J Mol Cell Cardiol 29, 2687–2698 (1997)

Basic FGF Enhances Calcium Permeable Channel Openings in Adult Rat Cardiac Myocytes: Implication in the bFGF-induced Increase of Free Ca^{2+} Content

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(Received 7 April 1997, accepted in revised form 17 June 1997)

P.-L. Merle, Y. Usson, M. Robert-Nicoud and J. Verdetti. Basic FGF Enhances Calcium Permeable Channel Openings in Adult Rat Cardiac Myocytes: Implication in the bFGF-induced Increase of Free Ca^{2+} Content. *Journal of Molecular and Cellular Cardiology* (1997) **29**, 2687–2698. Basic fibroblast growth factor (bFGF) has been implicated in the changes in gene expression that, under pathological conditions such as ischemia or volume overload, lead to adult cardiomyocyte hypertrophy. In many tissues, one of the first events following cell activation by growth factors is an enhancement of the intracellular free calcium concentration, generated by fluxes from internal storage compartments and through channels of the plasma membrane. The present study was undertaken to determine whether cardiac myocytes isolated from adult rat ventricles express Ca^{2+}-permeable channels activated by bFGF. Using the cell-attached mode of the patch-clamp technique, we observed that bFGF (from 0.1–10 nM) induced an increase of fast burst openings, mediated by Ca^{2+}-permeable channels with low conductance (15 pS) and voltage-independence. Inside-out patch-clamp experiments revealed that inositol 1,4,5-trisphosphate (5 μM) enhanced the opening of Ca^{2+}-permeable channels with similar properties as the bFGF-induced channels, indicating that IP_3 may be a second messenger of this process. Confocal fluorescence imaging of intracellular free calcium provided direct evidence that bFGF induced an increase of cytoplasmic and nucleoplasmic free Ca^{2+} concentrations which were generated, in part, by Ca^{2+} influx through the plasma membrane. In conclusion, this study supports the presence, in the plasma membrane of adult cardiac myocytes, of messenger-activated calcium channels which could play key roles in the calcium-dependent pathways that are activated in response to growth factors.
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Key Words: Cardiomyocyte; Basic fibroblast growth factor; Calcium channel; Inositol (1,4,5)-trisphosphate; Patch-clamp; Confocal microscopy.

Introduction

Basic fibroblast growth factor (bFGF or FGF_2) is a polypeptide implicated in a wide variety of physiological and pathological functions (Basilico and Moscatelli, 1992). The binding of bFGF to specific receptors initiates tyrosine phosphorylation of cytosolic target proteins and generates several cellular activation pathways (Jaye *et al.*, 1992). These cascades of activations ultimately lead to the translocation, into the nucleus, of proteins inducing the expression of oncogenes which further regulate

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either DNA replication, or specific gene expression (Ullrich and Schlessinger, 1990). In many cell types, an elevation of intracellular free calcium concentration ($[Ca^{2+}]_i$) has been shown to be one of the very first events following the binding of growth factors (Moolenar *et al.*, 1986). This increase is thought to both initiate and regulate subsequent phosphorylation of calcium-dependent kinases or phosphatases that are implicated in the mitogen cascade activation pathways (Soltoff and Cantley, 1988). One second messenger, synthesized in response to the binding of bFGF on tyrosine kinase receptors, is inositol 1,4,5-trisphosphate (IP_3), which is mainly responsible for an elevation of $[Ca^{2+}]_i$ through the release of calcium from internal stores (Berridge and Irvine, 1989; Jaye *et al.*, 1992). However, some studies have highlighted that IP_3 can also stimulate the influx of extracellular calcium through plasma membrane channels (Kuno and Gardner, 1987; Chapron *et al.*, 1989; Merle *et al.*, 1995; Vaca and Kunze, 1995). Several reports even state that this calcium influx is necessary to allow cell activation by mitogens (Estacion and Mordan, 1993; Mogami and Kojima, 1993). However, little data is available concerning the mechanisms involved in this particular activation pathway.

With respect to the heart, bFGF has been shown to play important roles in both normal development and pathological modifications of the myocardium. At an early stage of embryonic heart development, bFGF, synthesized by cardiac cells, first activates the division of myoblasts and, thereafter, participates in their differentiation into mature cardiac myocytes (Parker and Schneider, 1991). In this regard, we have recently shown, in cardiac myocytes cultured from neonatal rat ventricles, that bFGF induces, via phospho-inositol metabolism, the opening of a novel type of calcium permeable channel (Merle *et al.*, 1995). At the adult stage, bFGF is detected at relatively high levels in atria and ventricles, where it is located in extracellular, cytoplasmic and nuclear sites of the myocytes on which it could act in an autocrine, paracrine and also intracrine manner (Kardami and Fandrich, 1989; Speir *et al.*, 1992). In addition, and contrary to general belief, it has been established that adult cardiomyocytes express functionally, specific bFGF receptors (Liu *et al.*, 1995), which are different from those predominant in proliferative cells (Jin *et al.*, 1994). Although the participation of this growth factor in normal cardiac physiology remains uncertain, it is thought that, under patho-physiological conditions, such as volume overload or ischemia, bFGF may be involved in changes in gene expression that lead to cardiac myocyte hypertrophy (Cummins, 1993). *In vitro* experiments have shown that bFGF activates oncogene expression, inducing the synthesis of fetal-like proteins, comparable to what occurs in cardiomyocytes of hypertrophied hearts (Parker and Schneider, 1991). Some corroborating studies report that both the expression and the availability of bFGF are modified in the cases of volume overload or isoproterenol-induced cardiomyocyte injury and that bFGF is cardioprotective in the case of ischemia-reperfusion injury (Padua *et al.*, 1996). It has also recently been demonstrated that bFGF reduces myocardial infarct size after temporary coronary occlusion (Horrigan, 1996). However, despite much evidence implicating bFGF in cardiac myocyte activation, little is known about the initial events following bFGF binding to these cells.

