Insulin Facilitates the Induction of the Slow Na^+ Channels in Immature *Xenopus* Oocytes

G. Charpentier

Laboratoire de Physiologie Animale, Faculté des Sciences,
Université de Picardie Jules Verne, 33 rue Saint Leu, 80018 Amiens Cedex, France

Abstract. Endogenous slow sodium channels have been described in the membrane of immature *Xenopus laevis* oocytes. The opening of these channels is a complex process comprising an induction phase leading the channels from a state of electrical inexcitability into a voltage-dependent state. The mechanism by which the depolarization of the membrane causes the induction is dependent upon an enzymatic cascade implying a phospholipase C (PLC) and a protein kinase C (PKC). The existence of different isoforms of PLC has been described in the oocytes, each isoform being activated by distinct membrane receptors upon ligand binding. The present work investigated the effects of insulin known to bind to membrane receptor tyrosine kinases and to activate PLC-γ isoforms. Our results in current and voltage clamp experiments showed that insulin facilitated the induction of the slow Na^+ channels in a dose-dependent way. The current/voltage relationships indicated that the gating properties of the channels were not altered by the hormone. Lavendustins and tyrphostin, inhibitors of the epidermal growth factor signaling pathway, failed to block insulin effect as well as induction of the sodium channels. The results support the idea that some of the enzymes activated by insulin could also be involved in the acquisition of the channel voltage dependency and activated by sustained depolarization of the membrane.

Key words: *Xenopus* oocytes — Sodium channel — Insulin — Electrophysiology — Pharmacology

Introduction

Excitability is a state that is considered to be a characteristic of nerve, muscle and endocrine cells. An excitable cell is a cell that can generate an action potential in response to a small depolarization. It implies the existence in the mem-

Correspondence to: Gilles Charpentier, Laboratoire de Neurobiologie Cellulaire et Moléculaire, CNRS, 1 Avenue de la Terrasse, 91198 Gif-sur-Yvette Cedex, France
E-mail: Gilles.Charpentier@nbcm.cnrs-gif.fr

brane of electrically gated channels, selective for a single ion species, sodium or calcium. However, when looking for the onset of electrical excitability in a simple organism the tunicates *Halocynthia roretzi* and *Halocynthia aurantium,* Takahashi and co-authors found that the oocyte and the egg of these species were excitable (Takahashi et al. 1971). Further investigations of the electrical properties of egg cell membranes, from various species, showed that some other eggs display resting and action potentials very similar to those of nerve and muscle cells eggs (Hagiwara and Jaffe 1979). The emergence of a particular ionic channel population in reproductive cells can in some cases be clearly associated with a specific biological function. For example, the membrane potential of eggs from various species presents a transient shift at fertilization which confers to the fertilized cell a quick mean to avoid polyspermy, as in the case of the anuran amphibians (Jaffe 1976).

The oocyte membranes of immature *Xenopus laevis* are not excitable in the basal state, but they can be made to produce a long-lasting action potential (Kado et al. 1979). Earlier studies have shown that during a long-lasting depolarization, the oocyte slowly develops a conductance which does not exist at the very beginning of the depolarization. The appearance of this property is linked to the emergence of an atypical subpopulation of slow voltage-gated sodium channels (referred to here as sNa^+ channels) (Kado et al. 1979; Kado and Baud 1981; Baud et al. 1982; Baud and Kado 1984). The process by which the membrane of the *Xenopus* oocyte that was inexcitable becomes electrically excitable has been referred to as the induction (Baud and Kado 1984). Induction comprises the transformation of a population of channels, highly selective for sodium ions, from an inexcitable state to an electrically gatable configuration. Once induced, the sodium channels are voltage sensitive and their gating is driven by the variations of the membrane potential, as long as they remain induced. More recent reports have shown that the induction of the sNa^+ channels was dependent upon the activation of an enzymatic cascade involving a phospholipase C (PLC) and a protein kinase C (PKC) (Charpentier et al. 1993, 1995). Nevertheless, the link between the depolarization of the membrane and the activation of the enzymes remains to be elucidated. As a working hypothesis (Charpentier et al. 1995), it was proposed that increased PLC activity could result from the activation of a membrane receptor either linked to a G-protein or possessing a tyrosine kinase activity, or from the direct effect of depolarization on PLC, independently of a receptor activation. Indeed, some reports have shown that the production of inositol trisphosphate comprises a voltage-dependent step (skeletal muscle, Vergara et al. 1985; neurons, Audigier et al. 1988; smooth muscle, Ganitkevich and Isenberg 1993).

In the present study, we tested the hypothesis that activation of transmembrane receptor tyrosine kinases could be involved in the induction process of the sNa^+ channels by stimulating defolliculated oocytes with insulin. Our results showed that the hormone significantly increased the induction rate of the sNa^+ channels. The possible biological implications of these findings are discussed.

Materials and Methods

Isolation, culture of oocytes and treatments

Oocytes were obtained from mature, non-hormonally treated *Xenopus laevis* females (from Centre de Recherches de Biochimie Macromoléculaire, Montpellier, France). Pieces of ovary were excised from tricaine methane sulphonate (0.2%) anesthetized donors. Ovary pieces were treated with dispase II (0.5 mg/ml for 2 h) to remove the follicular envelopes and cells. Stage V oocytes were then chosen for the experiments and maintained in OR2 medium for up to 6 days at 16 ± 1°C. The composition of OR2 medium was in mmol/l: NaCl 82.5; KCl 2.5; $MgCl_2$ 1; $CaCl_2$ 1; Na_2HPO_4 1; 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) 5; pH adjusted to 7.4 with NaOH (Wallace et al. 1973), no antibiotics were added. Incubation medium was renewed daily.

