if the muscles are pretreated with μ -

conotoxin (Cruz *et al.*, 1985), the specific inhibitor of the channel (McGeoch and Guidotti, 1992) (Figures 4A and 3A3). Pretreating the muscles with 10 μ M TTX (the inhibitor of the classic Na⁺ channel of the action potential [Catterall, 1986]) has no effect on the insulin-dependent ion changes (not shown).

The Insulin-dependent Rise in [Ca²⁺]_i Is Dependent on [Ca²⁺]_o

The source of the Ca²⁺ for the rise is probably [Ca²⁺]_o entering via the channel, and the transient is dependent

on [Ca²⁺]_o at the millimolar level. Lowering [Ca²⁺]_o with 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) or ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) from 1 mM to <100 nM eliminated the insulin-dependent transient (Figure 4B). This demonstrates a dependence of the transient on millimolar [Ca²⁺]_o but does not prove that the [Ca²⁺]_i rise came via entry through the channel. We attempted to answer the question by depleting internal muscle Ca²⁺ stores with the endoplasmic reticulum Ca²⁺

Figure 4. Factors that regulate the initial insulin-dependent spikes in [Na⁺]_i and [Ca²⁺]_i. Inhibition by μ -conotoxin, the dependence of the [Ca²⁺]_i spike on [Ca²⁺]_o, and [Na⁺]_o and production of the [Ca²⁺]_i spike by 8-bromo cGMP. Neonatal soleus muscles were loaded with the ion probes as outlined in MATERIALS AND METHODS. (A) The inhibition of the insulin-dependent [Ca²⁺]_i transient by μ -conotoxin is shown in a pair of soleus muscles from a single rat. The right soleus trace (offset) shows a typical insulin-dependent [Ca²⁺]_i transient, to be compared with that of the left soleus blocked by pretreatment with 250 nM μ -conotoxin. To the right of each trace a response to a depolarizing dose of KCl is included. In 4 experiments with muscle treated with 250 nM μ -conotoxin, there was only 1 small insulin-dependent [Ca²⁺]_i transient (<20 nM) to 100 nM insulin. (B) The [Ca²⁺]_i transient to insulin is dependent on millimolar [Ca²⁺]_o as reducing [Ca²⁺]_o to <100 nM with BAPTA eliminates the transient. Average [Ca²⁺]_i in 23 fibers. (C) A comparison of insulin-dependent [Ca²⁺]_i transients with physiological [Na⁺]_o (---), and where [Na⁺]_o is replaced with *N*-methyl-D-glucamine (—), to show the increase in the decay constant of the return rate. In physiological [Na⁺]_o the decay constant = 17.2 ± 1.4 s (5 experiments), and in *N*-methyl-D-glucamine = 32.4 ± 5 s (7 experiments). (D) A representative [Ca²⁺]_i transient dependent on 8-bromo cGMP. The average [Ca²⁺]_i rise to 8-bromo cGMP in 2 experiments with a total of 18 fibers = 83 ± 40 nM. The righthand peak shows the [Ca²⁺]_i rise to KCl.

ATPase inhibitor, thapsigargin but were unable to get a rise in $[Ca^{2+}]_i$ to 2–10 μM of the compound (a standard $[Ca^{2+}]_i$ rise was obtained in CHO, P19, and N1E neuroblastoma cells to the thapsigargin as positive controls). We attribute this to the fact that muscle has a very high level of sarcoplasmic reticulum Ca^{2+} ATPase, and in whole muscle and muscle fibers it is difficult to reach an inhibitory ratio of thapsigargin to Ca^{2+} ATPase (Lytton *et al.*, 1991).

A Na^{+}/Ca^{2+} Exchanger Is Implicated in Returning the Insulin-dependent $[Ca^{2+}]_i$ Spike to Baseline

The return of $[Ca^{2+}]_i$ after insulin to baseline level was dependent on $[Na^{+}]_o$, suggesting Na^{+}/Ca^{2+} exchanger involvement. Replacing $[Na^{+}]_o$ with *N*-methyl-D-glucamine increased the exponential decay constant to the transient $[Ca^{2+}]_i$ rise from 17.2 ± 1.4 to 32.4 ± 5 s (Figure 4C). The other membrane proteins, which are also likely to be involved in the return of $[Ca^{2+}]_i$ to baseline level, are a plasma membrane Ca^{2+} ATPase and a sarcoplasmic reticulum Ca^{2+} ATPase.

