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# Potassium and calcium currents activated by foetal calf serum in Balb-c 3T3 fibroblasts

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In quiescent Balb-c mouse 3T3 fibroblasts, the application of whole or dialyzed 10% foetal calf serum elicits a biphasic electrical response, consisting of a transient outward current, flowing through $Ca^{2+}$-activated $K^+$ channels, followed by an inward one, lasting up to 15 min. On the basis of experiments with ion substitutions and blockers, the inward current can be attributed to the opening of cationic channels permeable to $Na^+$ and $Ca^{2+}$ ions. This current could mediate the calcium influx involved in the sustained elevation of $[Ca^{2+}]_i$ that has been observed in many preparations in response to mitogen stimulation and that is involved in triggering cell proliferation.

## Introduction

Mitogens and growth factors can increase the cytosolic free calcium concentration ($[Ca^{2+}]_i$) by two different mechanisms: $IP_3$-induced calcium release from intracellular stores and calcium influx from the extracellular medium [1,2]. Elevation of $[Ca^{2+}]_i$ by the latter mechanism can last longer than the transient rise due to the phosphoinositide pathway and in many preparations it has been shown to be a crucial step for cells to progress into the replicative cycle [2,3].

Calcium influx through agonist-activated channels has attracted the interest of many researchers in the last few years, particularly in blood cells and cells of neuronal origin [2,4]. Recently, direct electrophysiological data on mitogen-activated calcium-conducting channels have been made available [5–10] (see Discussion). These data have been obtained from single-channel recordings; usually the effects were of short duration (1–2 min) and thus cannot be easily compared with the long lasting increases in $[Ca^{2+}]_i$ they are supposed to induce and that can be recorded with fluorometric techniques [2,3,10]). Whole cell data on cationic channels activated by bradykinin (BK) have been obtained from human fibroblasts [11] and PC12 cells [12]; however, in fibroblasts the channels are poorly permeable to calcium, while in PC12 cells BK acted as a neurotransmitter rather than as a mitogen; moreover, in the latter preparation the inward current, mainly carried by calcium ions, was of rather small amplitude and could be clearly detected only in non-physiological conditions, e.g., by blocking the $K^+$ currents.

In the present work, using the whole-cell patch clamp technique, we report that in Balb-c mouse 3T3 fibroblasts foetal calf serum (FCS) elicits, in addition to an outward current flowing through $Ca^{2+}$-activated $K^+$ channels, an inward current that is due to the opening of calcium-permeable channels and can last for rather long times (up to 15 min). This current may be involved in the sustained $[Ca^{2+}]_i$ increase that has been observed in various fibroblasts in response to mitogen stimulation [2,3,10]. Part of the present results have been preliminarily reported in abstract form [13].

## Materials and Methods

### *Cell culture and incubation protocol*

Balb-c mouse 3T3 cells were mantained at 37°C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated FCS (Sigma, USA), 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% $CO_2$-air. Exponentially growing cultures were shifted to DMEM containing 1% FCS for 48–72 h before the electrophysiological