Chemicals and insulin were purchased from Sigma (St. Quentin Fallavier, France); lavendustin A and B, tyrphostin 23 were purchased from Biomol (Plymouth, PA, USA). Chemicals were applied externally by addition to the superfusate. Oocytes were incubated 10 to 120 min in medium containing chemicals before being tested. Appropriate vehicle controls were performed in each experiment.

Electrophysiological measurements

Electrophysiological recordings were done using a conventional two electrode voltage-clamp amplifier (Dagan Instruments, Minneapolis, MN, USA). Generation of clamp control voltages, data acquisition and analysis were done using the pCLAMP software, version 5.5 (Axon Instruments, Burlingame, CA, USA). Oocytes were impaled with 3 mol/l KCl-filled microelectrodes (0.8–1.5 MΩ) in a 50 μl recording chamber perfused at about 250 μl/min. After a stable resting potential was achieved, input resistance of the oocyte membrane was routinely measured by injecting a hyperpolarizing current (5 nA for 5 to 7 s) (Kado 1989). Induction of the sNa^+ channels was obtained either in current clamp or in voltage clamp conditions (Kado and Baud 1981). In current clamp experiments, the sNa^+ channels were induced by repetitive injections of depolarizing currents (up to 200 nA for 5 s). In voltage clamp experiments, the sNa^+ channels were induced by a single depolarization step to +50 mV of 90 to 180 s duration from a holding potential at either −60 or −20 mV. At −20 mV, the voltage-dependent Ca^{2+} channels are inactivated such that at +50 mV, Ca^{2+} entry and chloride channel activation are minimized (Charpentier and Kado 1999). Peak Na^+ currents were obtained at membrane potentials at +50 mV and measured as the peak value from the level of the holding current. The time at half-maximal inward current ($t_{1/2}$) has been chosen as the kinetic parameter to compare controls with insulin-treated oocytes. Measured parameters from individual control and experimental oocytes were averaged and expressed as means ± SEM. Significance between the means was tested using a Student's unpaired *t*-test; *p* values inferior to 0.05 were considered significant.

Each oocyte was used only for one induction, because the time courses for successive inductions were not identical, unless more than 1 h has elapsed between two inductions (Baud and Kado 1984). The current/voltage (I/V) relationship was performed on the same oocytes first without induced sNa^+ channels and then with induced sNa^+ channels. Investigated potentials ranged from −60 to +90 mV, from a holding potential at −60 mV, with 10 mV increments. In the non-induced oocytes, currents were elicited by 500 ms test pulses at 30 s intervals to avoid inducing the sodium channels. With induced sNa^+ channels, the I/V relations were obtained with test pulse duration of 1 s at 500 ms intervals. Elicited currents were measured at the end of the test pulses from the level of the holding current. All experiments were done at room temperature, 21–23 ℃.

Results

Effect of insulin and glucose on the oocyte membrane potential

The oocytes were empaled and current clamped at zero current. After the resting potential stabilized, insulin (10 μg/ml) or glucose (5 mmol/l) were superfused on the cells. The changes of membrane potential and resistance were investigated. Within minutes following exposure to insulin, the membrane potential depolarized; the mean amplitude of the depolarization was 8 ± 5 mV ($n = 13$) (Fig. 1A and B). The depolarization was accompanied by a decrease in the membrane resistance (0.47 ± 0.2 MΩ) ($n = 5$) and, in some cells, by oscillations of the membrane potential as illustrated on Fig. 1A. Oocytes bathed in the presence of insulin for 1 min showed similar responses occurring after the hormone was washed (Fig. 1B). These results were taken to mean that binding of insulin to membrane receptors activated a cascade of intracellular events resulting in calcium release from inositol trisphosphate sensitive stores. Ca^{2+}, in turn, activated the Ca^{2+}-dependent chloride channels (Miledi 1982; Barish 1983). Opening of the Cl^- channels accounted for the shift of the resting membrane potential towards the equilibrium potential of Cl^- ions (around −30 mV, see Barish 1983) and also for the decrease in the input resistance of the cells. The effects of insulin on the membrane potential and resistance were reversed after about 30 min of wash with standard OR2 medium (data not shown).

Addition of glucose in the superfusate also resulted in a depolarization of the membrane potential (Fig. 1C); the mean amplitude of the depolarization was 2.8 ± 0.5 mV ($n = 15$). The depolarization occurred without any change in the membrane resistance. The membrane potential returned within 1 min to its initial value following removal of glucose from the bathing medium. The presence of Na^+-glucose cotransport has been reported in *Xenopus* oocytes (Weber et al. 1989). Glucose uptake by the cells is therefore expected to be electrogenic and to depolarize the membrane due to the entry of Na^+ without any decrease in the membrane resistance. Our results are in agreement with these expectations.