The present study was undertaken to determine whether modifications of the membrane permeability to calcium could represent an early signal of the adult cardiomyocyte response to bFGF. We report that cardiomyocytes, taken from adult rat ventricles, express bFGF-activated calcium permeable channels, the activation of which is triggered by IP_3. Moreover, using confocal microscopy, we demonstrate that bFGF induces an increase of cardiomyocyte free calcium content in the cytoplasm as well as in the nucleoplasm. Finally, we observed a partial inhibition of this $[Ca^{2+}]_i$ enhancement by gadolinium, which also inhibits the bFGF-induced calcium channel openings. Taken together, these observations suggest that bFGF induces, through the plasma membrane of adult cardiomyocytes, calcium influx which could partly be responsible for the ensuing increase in intracellular free calcium content. We discuss the physiological relevance of this process in the cardiomyocyte response to growth factors.

Materials and Methods

Cell isolation

Adult rat cardiac myocytes were isolated using conventional enzymatic dissociation (Dubus, 1990). Briefly, hearts were excised from 4-month-old male Wistar rats and placed in ice-cold nominally Ca^{2+}-free solution (composition, in mM: $MgCl_2$, 0.5; KCl, 10; KH_2PO_4, 4; $NaHCO_3$, 15; NaCl, 70; Glucose, 11; Sucrose, 115; Pyruvate, 2; HEPES, 5; pH, 7.2). The hearts were cannulated and retrogradely perfused through the aorta for an initial wash-out period of 3 min with Ca^{2+}-free solution. Afterwards, the hearts were switched to a recirculating collagenase solution, made from calcium

free solution containing 0.01 g/l collagenase D (activity: 0.4 U/mg) and 0.1 g/l BSA, gassed with O_2 95%, CO_2 5%, pH 7.3 and perfused for 30 min, until the tissue became flaccid. At this stage, ventricles were teased into small pieces and left for 3 min in collagenase solution mixed (v/v) with BSA solution (made from calcium free solution containing 0.2 g/l BSA) in a rotating incubator. Ventricle tissue was then dispersed, filtered on a 200 μm Nylon mesh and slowly centrifuged (1 min, 25 g). Cells were resuspended three times in BSA solution and calcium concentration was gradually increased by addition of modified Tyrode's solution (containing, in mM: NaCl, 125; KCl, 5.6; $CaCl_2$, 1.2; $MgCl_2$, 1.2; HEPES, 10; $NaHCO_3$, 10; Glucose, 11). Between each step, cells were left in an incubator for a 5-min self-sedimentation, during which non-viable cells settled more slowly, thereby increasing the viable cell fraction. Cells were finally suspended in modified Tyrode's solution (final calcium concentration: 1.2 mM) and plated in tissue culture dishes (Falcon Becton Dickinson Co., Franklin Lakes, NJ, USA) at low density. Cells were placed in an incubator (95% air, 5% CO_2, 37°C) and allowed to adhere for at least 1 h before being used. Under these conditions, well separated and adhered, rod-shaped myocytes were obtained, as required for patch-clamp experiments.

Single channel recordings

Single channel currents from both cell-attached and inside-out membrane patches were recorded using standard patch-clamp techniques. All experiments were conducted at room temperature (25°C) and completed within 24 h of cell isolation. The culture dish on which cells were settled was placed upon the stage of an inverted microscope and target cells were carefully chosen using the following criteria: rod-shaped appearance, clear striations, sharp edges, no visible granulation, good adherence and the absence of spontaneous contractile activity. Single channel currents were recorded with a RK-300 amplifier and stored on digital audio tape (DTR-1200, Biologic, Claix, France). Experimental results were subsequently replayed on a Gould recorder and, after digitization (through an IEEE interface), data were analysed using Bio-Patch software (Biologic, Claix, France). Pipettes were fabricated on a pipette puller (P-87, Sutter Instrument Co, San Rafael, CA, USA.) from glass capillaries (Kimax-51 Kimble, Vineland, NJ, USA) and had resistances of 4–8 MΩ when filled with the barium pipette solution. Cell-attached recordings were conducted in modified Tyrode's solution, where sodium bicarbonate was substituted for HEPES (10 mM). Basic FGF was added at a final concentration of either 0.1 or 10 nM, in the presence of BSA (0.01 g/l). In the case of patch excision, the bath solution was changed to internal solution containing (in mM): KCl, 150; $MgCl_2$, 2; $CaCl_2$, 0.55; EGTA, 1.1; HEPES, 10; and inositol 1,4,5 trisphosphate was added at a final concentration of 5 μM. In most experiments, barium was used as the charge carrier to obtain the best resolution of differences in single channel amplitude, since calcium channels are generally more permeable to this ion, and barium is known to inhibit potassium currents (Tsien *et al.*, 1987). For some experiments, calcium was substituted for barium. The composition of either barium or calcium acetate pipette solutions were respectively (in mM) $Ba(CH_3OOH)_2$ or $Ca(CH_3OOH)_2$ 50; Sucrose, 150; HEPES, 10. In other experiments, gadolinium or lanthanum chloride were added at a final concentration of 200 μM in the barium pipette solutions. To determine channel specificity sodium pipette solution was also used (composition, in mM: $Na(CH_3COOH)$, 50; $MgCl_2$, 1.2; Sucrose, 180; HEPES, 10, with or without tetrodotoxin 200 nM).

Confocal microscopy and fluorescence measurements

Cardiac myocytes were plated on glass coverslips coated with laminin (5 $\mu g/cm^2$). Cells were loaded with Fluo-3 for 20 min, in the dark and at room temperature, by incubation in modified Tyrode's solution containing: 5 μM Fluo-3 acetoxymethyl ester, 50 mg/l BSA and 2.5 mg/l pluronic F-127. Experiments were performed after an equilibration period of at least 10 min to allow final dye cytosolic hydrolysis. Fluorescence images were obtained using a laser confocal microscope (Zeiss LSM 410, Iena, FRG). Fluorochrome was excited by the 488 nm line of an Argon laser and emitted light was collected through a 510–560 nm bandpass filter. The confocal detector apparatus provided an axial resolution of 0.6 μm and the optical cross-section was fixed at the upper half of a target cardiomyocyte which was chosen according to the same criteria as for patch-clamp experiments. Relative changes of free $[Ca^{2+}]_i$, were determined by defining a scanning line along the length of a cell. This permitted us to record time-dependent changes in fluorescence along this line. When using line-scan mode, illumination intensity was attenuated 300-fold to avoid fluorochrome photobleaching.