8-Bromo cGMP Causes the Same Transient $[Ca^{2+}]_i$ Spike as Insulin

The channel is gated by cGMP ($K_m = 3.7 \pm 0.6 \times 10^{-7}$ M) (McGeoch and Guidotti, 1992) and any increase in conductance of cations (in this case Ca^{2+}) should therefore also occur on addition of the membrane-permeable and phosphodiesterase-resistant form of the cyclic nucleotide, 8-bromo cGMP. A larger (83 ± 40 nM), but temporally similar $[Ca^{2+}]_i$ transient to 20–100 μM 8-bromo cGMP occurred (Figure 4D).

Insulin reduces Depolarization $[Ca^{2+}]_i$ Rise; Putative Involvement of Protein Kinase C

In the course of these experiments it became apparent that insulin reduced the $[Ca^{2+}]_i$ rise upon depolarization of muscle. KCl (200 mM) was added to all muscle preparations after the insulin-dependent phenomena had been observed to check the physiological state of the muscle. We expected an approximate 500-nM rise in $[Ca^{2+}]_i$ upon depolarization from a muscle in a "stable physiological state." In control experiments the rise in $[Ca^{2+}]_i$ was 491 ± 108 nM and in those pretreated with insulin 139 ± 101 nM (Figure 5A and B). If the protein kinase inhibitor HA1004 was given before insulin, then no reduction in the $[Ca^{2+}]_i$ rise to KCl occurred (436 ± 149 nM) (Figure 5C). Treatment of the muscle before the depolarizing dose of KCl, with the protein kinase C stimulator, TPA (10 nM) caused the same reduction as insulin to the $[Ca^{2+}]_i$ rise (155 ± 22 nM) (Figure 5D). Treatment with HA1004, a protein kinase inhibitor (Hidaka *et al.*, 1984), followed by TPA (the protein kinase C stimulator) eliminated the reduction in the $[Ca^{2+}]_i$ rise to KCl (795 ± 361 nM) (Figure 5E). The inset table of

Figure 5. Insulin reduced the $[Ca^{2+}]_i$ rise to a depolarizing dose of KCl. Neonatal soleus muscles were loaded with the ion probes as outlined in MATERIALS AND METHODS. The phorbol ester, TPA, gave the same effect as insulin and both the insulin reduction and that by TPA were blocked by the kinase inhibitor, HA1004. (A) Control $[Ca^{2+}]_i$ rose to 200 mM KCl. (B) Depressed $[Ca^{2+}]_i$ rise to KCl in muscle pretreated with 1 nM insulin. (C) Block of insulin depression of $[Ca^{2+}]_i$ rise to KCl, by kinase inhibitor, HA1004. (D) TPA produced the same depression of the $[Ca^{2+}]_i$ rise to KCl as insulin. (E) In the presence of the kinase inhibitor, HA1004, TPA did not depress the rise in $[Ca^{2+}]_i$ to KCl. (A tabulated summary of the data for the experimental conditions depicted in graphs A–E is given top left. n = number of experiments, each of which averaged $[Ca^{2+}]_i$ in 4–20 muscle fibers.)

Figure 5 (top left) gives the number of experiments for monitoring the reduction of $[Ca^{2+}]_i$ rise upon depolarization with insulin.

Because the $[Ca^{2+}]_i$ transient spike to insulin in polarized muscle is associated with a cGMP-gated channel, it was necessary to consider this spike's association with the insulin-dependent reduction of Ca^{2+} release upon depolarization. 8-bromo cGMP, which elicits a transient $[Ca^{2+}]_i$ spike in the absence of insulin (Figure 4D), did not reduce the $[Ca^{2+}]_i$ rise upon depolarization (In 2 muscle averaging 18 fibers the depolarization $[Ca^{2+}]_i$ rise after 8-bromo cGMP = 556 ± 181 nM).