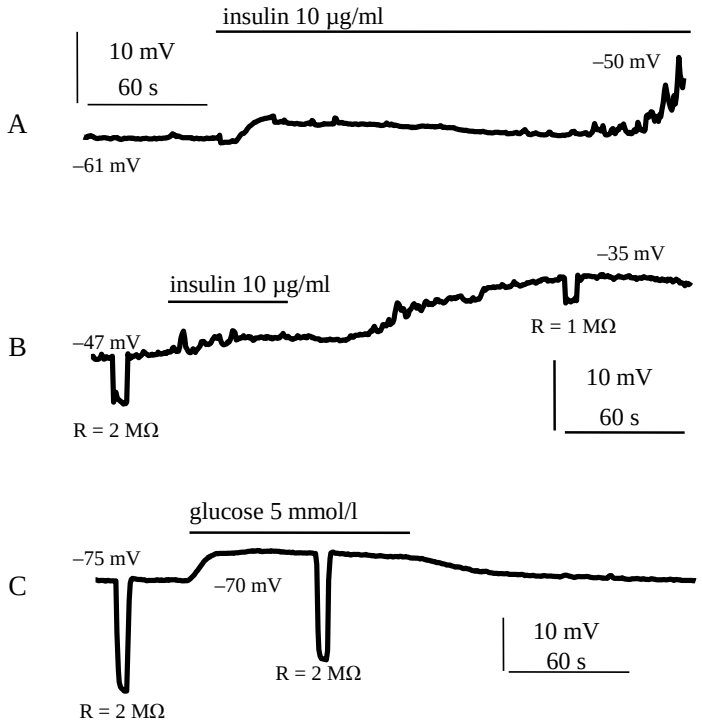

Figure 1. Effects of insulin and glucose on the oocyte resting membrane potential. **A.** Insulin (10 μg/ml) added to the OR2 superfusion medium caused a depolarization accompanied by fluctuations of the membrane potential. **B.** Superfusion of insulin for 60 s resulted in a depolarization and a decrease in the input membrane resistance (R). **C.** Addition of glucose (5 mmol/l) in the medium produced a depolarization but no change in the membrane resistance. Records shown are from different oocytes.

Divalent cations have been shown to inhibit some endogenous channels of the oocytes such as the voltage-dependent calcium channels and the sNa^+ channels (Quinteiro-Blondin and Charpentier 2001). Addition of Zn^{2+} (3 mmol/l) did not prevent the glucose-induced depolarization ($n = 3$; data not shown) and gives support to the findings that the glucose-induced depolarization could not be explained by the opening of voltage-dependent Ca^{2+} channels or Na^+ channels.

Effect of insulin on current clamp membrane responses

The induction of the sNa^+ channels of the oocyte membrane can be achieved either in current or in voltage clamp conditions (Kado and Baud 1981). In current clamp experiments, the sNa^+ channels were induced by injecting iterative depolarizing currents (Fig. 2A). With repeated depolarizing current pulses, the potential responses increased but returned to the resting value at the time the current pulse

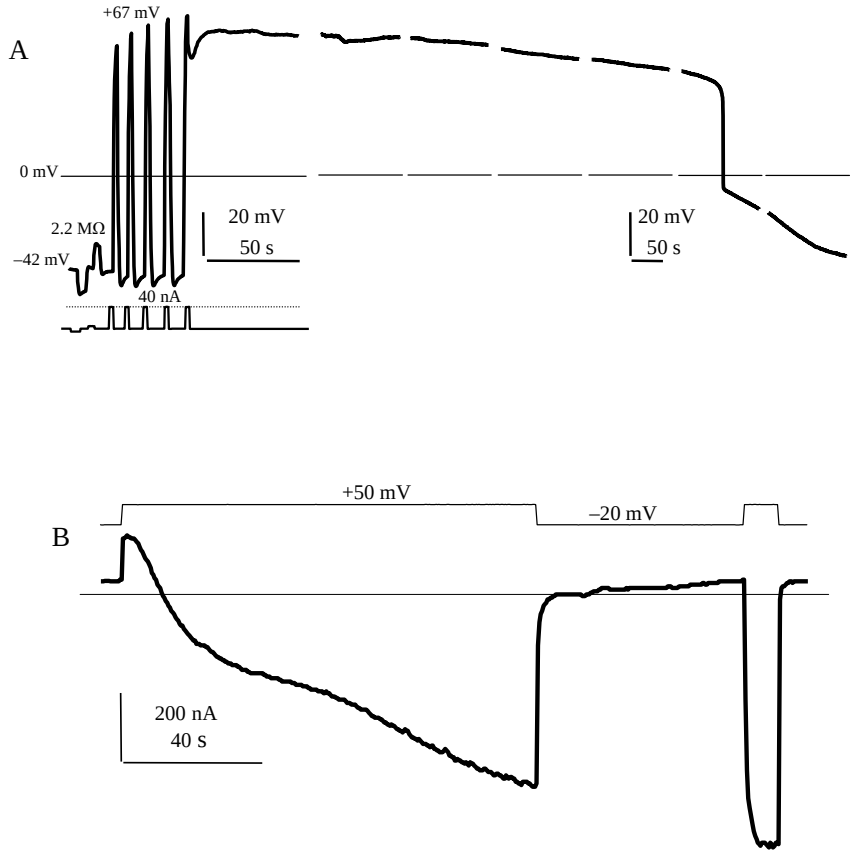

Figure 2. Standard procedures for inducing the sNa^+ channels. **A.** Membrane potential responses (upper traces) to 5 s injected current pulses (lower traces). At the end of the fifth pulse, the membrane potential remained depolarized for about 13 min due to sNa^+ channel opening before returning to resting level. **B.** Membrane current responses to voltage clamp steps to +50 mV from holding at −20 mV. Note that the elicited current was first outward and became slowly inward following a sigmoidal time course. When membrane potential was stepped to holding level, the current returned to zero. In response to the subsequent test pulse, the current was immediately inward due to the opening of the channels induced by the long-lasting depolarization.

was interrupted. Previous works have indicated that this increase in the potential response, during the injection of the depolarizing current, was due to the opening of sodium channels which did not inactivate (Kado et al. 1979; Baud and Kado 1984). At some point, the number of channels that opened and remained in a permeable state increased in such a way that the membrane potential remained depolarized for a long time before returning to more hyperpolarized level. The duration of the

plateau phase varied greatly between oocytes, some could stay depolarized for as long as 30 min (Kado 1983).