Line-scan images were then constructed, by assembling individual line-scans. The x-axis represents the position along the cell and the y-axis is time (scanning rate was 20 ms/line). Time-dependent changes of fluorescence were then calculated by averaging the fluorescence intensity in specific regions or the totality of each line-scan. Data were normalized by dividing values by the mean resting fluorescence observed at the beginning of each experiment. Percentage increase is expressed $\pm$ S.E.M. Thapsigargin (20 μM), cyclopiasonic acid (10 μM) and genistein (50 μM) were prepared in dimethyl sulfoxide so that the final concentration of the solvent in experimental solutions was $\leq 0.5\%$.

Materials

All chemicals were from Sigma (St Louis, MO, USA). BSA (fraction V, fatty acid free) collagenase (type D) and bFGF (human, recombinant) were from Boehringer (Mannheim, Germany).

Results

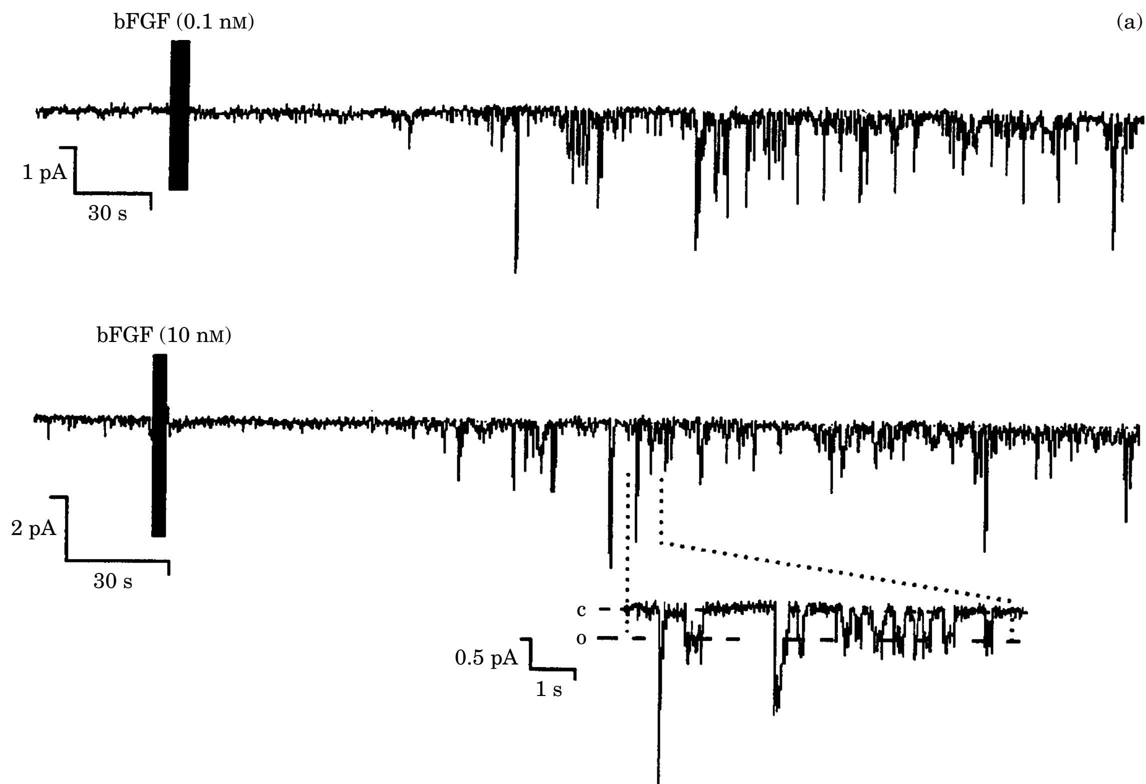
Effects of bFGF on membrane channel activation

In order to record changes of membrane permeability to calcium, patch-clamp experiments were carried-out on adult rat cardiac myocytes, which resting potential was -68 ± 3 mV (as measured, using intracellular microelectrodes filled with 3 M KCl, from 15 independent experiments). Figure 1(a) shows a typical records obtained using a barium pipette solution from cell-attached patches held at -35 mV of membrane potential (membrane potential $= -68 -$ holding potential). The addition of 0.1 or 10 nM bFGF induced, in the following 2 min, the openings of channels exhibiting fast bursts activities [Fig. 1(a)]. Distribution analysis of the trace obtained using 10 nM bFGF revealed that the amplitude of the corresponding single inward current was 0.5 pA [Fig. 1(b)]. The value of the single current amplitude varied linearly with the holding potential and the openings were found to display a voltage-independent behavior. The current-voltage relation [Fig. 1(c)] was fitted by a straight-line corresponding to channels exhibiting a slope conductance of 17 ± 5 pS and a reversal potential of 10 ± 5 mV (each point is the mean of 10–17 experiments, voltages are membrane potentials). Figures 1(d) and 1(e) show the histograms of distribution for the mean open and closed times, fitted by the sum of two exponentials. The fast and slow time constants were, respectively, 1.3 and 12.1 ms for the open times and 1.7 and 8.5 ms for the closed times.

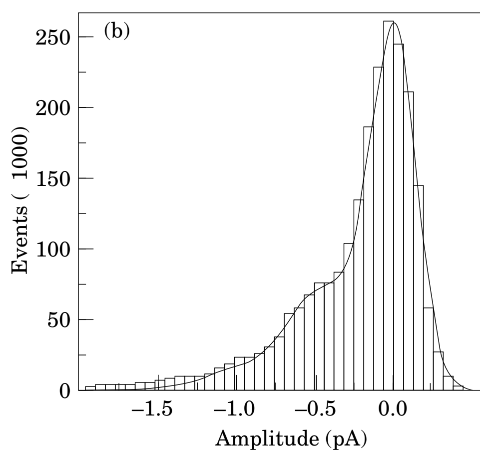
From the calculation of the fluxes of the bFGF-induced currents (expressed in pA/s) a curve representing time-dependent modifications of the fluxes of current intensity before and after bFGF addition was drawn (Fig. 2). this curve, representative of seven experiments, revealed that the time-course evolution of the bFGF-induced currents often displayed cyclic periods of intense channel openings (slope a and parallels) between which membrane permeability returns to basal value (slope b and parallels).