The kinase inhibitor, HA1004, which blocked the insulin-dependent reduction in $[Ca^{2+}]_i$ release upon de-

polarization, did inhibit the transient insulin-dependent [Ca²⁺]_i spike (Figure 5C).

The Relationship of [Ca²⁺]_o to the [Ca²⁺]_i Rise upon Depolarization

The [Ca²⁺]_i rise upon depolarization was dependent on [Ca²⁺]_o. A small rise in [Ca²⁺]_i occurred when 200 mM KCl was applied to neonatal muscles in <100 nM [Ca²⁺]_o and increased significantly when the [Ca²⁺]_o was returned to the millimolar level (Figure 6).

DISCUSSION

These experiments depict insulin-dependent changes in [Na⁺]_i and [Ca²⁺]_i that relate to phases in the functioning of a cation channel taking Na⁺ and Ca²⁺ into muscle. We have used 1) the channel activators, insulin and 8-bromo cGMP; 2) the channel inhibitor μ -conotoxin; 3) the Na⁺ pump inhibitor, ouabain; and 4) the Na⁺/Ca²⁺ inhibitor, lack of external Na⁺, to reveal the phases in the channel action that regulate [Na⁺]_i and [Ca²⁺]_i and the interaction of other membrane proteins with the channel. We have demonstrated in these experiments how finely controlled is the channel by Ca²⁺. The previous voltage-clamp experiments on muscle preparations characterized the activators and inhibitors of the channel and indicated that the oscillating current could represent a highly regulated entry mechanism of Na⁺ into muscle (McGeoch and Guidotti, 1992). Here we provide supporting evidence for this.

The Fluctuating [Na⁺]_i Baseline

We propose that the cation channel under study via its inward cation current constantly "tops up" the cell's cations. The fluctuating [Na⁺]_i seen in these experiments could reflect the constant inhibition of the "top up" process via the inhibitory Ca²⁺ component in the inward cation current. The [Na⁺]_i fluctuations are not due to apparatus artifacts because one fiber preparation will display the phenomenon and another with precisely the same conditions on the same day will not. Good focusing with a small flattened muscle very near to the bottom of the dish are probably needed, and these optimal conditions were not achieved in every experiment. In the absence of clear fluctuations the changes in [Na⁺]_i to insulin and changing [Ca²⁺]_o are however observed (Figure 1A).

Regulation of [Na]_i

Baseline [Na⁺]_i is controlled by [Ca²⁺]_o. Millimolar [Ca²⁺]_o maintains a steady [Na⁺]_i and [Ca²⁺]_i and can be viewed as a repressive control on channel conductance. A reduction in [Ca²⁺]_o to <100 nM relieves the repression, resulting in dramatic changes in internal ion concentrations. The steady but fluctuating [Na⁺]_i in the

Figure 6. [Ca²⁺]_i rise to a depolarizing dose of KCl is dependent on [Ca²⁺]_o. Neonatal soleus muscles were loaded with the ion probes as outlined in MATERIALS AND METHODS. The average [Ca²⁺]_i rise to a depolarizing dose of KCl in 4 soleus muscle fibers loaded with Fura-2,AM was 200 nM with low [Ca²⁺]_o (<100 nM). It increased to 550 nM when [Ca²⁺]_o was returned to 2 mM.

presence of millimolar [Ca²⁺]_o is a manifestation of a finely tuned channel. When insulin increases the channel conductance, this is particularly important. Na⁺ can continuously enter the cell via the channel to supply Na⁺ to the Na⁺ pump. The Ca²⁺ control on the inward current ensures that depolarization does not occur and that the classic TTX Na⁺ channel of the action potential is not activated. The possible "signal" from the inward current of the cation channel is carefully separated from that of the TTX channel via the repressive [Ca²⁺]_o control. When insulin is applied and the Na⁺ pump is inhibited by ouabain, there is an increase in [Na⁺]_i, which is blocked by μ -conotoxin. In the absence of ouabain, however, insulin brings about a decrease in [Na⁺]_i because the Na⁺ pump is activated and outstrips the increased Na⁺ entry (McGill and Guidotti, 1991).