In voltage clamp experiments, the sNa^+ channels were induced by a long-lasting depolarization step to +50 mV of 100 s duration from a holding potential at −20 mV (Fig. 2B). In response to the depolarization step, the membrane current showed a sigmoidal time course. The current was first outwardly directed. The amplitude of this outward current is equal to the voltage step divided by the input resistance of the oocyte. Then, the current became inward and increased as more sodium channels were induced and opened. At the end of the depolarization step, the current rapidly returned to zero, indicating a fast closure of the channels. Once induced, the sNa^+ channels were voltage dependent, they can open or close with potential variations. Indeed, if another depolarization step was delivered, the current was immediately inwardly directed and rapidly reached its maximum. Thus, the sNa^+ channels can exist in at least three distinct states: i) a non-induced state before the cell is depolarized; ii) an induced and closed state after the cell has been depolarized for some time and then repolarized; iii) an open state at positive potentials (Baud and Kado 1984).

The possible effects of the enzymatic cascade activated by insulin were tested on the induction process of the sNa^+ channels in current clamp conditions. In the following experiments, each oocyte was exposed to two successive series of iterative depolarizing current pulses of 5 s duration at 5 s intervals until the first sNa^+ channels were induced. However, the series were interrupted before full induction was obtained, that is before the occurrence of the plateau phase. The two series were separated by a 10 min interval during which the cells were current clamped at zero current. On each oocyte, the first series were performed in OR2 medium whereas the second series were performed either in standard OR2 or in OR2 supplemented with insulin (2 μg/ml).

In the first series in OR2 medium, the injection of depolarizing current pulses into the oocyte resulted initially in purely passive membrane potential changes corresponding to the oocyte input resistance and membrane capacitance. However, the later current pulses gave rise to a depolarization which increased during the pulse indicating that the first sNa^+ channels were induced. The induction of the first sNa^+ channels was obtained after 10 ± 1 depolarizing current pulses ($n = 32$; Fig. 3). After 10 min, the second series were delivered in OR2 medium and the induction occurred after 11 ± 2 current pulses ($n = 16$; see Fig. 3A). The number of current pulses required to induce the sNa^+ channels in the second series has always been equal or superior to the number obtained in the first series. On the other hand, when the oocytes were incubated for 10 min in the insulin-supplemented medium between the two series, the second series of depolarizing currents caused the induction of the first sNa^+ channels in 3 ± 1 pulses ($n = 16$; Fig. 3B and C). Moreover, the induction process seemed to be facilitated by the presence of insulin, since the plateau phase during which the potential remained positive was rapidly obtained.