When calcium was substituted for barium, no significant modification of the bFGF-induced currents was observed. Similarly, using chloride instead of acetate, did not affect current characteristics. However, the reversal potential shown in Figure 1(b) is lower than the theoretical calcium reversal potential evaluated from the Nernst equation, suggesting that the specificity of the observed channels for calcium or barium is weak. Consequently, sodium pipette solutions were used in order to test the channel permeability to this cation and tetrodotoxin (200 μM) was added to inhibit voltage-dependent sodium channels. When cell-attached experiments were conducted in same conditions as previously, but using sodium pipette solutions, no channel activation was observed in response to bFGF addition (10 nM; n = 12; data not shown). Furthermore, it was noticed that bFGF-activated channels

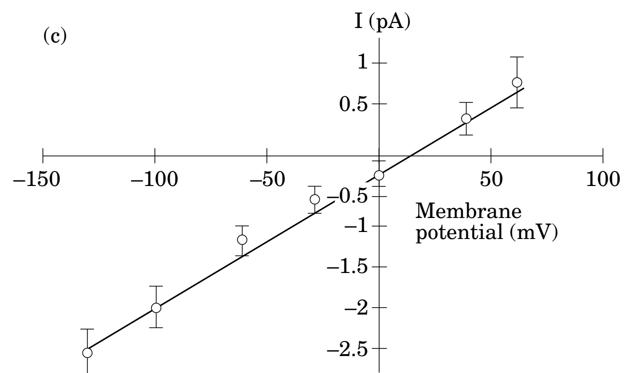
Figure 1 Effect of bFGF on cell-attached Ca^{2+} channel currents. (a) Representative traces of bFGF-induced Ca^{2+} currents recorded from an adult rat cardiomyocyte, using the cell-attached configuration and barium pipette solution. At basal level, membrane permeability was low and stable. Addition of bFGF (0.1 or 10 nM) to the bath solution enhanced inward currents in the following minutes (from 1–5 min). Open and closed states of the 10 nM bFGF-induced channels are indicated in the enlargement (membrane potential $= -35$ mV, record sampled at 1 kHz). (b) The amplitude distribution of the observed openings reveals an inward unitary current of 0.5 pA. (c) The current–voltage relation was plotted after conversion of the holding potentials into membrane potential according to the resting potential of -68 ± 3 mV as measured from 15 independent experiments. Each point represents the mean of 10–17 experiments. The slope conductance is 17 ± 5 pS and the reversal potential equals 10 ± 5 mV. (d) and (e) Histogram distributions of the open and closed times were fitted by the sum of two exponentials. Fast and slow time constants are respectively 1.3 and 12.1 ms for the open times and 1.7 and 8.5 ms for the closed times.



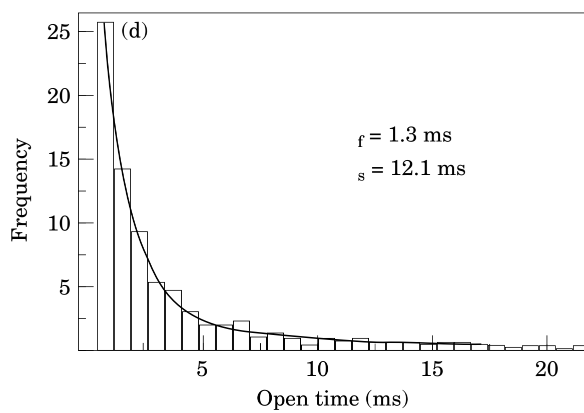
bFGF (0.1 nM)
(a)
1 pA
30 s
bFGF (10 nM)
2 pA
30 s
c
o
0.5 pA
1 s



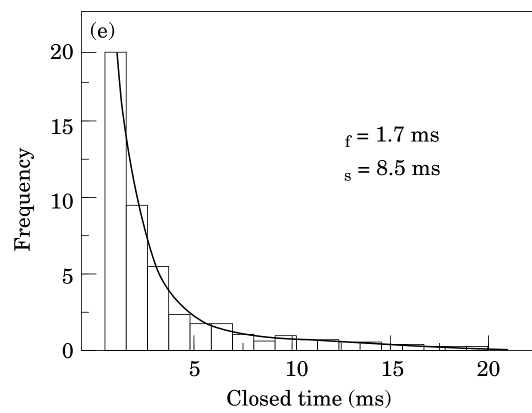
(b)
Events (1000)
Amplitude (pA)



(c)
I (pA)
Membrane
potential (mV)



(d)
f = 1.3 ms
s = 12.1 ms
Frequency
Open time (ms)



(e)
f = 1.7 ms
s = 8.5 ms
Frequency
Closed time (ms)

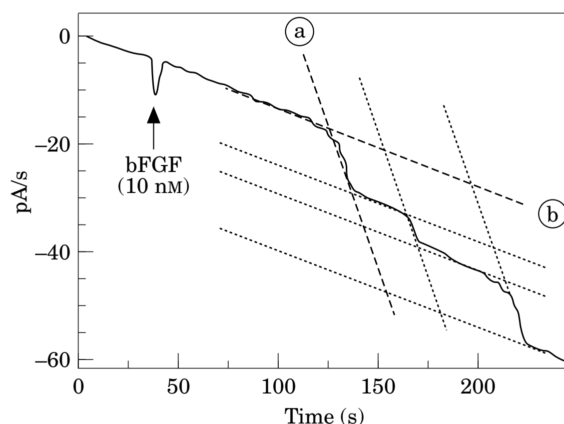


Figure 2 Time-course variations of the bFGF-induced current fluxes. Fluxes of calcium currents were estimated by computing the value of the calcium current amplitude per second (pA/s). From the trace representing the time-course evolution of current fluxes in response to bFGF (10 nM), intense periods of current activity were observed (slope a and parallels), followed by moments of lower activity, during which membrane permeability to Ca^{2+} returns to basal level (slope b and parallels).

were still recorded when tetrodotoxin was added in the pipette barium or calcium solutions. Since gadolinium and lanthanum are known to inhibit different types of calcium channels (Boyett *et al.*, 1996), these ions were added to barium pipette solution (200 μM). The presence of La^{3+} partially inhibited the bFGF-activated openings, while Gd^{3+} led to a complete inhibition (data not shown).