The first [Na⁺]_i changes to insulin (within the first few minutes) indicate direct involvement of a Ca²⁺-regulated cation channel. The closure of the channel via the Ca²⁺ component of the conductance gives a dip in [Na⁺]_i, followed by a return to baseline, as [Ca²⁺]_i decreases and the channels reopen. Frequently the new baseline is transiently raised (Figure 3C) before the long-term decrease in [Na⁺]_i to insulin starts. This transiently raised baseline could be due to statistical variation in channel open time. Only 10 open channels of 150-pS conductance are required to supply sufficient Na⁺ to allow full activity of Na⁺ pumps in the viewed area of 25 × 25 μ m. The channel has rather slow rates of opening and closing due to its cooperativity, and this coupled with the small number of channels involved could cause some of the variability of the Na⁺ transient. The number of active Na⁺ pumps within the muscle fiber could also influence the [Na⁺]_i baseline.

This ubiquitous cation channel may be responsible, when mutated, for disease states related to abnormal Na^+ entry and $[\text{Na}^+]_i$ level. Hypertension is common in the population and is such a disease. In the smooth muscle lining of blood vessels, a channel phenotype coding for lowered Ca^{2+} binding would result in raised Na^+ entry, increased water entry, cell swelling, and decreased vessel lumen/increased intraluminal pressure. We already have evidence that the Ca^{2+} binding of the channel is tissue specific (McGeoch and McGeoch, 1994) and therefore several isoforms may exist. A predisposition to hypertension may reflect overexpression of low Ca^{2+} affinity forms in smooth muscle. Hypertension is also common in diabetes where there are raised insulin levels, and the mechanism here would be similar, particularly if associated with low serum Ca^{2+} .

The frequency of the fluctuations in $[\text{Na}^+]_i$ in the whole soleus in these fluorescence experiments is two to three per minute (sampling resolution = 60/min) compared with current oscillations of several hundred per minute in patch-clamped reconstituted channel protein (sampling resolution = 600 000/min) (McGeoch and Guidotti, 1992). The slow $[\text{Na}^+]_i$ fluctuations in intact soleus are most likely to reflect the true rate in vivo where there is no clamp on voltage and Ca^{2+} diffusion is slower.

It is possible that the Ca^{2+} component of the cation current through the channel, manifest here as a transient, may have a function in insulin action per se, because a 50-nM transient is a significant increase over the background level. There are few reports in the literature of cation transients similar to those reported here. Insulin-like growth factor-2 caused a 20-s $[\text{Ca}^{2+}]_i$ transient in 3T3 cells (Nishimoto *et al.*, 1987). Na^+ oscillations with a dependence on $[\text{Ca}^{2+}]_o$ have been reported in acinar cells (Wong and Foscett, 1991). We can demonstrate the $[\text{Ca}^{2+}]_i$ transient to insulin in many Fura-2-loaded cells: skeletal muscle, heart muscle, adipocytes and white blood cells, and we have also monitored the $[\text{Na}^+]_i$ transient in a neuroblastoma cell line.

Effect of Insulin on Ca^{2+} Release by Depolarization

The reduction of the $[\text{Ca}^{2+}]_i$ rise of muscle depolarization by insulin is interesting because the greatest tissue in the body in terms of mass is skeletal muscle. Insulin would appear to make muscles more efficient by tempering the $[\text{Ca}^{2+}]_i$ rise of excitation-contraction (E-C) coupling. Functionally there is no requirement for micromolar rises in $[\text{Ca}^{2+}]_i$ for muscle contraction, as Ebashi demonstrated in 1959 that a rise in $[\text{Ca}^{2+}]_i$ of only 200 nM elicits effective contraction (reviewed by Ebashi, 1991). The reduction of the $[\text{Ca}^{2+}]_i$ rise upon depolarization by insulin could render the muscle more energy efficient, because less ATP-consuming Ca^{2+} pump action is required to return a smaller $[\text{Ca}^{2+}]_i$ rise to baseline.