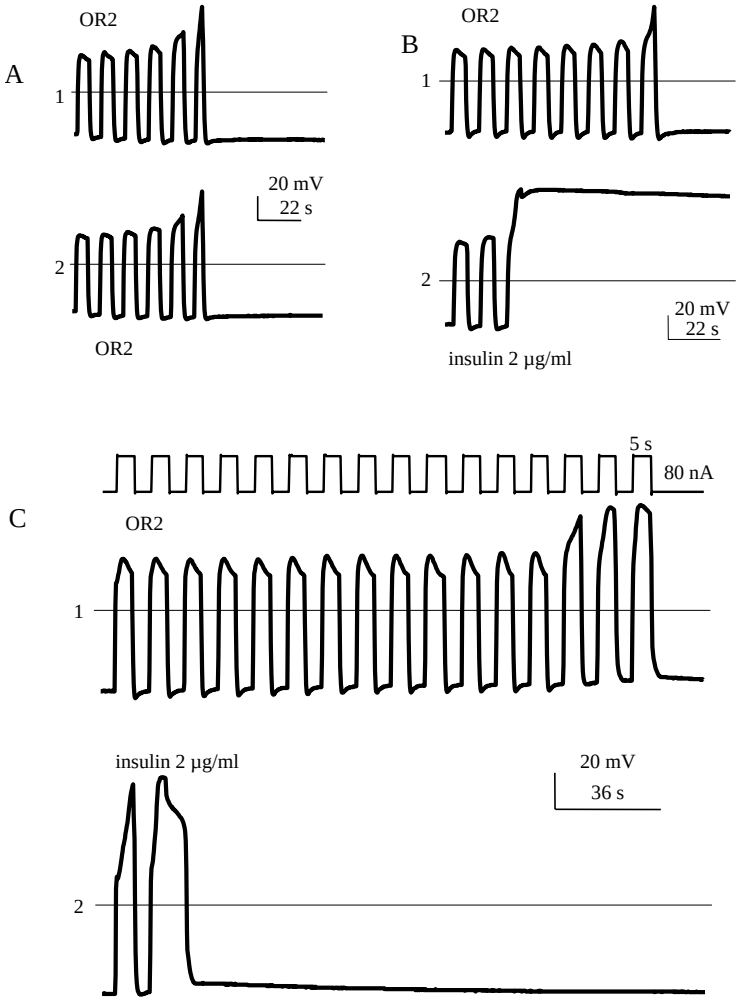

Figure 3. Effects of insulin on sNa^+ channel induction in current clamp experiments. Two series of depolarizing current (see upper trace in C) were injected at a 10 min interval. **A.** Control oocyte in OR2 medium. The number of depolarizing current pulses required to induce the first sNa^+ channels in series 1 and 2 was exactly the same. **B** and **C.** Series 1 were performed in OR2 medium and series 2 after 10 min incubation with insulin (2 μg/ml). Insulin caused the induction of the sNa^+ channels to be more rapidly obtained. Two traces are presented to illustrate the variability in the response of insulin-treated oocytes.

Effect of insulin on membrane currents in voltage clamp experiments

Concentrations of insulin varying from 0.1 to 10 μg/ml were tested on the oocytes in voltage clamp conditions. Two parameters were used to evaluate the effect of

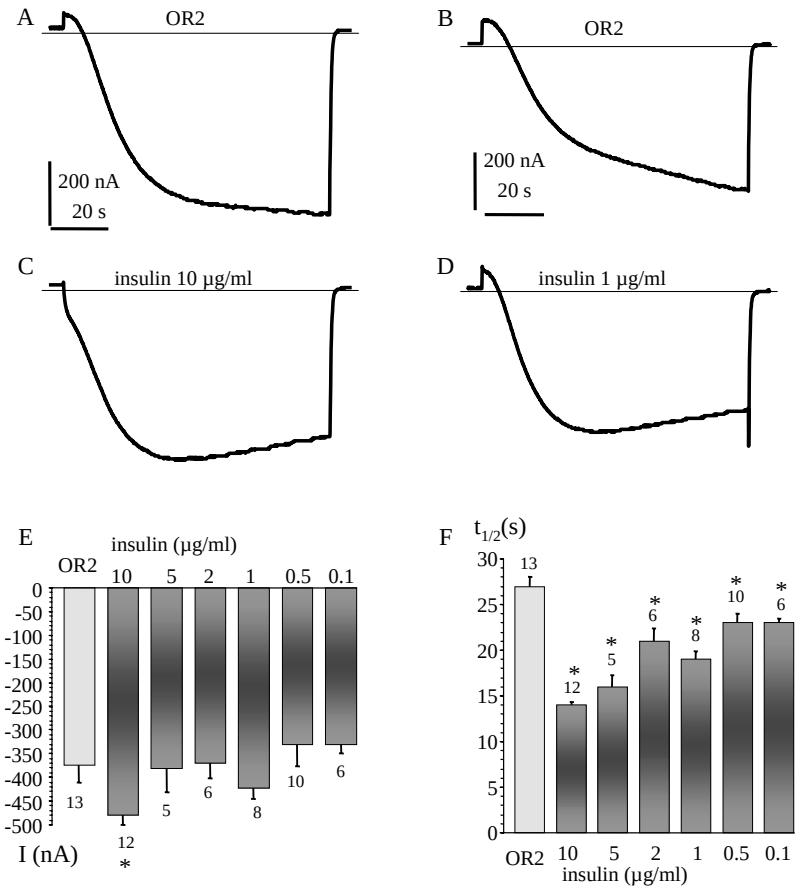

Figure 4. Effects of insulin on sNa^+ channel induction in voltage clamp experiments. **A** and **B.** Control oocytes from different batches in OR2 medium. **C** and **D.** Addition of insulin facilitated the rate of induction of the Na^+ current. With increased insulin concentrations, the magnitude of the initial outward current became smaller than the magnitude of the inward (sodium) current, in such a way that in the presence of insulin at a concentration of 10 μg/ml, the elicited current was immediately inward. **E** and **F.** Dose-dependent effect of insulin on the Na^+ current amplitude and on the time for half-maximal inward current ($t_{1/2}$). The number of oocytes tested is indicated near the error bar. Asterisk indicates a significant ($p < 0.05$) change as compared to control means.

the hormone: the amplitude of the sodium current and a kinetic factor $t_{1/2}$ used to compare induction rates. In OR2 medium, the average current amplitude was -375 ± 37 nA ($n = 13$) and $t_{1/2}$ was 26.6 ± 1.1 s. Typical sigmoidal time courses of the sNa^+ channel induction in oocytes from different donors are illustrated in Fig. 4A and B. When oocytes were incubated for 10 min in OR2 medium supple-

mented with insulin (10 μg/ml), the most conspicuous effect was the absence of any outward current evoked in response to the depolarizing step (Fig. 4C). The elicited current was immediately inward as if the sNa^+ channels were already induced, before the beginning of the depolarizing shift. Indeed, $t_{1/2}$ was significantly decreased to 14.3 ± 0.3 s ($p = 0.0023$). In addition, the average current amplitude was significantly increased (-479 ± 20 nA; $n = 12$; $p = 0.0148$). Decreased concentrations of insulin resulted in reduced effects on the amplitude of the sodium current as well as on $t_{1/2}$ (Fig. 4D,E and F).