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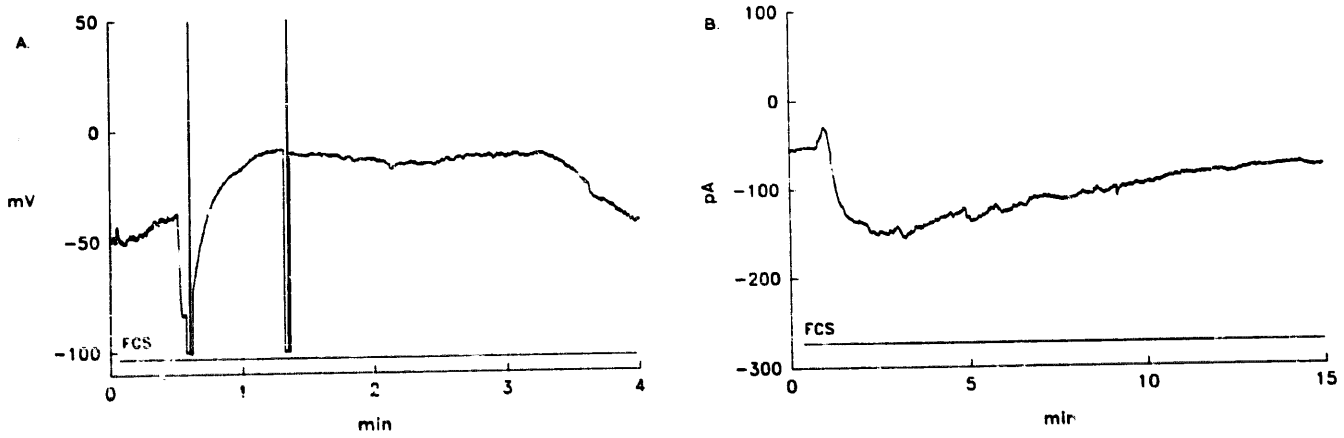


Fig. 1. Electrical reponse of Balb-c 3T3 fibroblasts to 10% FCS stimulation. (A) Response recorded in current clamp conditions. In this experiment, the depolarizing component apparently starts before the onset of the transient hyperpolarization. Large deflections are voltage ramps from −100 to +50 mV applied in order to monitor the *I–V* relationship. (B) Current response recorded in voltage clamp conditions ($V_h$ = −50 mV).

experiments [14]. Under such conditions more than 95% of the cells were in $G_0/G_1$ state, as evaluated by DNA flow cytometry.

*Electrophysiology*

Cells used in the experiments were flat and firmly attached to the substrate (35-mm dishes, Nunc, Denmark). Unless otherwise indicated, cells were superfused at 3 ml/min with a standard Tyrode solution of the following composition (in mM): 154 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 NaHepes, 5.5 glucose, NaOH to pH 7.4. Cells were then stimulated by switching to the same solution with 10% FCS added; since the dish contained about 1 ml of solution, the change was complete in less than 1 min. Pipette solutions contained (in mM) either 133 KCl, 3 $MgCl_2$, 0.1 EGTA, 5 Hepes, 0.4 NaGTP, 5 $Na_2ATP$, 5 $Na_2PC$, KOH to pH 7.3, or (in experiments with low internal $Cl^-$) 60 $K_3$-citrate, 10 KCl, 10 NaCl, 1 $MgCl_2$, 10 Hepes, 0.1 EGTA, 5 MgATP, KOH to pH 7.3. Charybdotoxin was from Latoxan (France). Unless otherwise specified the experiments were performed at 32–34°C.

Whole cell patch clamp recordings were performed using electrodes of 3–8 MΩ impedance connected to a RK 300 patch clamp amplifier (Bio-Logic, France). Data were digitized and stored on a VCR video recorder. Off-line analysis and generation of the voltage ramps used to record the *I–V* relationships (speed: 0.4 V/s) were performed with pCLAMP software (AXON Instruments, USA). Capacitance measurements were performed by analog compensation.

Values are given as means ± S.D.

## Results

*Voltage and current responses to FCS*

The membrane potential ($V_m$) of 3T3 cells bathed in standard Tyrode solution, after 48–72 h of serum star-