Role of IP_3 in the bFGF-activated calcium channels

Inositol 1,4,5 trisphosphate has previously been proposed as a messenger in mediating the openings of plasma membrane Ca^{2+} channels. Consequently, the inside-out configuration of the patch-clamp technique was used to determine the effects of IP_3 on the channel activity of membranes excised from adult cardiomyocytes. Addition of IP_3 (5 μM) promoted openings of calcium or barium channels in 70% of the experiments (36 out of 51 patches). Figure 3(a) is a representative example of the IP_3-induced currents recorded using a barium pipette solution (membrane potential = −30 mV). The corresponding elementary inward current amplitude, evaluated from the histogram amplitude distribution, was 0.5 pA [Fig. 3(b)]. The current-voltage relation [Fig. 3(c)] indicates that the observed channel has a conductance of 15 ± 5 pS and a reversal potential of 12 ± 5 mV ($n = 15$). Finally, the fit of the mean open and closed times yielded time constants of 1.5 ms and 10.0 ms for open states and 1.6 ms and 9.2 ms for closed states [Figs 3(d) and 3(e)]. In order to assess the specificity of these IP_3-induced channels, different pipette solutions were used. Similar to that observed in cell-attached records, no significant difference was observed between barium and calcium. Moreover, for records performed under same conditions, but using sodium pipette solution, IP_3-activated openings were no longer observed ($n = 7$; data not shown). It is important to note that, in the absence of IP_3, addition of calcium in the bath solution did not induce calcium channels openings. Furthermore, no voltage-dependence was observed and addition of calcium channel blockers such as verapamil, nicardipine or nifedipine had no effect on the IP_3-induced channels openings.

Confocal calcium imaging

Modifications of the intracellular free calcium concentration of adult cardiomyocytes were analysed by confocal scanning microscopy in the line-scan mode. The line-scan was chosen along a cross-section of an adult cardiomyocyte loaded with Fluo-3/AM [Fig. 4 (a), images 1 and 2] and pictures representing time-dependent modifications of the fluorescence across the cell were obtained [Fig. 4(a), image 3]. The upper trace of Figure 4(b-1) (Cell), corresponds to the time-dependent changes of normalized fluorescence along the line-scan. This trace is a representative experiment, showing that addition of bFGF led to a significant increase of the fluorescence intensity, which slightly decreased in

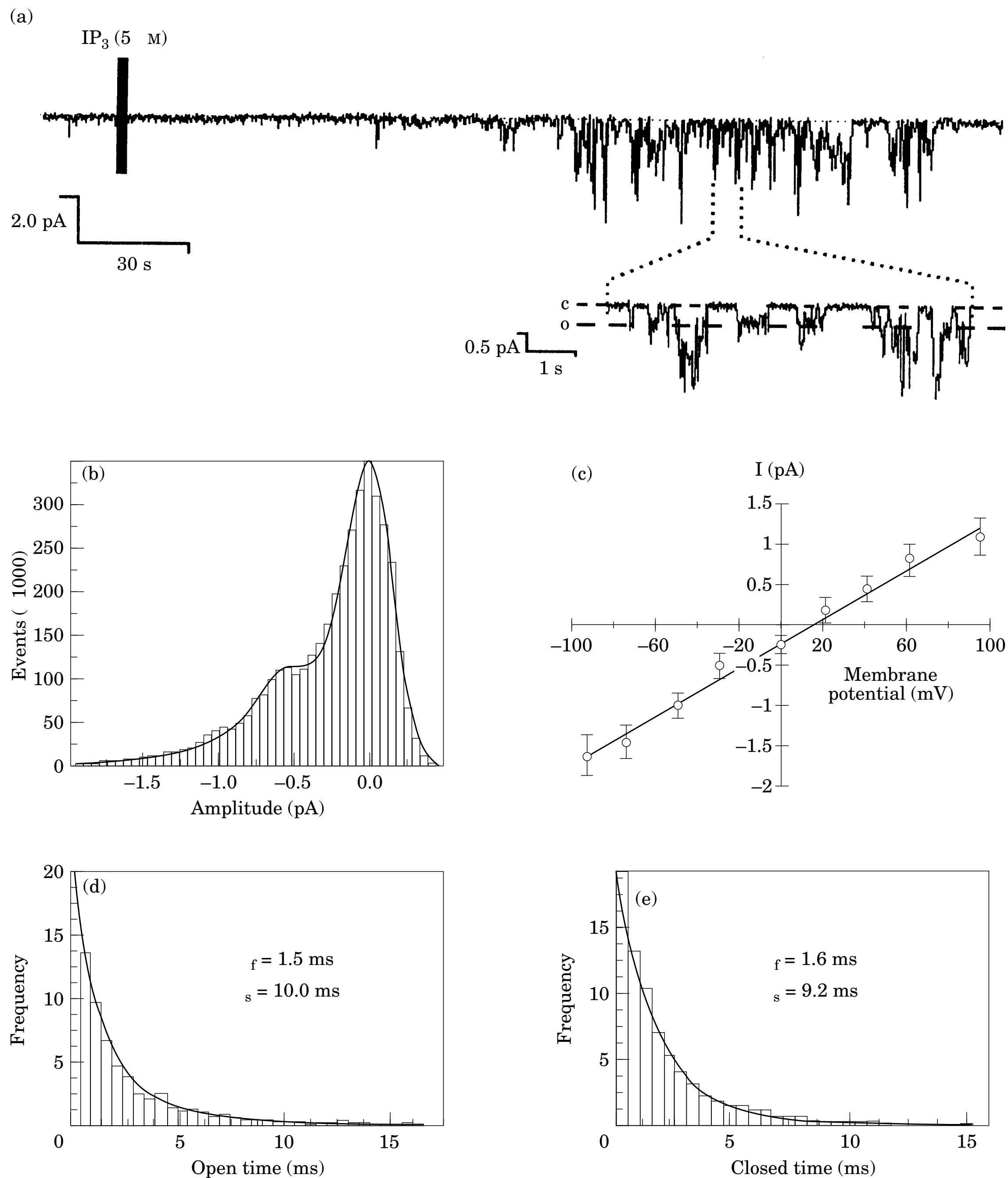
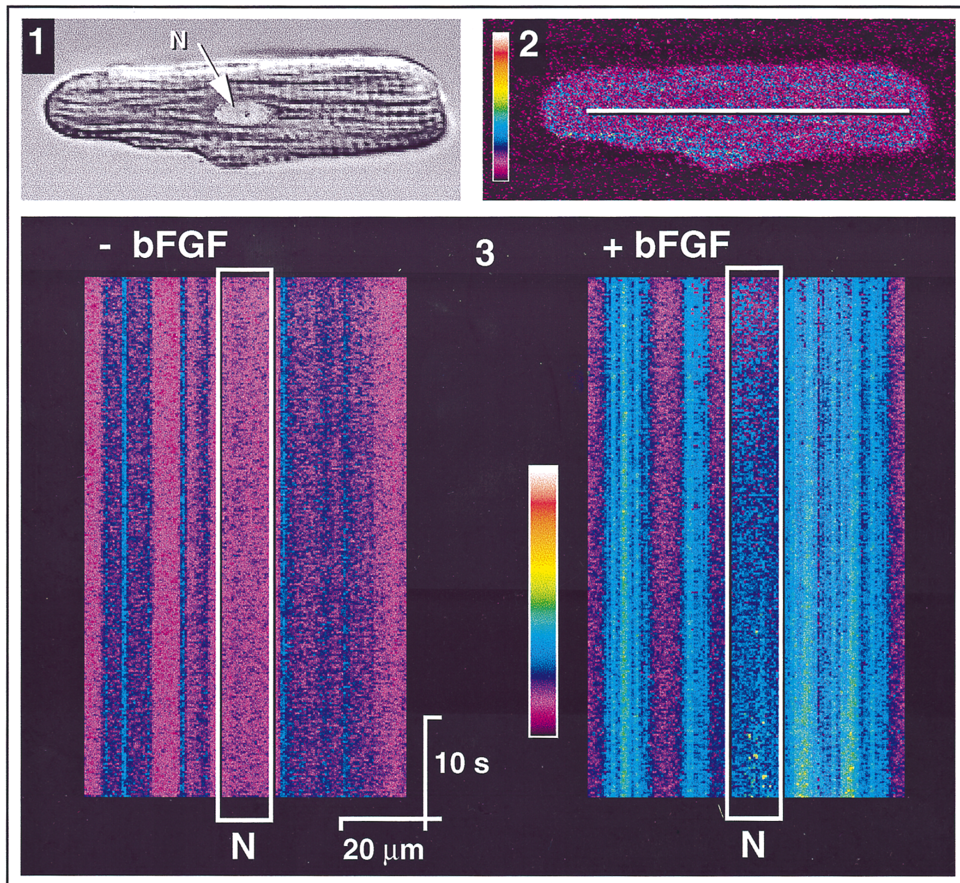