The I/V relationships were performed before and after induction of the sNa^+ channels, from a holding potential at -60 mV with depolarization steps of 10 mV increment. Before the induction of the sNa^+ channels (Fig. 5A), two conductances were evoked: one activated at -20 mV and peaked at $+20$ mV. It was due to the opening of chloride channels in response to Ca^{2+} entry through voltage-dependent Ca^{2+} channels (Barish 1983). The other activated at about $+60$ mV, it was due to the opening of non-selective cationic channels and K^+ is the main ion transiting through these channels (Arellano et al. 1995). After the induction of the sNa^+ channels (Fig. 5A), an inward current was activated at about -10 mV, it peaked at $+50$ mV and reversed direction for potentials more positive than $+70$ mV. Part of the sodium inward current is expected to be masked in the range of potentials of -20 to $+50$ mV by the Ca^{2+}-dependent chloride current which is outwardly directed (Fig. 5A). When the oocytes were bathed in a medium supplemented with insulin (5 μg/ml), the I/V curve superimposed with that of the controls until about

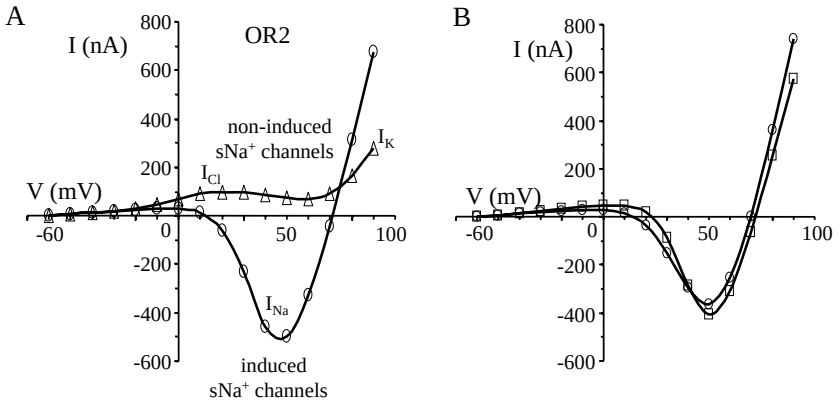

Figure 5. I/V relationships. Typical I/V relations obtained in different conditions of bathing media. The same oocyte was used for the experiments in each panel. **A.** Current activation before (open triangles) and after (open circles) sNa^+ channel induction in OR2 medium. Note that the Cl^- current partly masks the Na^+ current in the range of potentials from -20 to $+50$ mV. **B.** In the presence of insulin (5 μg/ml) (open squares) the Cl^- current and the Na^+ current have a slightly greater amplitude than that of the control (open circles).

0 mV. In the presence of insulin, the amplitude of the chloride current was larger; whereas the amplitude of the sodium current was about similar to that of the controls (Fig. 5B).

Effect of some tyrosine kinase inhibitors on the sNa^+ channel induction

PLC has been shown to be implicated in the sNa^+ channel induction; involvement of PLC in this process was proposed to be possibly linked to the activation, by the membrane depolarization, of a membrane receptor either coupled to a G-protein or possessing a tyrosine kinase activity (Charpentier et al. 1995). Experiments were performed to block the tyrosine kinase pathway downstream membrane receptors with three different inhibitors: tyrphostin 23, lavendustins A and B. Oocytes were incubated for at least 1 h in OR2 medium supplemented with lavendustin A (50 μmol/l) and then incubated for 10 min in the presence of insulin (5 μg/ml). The

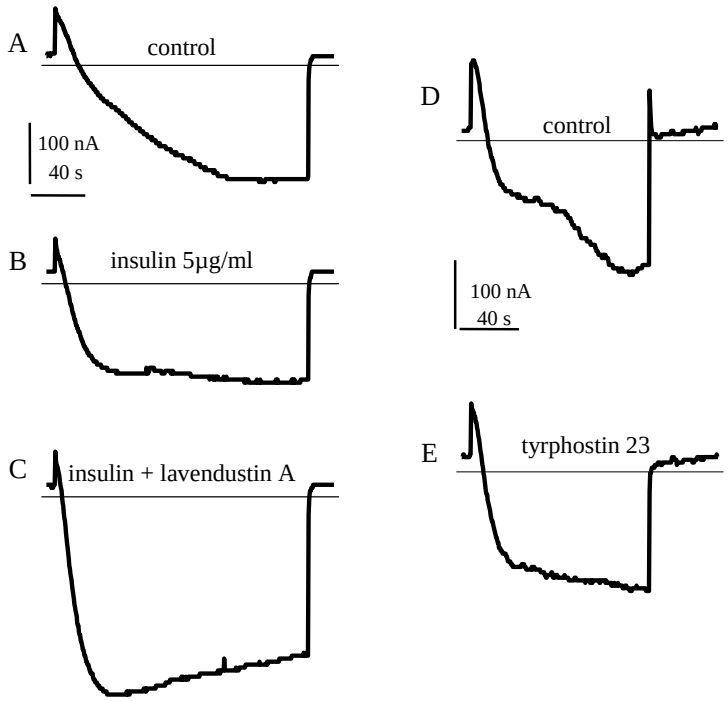

Figure 6. Effects of tyrosine kinase inhibitors on insulin facilitation and on the sNa^+ channel induction. **A.** Control oocyte in OR2 medium. **B.** 10 min incubation with insulin (5 μg/ml) resulted in facilitated induction of the sNa^+ channels. **C.** Pre-incubation for about 60 min with lavendustin A (50 μmol/l) failed to inhibit insulin effect on the induction. **D.** Control oocyte in OR2 medium. **E.** Tyrphostin 23, which is known to inhibit the tyrosine kinase pathway activated by epidermal growth factor receptors, had no blocking effect on the inward current at 150 μmol/l concentration.