It was not possible to determine whether insulin reduced Ca^{2+} entry from the external solution or from internal stores because thapsigargin failed to cause Ca^{2+} release in these skeletal muscles. The resistance of the sarcoplasmic reticulum Ca^{2+} ATPase to thapsigargin has been documented (Thastrup *et al.*, 1990; Lytton *et al.*, 1991). In cultured smooth muscle cells when an attenuation of the contractile response to vasopressin was produced by insulin, both extracellular and internal Ca^{2+} were the source of the attenuated $[\text{Ca}^{2+}]_i$ rise (Standley *et al.*, 1991).

That TPA produces the same degree of reduction in the $[\text{Ca}^{2+}]_i$ rise as insulin indicates that insulin may initiate the following cascade of events: Occupancy of the insulin receptor activates the receptor tyrosine kinase, which in turn stimulates phospholipase C of the inositol phosphate pathway to generate an increase in diacylglycerol (DAG). This stimulates protein kinases, which inhibit the activity of the dihydropyridine binding protein, resulting in depression of the putative mechanical signal to the foot of the ryanodine binding protein and Ca^{2+} release channel. At some point the kinase inhibitor HA1004 can block this cascade so that the $[\text{Ca}^{2+}]_i$ rise of E-C coupling is then not reduced by insulin. Because TPA is well documented as stimulating protein kinase C (Nishizuka, 1988), this kinase may be in the cascade after the generation of DAG. However, it has been reported that protein kinase C *activates* the Ca^{2+} channel activity of the dihydropyridine receptor (Chang *et al.*, 1991). The dominance of HA1004 over TPA indicates that the inhibitor acts either on the same kinase as TPA or "downstream" in the cascade from TPA. Insulin has been documented as stimulating the phosphorylation of the inositol pathway (Cooper *et al.*, 1990) and is involved in kinase-activating pathways (Kyriakis *et al.*, 1992), but its activation of protein kinase C remains controversial (Blackshear *et al.*, 1991). The occupied, phosphorylated insulin receptor may alternatively activate an extracellular signal-regulated kinase-kinase (ERK kinase) (Robbins and Cobb, 1992; Boulton *et al.*, 1991), and the ERK may then inactivate the dihydropyridine binding protein. Recent work has documented insulin-stimulated seryl/threonyl protein kinases in intact rat skeletal muscle (Hei *et al.*, 1993). Future work, monitoring specific kinases as a function of insulin, will answer this question.

It is of interest to note that the kinase inhibitor, HA1004, as well as blocking the insulin-dependent reduction of the $[\text{Ca}^{2+}]_i$ rise upon depolarization, actually prevented the insulin-dependent $[\text{Ca}^{2+}]_i$ transient (Figure 4C). This would indicate the possible existence of two kinase cascades, one to the cGMP-gated insulin-sensitive channel and the other to the dihydropyridine binding protein, and the kinase inhibitor, which is not specific, inhibits both.

In summary we have demonstrated in skeletal muscle that $[\text{Na}^+]_i$ is regulated by the inward current of a cation

channel. The channel has a repressive Ca²⁺ control visualized here as fluctuations in [Na⁺]_i in SBFI-loaded muscle and previously as an oscillating Na⁺ current in voltage-clamped muscle (McGeoch and Guidotti, 1992). When insulin increases the conductance of the channel, transient spikes to [Na⁺]_i and [Ca²⁺]_i occur and the latter may be important as a "signal" because a 50-nM rise in [Ca²⁺]_i is significant when the baseline level is only 100 nM. It is possible that this signal operates in a "50-nM range window" avoiding cross signalling with larger rises in [Ca²⁺]_i associated with contraction. Insulin also reduces the [Ca²⁺]_i rise upon muscle depolarization and the effect involves insulin stimulation of a kinase, possibly protein kinase C.

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