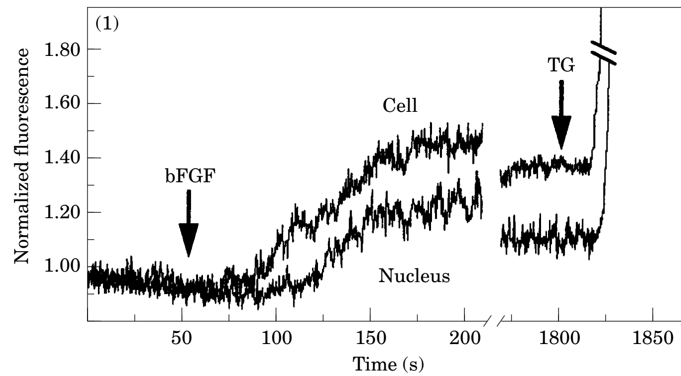
Figure 3 Effect of IP_3 on inside-out Ca^{2+} channel currents. (a) Representative example of IP_3-induced currents recorded from cardiomyocytes inside-out patch membrane held at −30 mV. From 1–5 min after IP_3 addition into internal bath, downward deflections were observed corresponding to open and closed states as indicated in the close up. (b) The resulting density histogram of current amplitudes revealed an inward unitary current of 0.5 mV. (c) The current–voltage relation of the single IP_3 induced currents defined a straight line having a slope conductance of 15 ± 5 pS and a reversal potential of 12 ± 5 mV. (d) and (e) Histogram distributions of the open and closed times were fitted by the sum of two exponentials which fast and slow time constants are respectively 1.5 and 10.0 ms for the open times and 1.6 and 9.2 ms for the closed times.

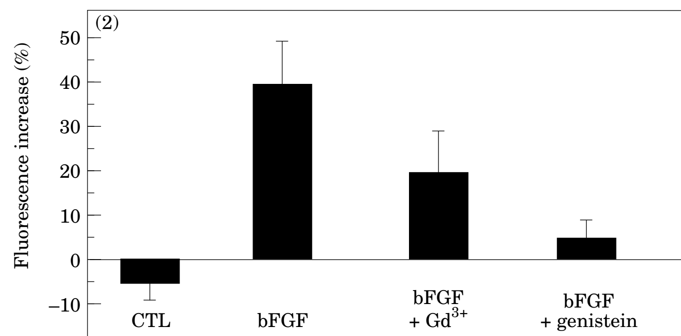
the following 10–15 min, but remained at a level higher than the resting level even 30 min after bFGF injection. Heterogeneity of the bFGF-induced increases of the calcium concentration was often observed along the line-scan and the increase of the basal level varied from 10–100% in different

(a)



(b)





parts of the cell. For some experiments, as shown in Figure 4(a), the line-scan was drawn across the nucleus (N) of the target cardiomyocyte, in order to evaluate the time-dependent evolution of fluorescence in a region corresponding to the nucleoplasm. The lower trace of Figure 4(b-1) (Nucleus) shows that, after bFGF addition, the increase of calcium concentration was also observed in the region corresponding to the nucleus. However, this increase was delayed and smaller than that observed from the cell. Thapsigargin was used to verify whether internal stores of calcium were depleted following bFGF addition. When added a few minutes to half an hour after bFGF, thapsigargin (20 μM) always led to a massive increase in fluorescence intensity, reaching saturating values. These results indicates that intracellular stores of calcium were not significantly depleted in response to bFGF.