Table 1. Sodium current amplitude and time for half-maximal inward current ($t_{1/2}$) in control and treated oocytes

Oocyte treatment	Peak Na^+ current	$t_{1/2}$	n
Control	-266 ± 32 nA	81.4 ± 13 s	6
Insulin (5 μg/ml)	-175 ± 16 nA^{s}	27.1 ± 3 s^{s}	6
Insulin + lavendustin A (50 μmol/l)	-215 ± 44 nA^{ns}	24.8 ± 3 s^{s}	4

Values are means ± SEM; n, oocyte number; ^{s} significant; ^{ns} non-significant.

Table 2. Sodium current amplitudes in control and treated oocytes

Oocyte treatment	Peak Na^+ current	n
Lavendustin A (50 μmol/l)	-507 ± 41 nA ^{ns}	8
Control	-509 ± 44 nA	5
Lavendustin B (50 μmol/l)	-270 ± 24 nA ^{ns}	9
Control	-306 ± 28 nA	4
Tyrphostin 23 (150 μmol/l)	-222 ± 25 nA ^{ns}	7
Control	-249 ± 60 nA	5

Values are means ± SEM; n, oocyte number; ^{ns} non-significant.

sNa^+ channels were induced in voltage clamp conditions. Results showed that the time course of the sodium current were very similar in the oocytes bathed in the media containing either insulin or insulin and lavendustin A (Fig. 6B and C). In controls, the average amplitude of the current was -266 ± 32 nA and $t_{1/2}$ was 81.4 ± 13 s ($n = 6$; Fig. 6A and Table 1). In the presence of insulin, the average amplitude of the current was -175 ± 16 nA and $t_{1/2}$ was 27.1 ± 3 s ($n = 6$; Fig. 6B). In the presence of insulin and lavendustin A, the average amplitude of the current was -215 ± 44 nA and $t_{1/2}$ was 24.8 ± 3 s ($n = 4$; Fig. 6C).

In current clamp conditions, the induction of the sNa^+ channels was tested in two series of injections of depolarizing currents separated by a 10 min interval as described above. The first series was performed on oocytes treated for 1 h with lavendustin A, induction was obtained in response to the third pulse ($n = 4$; data not shown). The second series was performed after incubation for 10 min in a medium containing insulin and lavendustin A, induction was obtained in response to the first depolarizing current pulse in the four cells tested. Current and voltage clamp experiments indicated that in spite of the presence of the tyrosine kinase inhibitor, insulin accelerated the induction rate of the sNa^+ channels.

In another series of voltage clamp experiments, the tyrosine kinase inhibitors were tested on the induction of the sNa^+ channels in OR2 medium without insulin. Lavendustin A (50 μmol/l), lavendustin B (50 μmol/l) and tyrphostin 23 (150 μmol/l) did not significantly change the average amplitude of the Na^+ current (see Table 2 and Fig. 6D,E).

Discussion

The membrane of immature *Xenopus* oocyte is known to possess a wide variety of receptors and channels (Arellano et al. 1996; Weber 1999). One of these channels, highly selective for sodium ions (the sNa^+ channels), exhibit rather unusual electrophysiological and pharmacological properties (Kado et al. 1979; Kado and Baud 1981; Baud et al. 1982; Baud and Kado 1984). Indeed, from a pharmacological point of view, the sNa^+ channels are not blocked by tetrodotoxin even in the micromolar range (Kado and Baud 1981; Baud et al. 1982). They also are insentitive to saxitoxin (1 μmol/l), amiloride (1 mmol/l), ouabaine (500 nmol/l), veratridine and to the toxin II from *Anemonia sulcata* (Baud and Kado 1981; Baud et al. 1982; Quinteiro-Blondin and Charpentier 2001). More recent studies have shown that the sNa^+ channels were blocked by divalent cations and by the local anaesthetic lidocaine (Quinteiro-Blondin and Charpentier 2001; Charpentier 2002). One of the most striking peculiarities of the sNa^+ channels is that unlike other voltage-dependent channels, the channels do not respond to a depolarization until they have been made voltage-dependent, or induced. Induction of the channels is accomplished by prolonged depolarizing shift in potential (Fig. 2A and B). Once induced the sNa^+ channels open and close with the variations of potential. Such a modification of the membrane channel properties produced by the simple depolarization of the membrane leads to examine what is happening to the channels to make them voltage sensitive, and how this is coupled to the depolarization. Previous reports have shed light on the induction of the sNa^+ channels. They showed that sustained depolarization of the oocyte membrane resulted in the activation of PLC and PKC, which in turn phosphorylated the sNa^+ channels or a closely associated regulatory protein (Charpentier et al. 1993, 1995). Hence, the channels became potentially gatable. Three possible mechanisms were proposed to explain the link between depolarization of the membrane and the activation of PLC (Charpentier et al. 1995). Activation of PLC could result: i) from the activation of a G-protein-linked membrane receptor; ii) from the activation of a receptor possessing an intrinsic protein tyrosine kinase activity; iii) from the direct effect of depolarization on PLC, independently of a receptor activation (Rhee and Choi 1992). In this paper, we investigate the possible effects on the sNa^+ channel induction of insulin, a hormone known to activate intracellular signaling events in *Xenopus* oocytes (Stefanovic and Maller 1988). Eventhough, the oocyte membrane seems to be deprived of specific insulin receptors (Hainaut et al. 1991; Janicot et al. 1991), insulin can bind to insulin-like-growth-factor-I (IGF-I) receptors albeit with a different affinity. Nonetheless, insulin and IGF-I receptors share common features; both are trans-membrane glycoproteins, ligand binding on the receptor stimulates a common signaling pathway comprising a tyrosine-specific protein kinase and thereby enhances phosphorylation of protein substrates (White and Kahn 1994).

We focused on the possible effects of insulin on the sNa^+ channel induction. We found, in current clamp experiments, that the induction rate was higher in

response to insulin. Indeed, the number of current pulses required to induce the first sNa^+ channels was significantly reduced (3 ± 1 compared to 11 ± 2 in controls, see Fig. 3). Moreover, in voltage clamp experiments, the time course of the current evoked by the depolarization step in the presence of insulin displayed conspicuous changes. In controls, the depolarizing pulse resulted in an initially outward current, occurring immediately after the capacitative transient. Afterwards, the clamping current became inwardly directed, with a sigmoidal time course, reached its maximum and could remain inwardly directed as long as the depolarization was maintained (Fig. 4). On the contrary, in the presence of high amounts of insulin (10 μg/ml), there was no outward current. The elicited current was immediately inward as if the sNa^+ channels were already in their induced state albeit closed, before the long-lasting depolarization was applied. As described above, the number of induced sNa^+ channels in a given cell depends upon the duration of the inducing depolarization (Fig. 2B). In the presence of insulin, the oocyte behaved as if it had undergone a preliminary inducing depolarization. The enzymatic cascade activated by the hormone seems to target either the sNa^+ channels, or a closely associated and regulatory protein, in such a way that the time needed by the channels to become voltage sensitive was conspicuously diminished. The significant reduction of $t_{1/2}$ also supports this explanation.

The detailed comparison of receptor tyrosine kinases has led to a classification of this family into three groups: the epidermal growth factor (EGF) group, the insulin and IGF-I group and the platelet-derived growth factor (PDGF) group (Yarden and Ullrich 1988). The sequence of events activated by EGF and PDGF receptors involves different steps among those phosphorylation of tyrosine residues which provides specific docking sites for various proteins possessing SH2 domains and mediating the signaling cascade (Cantley et al. 1991). Thus, tyrosine phosphorylation regulates the activity of various enzymes such as phosphatidyl inositol 3-kinase (PI3-K) or phospholipase C-γ (PLC-γ) (Schlessinger and Lemmon 2003). Some drugs can be used to inhibit this sequence of events: lavendustins and tyrphostins are such inhibitors (Lyall et al. 1989; Hsu et al. 1991). Our experiments in current or voltage clamp conditions showed that oocyte treatment with lavendustin A failed to block the effects of insulin (Fig. 6C). $t_{1/2}$ was significantly reduced compared to controls (Table 1). In addition, pretreatment of the oocytes with tyrphostin, known to block the EGF-activated tyrosine kinase pathway in oocytes (Lorenzo et al. 2001) and to block EGF-induced tyrosine phosphorylation of PLC (Margolis et al. 1989), in spite of the absence of insulin, also failed to inhibit the induction of the sNa^+ channels (Fig. 6). These results suggest that the enzymatic cascade involved in the sNa^+ channel induction seems to be independent of EGF and PDGF signaling pathway.

As pointed out by Baud (1983), a shift of potential to a value sufficient to induce the sNa^+ channels (that is a potential at least equal to +20 mV) has no chance to occur during the life of the immature or mature oocyte. Even the fertilization potential would not change the potential to such a high level, since it is partly due to a movement of chloride ions in Amphibians (*Rana pipiens*, Jaffe et

al. 1985; *Xenopus laevis*, Peres and Mancinelli 1985). Therefore, no natural event would depolarize the membrane to a potential allowing the induction of the sNa^+ channels. However, when the channels are induced, the activation threshold of the channels is negative (about −20 mV). Thus, it is no longer necessary to depolarize the membrane to positive values; a shift to −20 mV would be enough. During progesterone-induced meiotic maturation, the oocyte undergoes many changes: the membrane potential shifts to about −10 mV, at the time of early germinal vesicle breakdown. Moreover, the membrane potential of the oocytes has been shown to undergo transient depolarizing shifts at about 0.5 Hz (Kado et al. 1981). In addition, it is known that the sodium content continuously increases during the growth of the oocytes, from 1–6 mmol/l to 20 mmol/l before fertilization (see the review of Dascal 1987). There is the possibility that Na^+ would enter the cell through the sNa^+ channels during maturation. The hypothesis that the induction of these channels, by a shift of potential, could be an artifact, is plausible. In this case, induction would be due to the activation of the PKC cascade in response to biochemical processes triggered by some external factor such as a hormone. Progesterone is a likely *in vivo* candidate; whereas *in vitro* insulin might exert similar effects, since it can induce meiotic maturation (El-Etr et al. 1979) *via* PLC activation (Garcia de Herreros et al. 1991). Therefore, the induction of the sNa^+ channels might not be electrically activated, but the gating of the channels could be. Further experiments are needed to clarify these points.

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