As shown in the histogram [Fig. 4(b-2)] the average bFGF-induced increase was $40 \pm 10\%$ of the normalized fluorescence resting level ($n = 12$). When cells were placed in Tyrode's solution containing gadolinium (200 μM), the bFGF-induced calcium-increase was significantly inhibited and corresponded to a mean enhancement of $20 \pm 10\%$ ($n = 5$) above resting fluorescence. However, in these conditions, subsequent addition of thapsigargin still led to an increase of the fluorescence to saturating levels, indicating that internal stores were not affected by extracellular gadolinium. In contrast to what was observed with gadolinium, addition of lanthanum (200 μM) had no inhibitory effects on the increase in fluorescence levels in response to bFGF (data not shown). When cells were pre-incubated for 30 min in Tyrode's solution containing 50 μM of genistein, a tyrosine-kinase inhibitor, the bFGF-induced increase of the fluorescence level was strongly inhibited ($5 \pm 5\%$ of the resting level, $n = 6$). However, genistein did not lead to a reduction of the subsequent increase of the fluorescence level induced by thapsigargin. It is worth noting that, for all the above experiments, the use of cyclopiasonic acid (10 μM) instead of thapsigargin led to similar results (data not shown).

Discussion

Adult cardiac myocytes have lost the capability to divide. However, they express growth factors and growth factor receptors the roles of which in the normal heart work remain largely unknown (Kardami and Fandrich, 1989; Speir *et al.,* 1992; Jin *et al.*, 1994). In patho-physiological situations, bFGF and other growth factors are thought to be involved in the processes leading to cardiac myocyte hypertrophy (Parker and Schneider, 1991; Cummins, 1993). The influx of calcium through plasma membrane channels has been shown, in several tissues, to be a crucial step in growth factor activation pathways (Estacion and Mordan, 1993; Mogami and Kojima, 1993). However, little data is available concerning the first events following the activation of cardiomyocytes by bFGF. The aim of this work was to determine whether bFGF could induce an enhancement of membrane permeability to calcium in cardiomyocytes isolated from adult rat ventricles.

In this paper, we demonstrate the presence of calcium permeable channels, in cardiomyocyte membranes, that are activated by bFGF [0.1 or 10 nM; Fig. 1(a)]. The bFGF-induced currents displayed low conductance, voltage-independence and low reversal potential [Figs 1(b)–(e)]. All of these characteristics do not correspond to calcium channels classically depicted in the cardiomyocyte plasma membrane (Boyett *et al.*, 1996). However, the channels described here share many common

Figure 4 Effect of basic FGF on intracellular free calcium concentration. (a) Differential interference contrast microscopy (1) and confocal fluorescent image (2) of an adult cardiac myocyte loaded with fluo-3/AM. The location of the nucleus (N) is pointed out by an arrow and the white straight-line drawn in (2) represents the location of the laser spot scanning line. Along this line, time-dependent changes of fluorescence intensity were recorded which permitted the reconstruction of line-scan pictures (acquisition rate = 20 ms/line). The line-scan images (3) represent time-course evolution of the fluorescence at the resting state (left side) and 1 min after addition of 10 nM bFGF (right side). Fluorescence colors in the black/blue range correspond to fluorescence in absence of calcium and the red/white colors correspond to fluorescence in presence of maximum cytoplasmic calcium concentration, calibrated on active cardiomyocytes during contraction wave. (b) (1) Time-course evolution of the normalized fluorescence before and after bFGF addition evaluated by averaging the fluorescence intensity for the totality of the line-scan (Cell) or for a region of interest corresponding to the nucleoplasm (Nucleus). These traces, which are representative examples, show that the addition of bFGF rapidly led to an increase of the normalized fluorescence intensity. This increase could also be specifically observed in the region corresponding to nucleoplasm, but it was delayed and less pronounced. When thapsigargin (TG) was added either 30 min after bFGF (as indicated in traces 1) or a few minutes after bFGF (not shown), the fluorescence level reached saturating values, corresponding to a complete depletion of calcium from the intracellular stores. (2) Mean increases of the normalized fluorescent level (expressed as % of increase $\pm$ S.E.M.) observed in the following conditions: control ($n = 6$), bFGF 10 nM ($n = 12$), gadolinium 200 μM ($n = 6$) and genistein 50 μM ($n = 5$). In all cases, subsequent addition of thapsigargin, led to a massive increase of the fluorescence level.

properties with other growth factor-activated channels, observed on different cell types (Kuno and Gardner, 1987; Chapron *et al.*, 1989; Merle *et al.*, 1995). When barium was substituted for calcium, no significant modification of the channel's characteristics was detected. As a result, the bFGF-induced currents, displaying intense burst activity, may induce local and significant influxes of calcium through the plasma membrane of cardiomyocytes. However, the reversal potential of the observed currents is positive (12 ± 5 mV), but lesser than the theoretical calcium reversal potential, indicating that the selectivity of the channel for calcium is low. Cell-attached experiments have be reproduced under the same conditions as the barium patches (10 nM bFGF; -35 mV membrane potential) with the exception that sodium was the cation of the pipette solution. Under these conditions, no bFGF-induced currents were observed, suggesting that the channels were not permeable to this monovalent ion. Addition of lanthanum into pipettes containing barium or calcium solution led to partial inhibition of the bFGF-induced currents, which were inhibited in the presence of gadolinium. Gadolinium is a blocker of stretch-activated channels (Boyett *et al.*, 1996), but has also been shown to block L-type voltage activated channels (Lacampagne *et al.*, 1994). In our experiments, application of positive or negative pressure in the pipette compartment did not generate channel openings comparable to those induced by bFGF. Consequently, the observed channels were not thought to be stretch-activated channels. From the above results we conclude that the bFGF-induced channels are indeed permeable to calcium, but further studies are required to identify the nature of these channels and to clarify their actual selectivity to calcium and other ions. Finally, since the presence of bFGF in the pipette was not necessary to enhance calcium influx, the corresponding channels were thought to be second messenger-operated channels, and not receptor-operated channels.

One of the activation pathways of the bFGF tyrosine kinase receptors expressed by adult cardiomyocytes (Jin *et al.*, 1994; Liu *et al.*, 1995) is known to lead to the synthesis of inositol 1,4,5 trisphosphate (Jaye *et al.*, 1992). Addition of this second messenger to channels, recorded in the inside-out patch-clamp configuration, led to an enhancement of channel openings [Figs 3(a) to (e)]. Comparison of the bFGF-induced currents, observed in the cell attached mode, with the ones activated by IP_3 confirms that, in both cases, similar channels were concerned (Table 1). This observation is in agreement with independent reports which have previously demonstrated the presence of IP_3-activated Ca^{2+} channels in the plasma membrane of several tissues (Kuno and Gardner, 1987; Chapron *et al.*, 1989; Merle *et al.*, 1995; Vaca and Kunze, 1995). However, the precise mechanisms regulating IP_3 synthesis and calcium influxes are not fully understood. It has been shown, in other cell types, that the phosphoinositide messenger system oscillates, leading to cyclic increases in inositol trisphosphate (Ciapa, 1994). This observation leads us to suspect that such a process might be responsible for the time-dependent evolution of the current fluxes displayed in Figure 2. It is clear that more studies will be required to clarify the precise mechanisms controlling openings of IP_3-activated plasma membrane channels. However, considering the patch-clamp results presented here raises two main questions: firstly, is the free calcium content significantly increased in response to bFGF; and, secondly, what is the implication of the observed channel openings in this potential calcium increase? In order to better assess these questions, confocal imaging of intracellular free calcium content were performed.

Time-dependent modifications in the fluorescence intensity of adult cardiomyocytes, loaded with Fluo-3, revealed that bFGF induced an increase of the $[Ca^{2+}]_i$ resting level [Figs 4(a) and (b)]. According to the pseudo-ration equation previously established on adult cardiomyocytes, it could be estimated that $40 \pm 10\%$ increase in the normalized fluorescence correspond to a $56 \pm 15\%$ of increase in intracellular free calcium concentration (Cannell *et al.*, 1994). This result corroborates previous data showing a rise of the $[Ca^{2+}]_i$ on many other tissues in response to different growth factors (Moolenar *et al.*, 1986). In addition, when line-scan was set to cross the nucleus area, an increase of the fluorescence level was detected in the corresponding region [Fig. 4(a)]. These observations suggest that, very soon after bFGF addition, an enhancement of the calcium content in both the cytoplasm and the nucleus could represent a crucial step in the growth factor-induced activation pathways. Genistein, a tyrosine kinase inhibitor, was found to strongly inhibit the bFGF-induced free calcium augmentation. This indicates that activation of bFGF receptors having tyrosine kinase activity is required to trigger the calcium raise. This increase was not transient, but was maintained even 30 min after cell activation. This time-course does not correspond to classical IP_3-induced calcium release from internal stores, which provoke an intense but transient increase of cytosolic free calcium content. Moreover, the fluorescence increase induced by addition of

Table 1 Comparison of the Ca^{2+} currents. This table summarizes the characteristics of the currents recorded after bFGF addition (cell-attached configuration) and after IP_3 adjunction (inside-out configuration). In both cases pipettes were filled with barium acetate solution (50 mM)

Patch-clamp configuration	Cell attached	Inside-out
Agonist (concentration in M)	bFGF (1×10^{-8})	IP_3 (5×10^{-6})
Conductance (pS)	17 ± 5	15 ± 5
Reversal potential (mV)	10 ± 5	12 ± 5
Voltage independence	++	++

thapsigargin (or cyclopiasonic acid) indicated that internal stores were not significantly depleted subsequent to bFGF. This observation suggest that the IP_3-induced $[Ca^{2+}]_i$ increase may not only depend upon internal calcium release, but could partly be generated by external calcium entry.

In order to better evaluate the implication of external calcium influxes in the $[Ca^{2+}]_i$ increase, cardiomyocytes were placed in Tyrode's solution containing gadolinium, which was found to inhibit the bFGF-induced channel openings. In this condition, the intracellular calcium content elevation induced by bFGF was significantly reduced. Thapsigargin was used to verify that gadolinium was without effect on the intracellular stores, which otherwise could explain the observed inhibition. Since intracellular stores were not depleted by gadolinium, we hypothesized that the half-reduction of the bFGF-induced raise was indeed due to the inhibition of the channels previously described in the patch-clamp experiments.

Calcium signalling pathways, including calcium influxes through plasma membrane channels, are commonly schematized in two distinct conceptual models (Tsunoda, 1993). In the first scheme, valid for excitable cells, external calcium entry, through voltage-activated channels, stimulates the release of calcium from internal stores, generating calcium-induced calcium-release (CICR). The second model, which concerns non-excitable cells, calls for an agonist-stimulated synthesis of IP_3, inducing the release of calcium from intracellular organelles. This $[Ca^{2+}]_i$ enhancement could then generate a capacitative calcium entry, through channels of the plasma membrane, leading to the activation of I_{CRAC} (calcium release-activated calcium currents). Nevertheless, over the past few years, many independent studies have highlighted that calcium signalling mechanisms are in fact much more complicated than initially thought (Putney, 1993). In particular, calcium signalling in excitable and inexcitable cells may be more similar than previously suspected. The present results strengthen this point of view, since the observed currents appear to be generated in response to bFGF by direct IP_3-activation of voltage-independent channels. This activation pathway represents an interesting alternative to the classical calcium signal activation mechanisms. Since channel activations were observed even using low dose of exogenous bFGF (0.1 nM), such a process might be of physiological relevance. It is clear that extensive work will be required to answer the questions concerning $[Ca^{2+}]_i$ changes in response to bFGF or other growth factors in these excitable, but quiescent, isolated cardiomyocytes. Nevertheless, the present observations strengthen the possibility that bFGF-induced calcium-influx through plasma membrane channels could significantly affect the cardiomyocyte function, under physiological or pathological situations.

Acknowledgements

We warmly acknowledge Dr José Olivares-Monares who has shown us how to isolate adult cardiomyocytes and who kindly allowed us to work on his cell preparation equipment. This study was partly supported by a Région Rhône Alpes grant (No. H099400812).

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