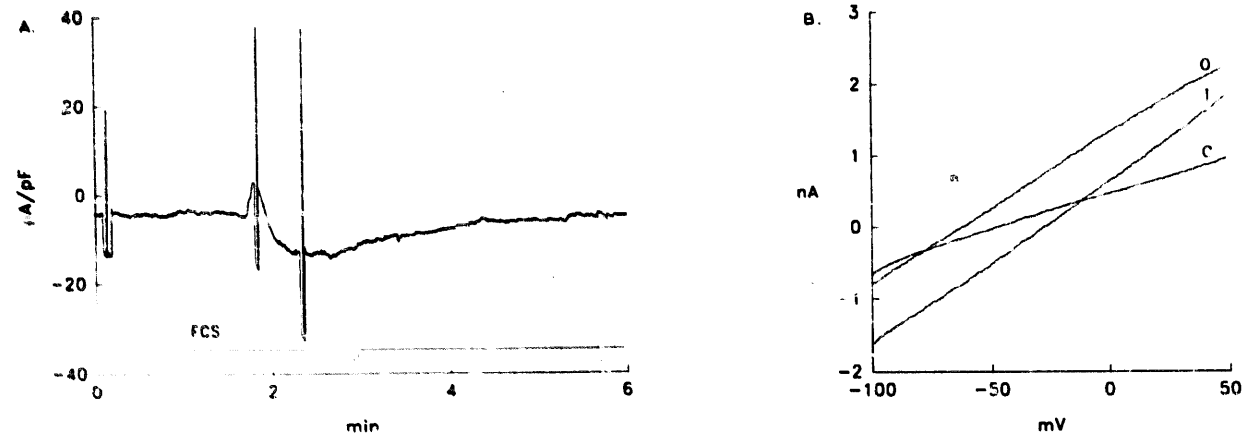


Fig. 2. Current-voltage relationships for the outward and inward currents. (A) Large deflections are current responses to voltage ramps from −100 to +50 mV applied to a cell voltage clamped at $V_h$ = −50 mV. (B) *I–V* curves for the control current (C) and the currents at the outward (O) and inward (I) peaks, from the same experiment of (A).

vation, was $-30 \pm 14$ mV (77 cells). In current clamp mode (8 cells) the response to 10% FCS application was usually biphasic, consisting of a hyperpolarization (up to $-86$ mV) followed by a depolarization (up to $-10$ mV) that could last several minutes. In some cells, the onset of the depolarizing component shortly preceded the hyperpolarization (Fig. 1A).

Likewise, the response recorded in voltage clamp mode at a holding potential ($V_h$) of $-50$ mV was mostly biphasic, with a transient outward current followed by an inward one whose duration was of 2 to 15 min (Fig. 1B; 43 cells). The response was obtained in about 90% of the cells tested. Some experiments were performed at 21°C, with no detectable differences. The biphasic current was still observed in 18 experiments performed using dialyzed serum, containing only substances with molecular weight > 3 kDa (such as growth factors), with no apparent difference with respect to the data obtained with undialyzed FCS.

Cell capacitance was measured in 29 cells and current densities were obtained: the mean peak outward current density was $5.2 \pm 3.4$ pA/pF, the mean peak inward current density was $6.9 \pm 5.9$ pA/pF.

*Ionic characterization of the currents*

To evaluate the current/voltage ($I/V$) relationships for the serum-induced currents, voltage ramps from $-100$ to $+50$ mV were applied before FCS stimulation as well as at the peak of the inward and outward current responses (Fig. 2A). The reversal potential ($V_{Rev}$) for the two currents is given by the voltage at which the peak $I$–$V$ curves cross the control one (Fig. 2B). $V_{Rev}$ at the peak of the outward current was $-80.4 \pm 16.4$ mV (five cells), near the theoretical equilibrium potential for $K^+$ ions ($V_K$) in our experimental conditions ($V_K = -93$ mV). The outward current could be completely abolished by 50 nM charybdotoxin (three cells; not shown), a blocker of $Ca^{2+}$-activated $K^+$ channels [15], without affecting the inward component. Therefore, its properties are similar to those of the $Ca^{2+}$-activated $K^+$ currents observed in response to mitogen stimulation in several preparations [7,11,14,16].

At the peak of the inward current, $V_{Rev}$ was $+2.5 \pm 9.6$ mV (Fig. 2B; 22 cells). This value could imply that the current was carried through cationic channels. In order to test this hypothesis, ion substitutions and blockers were employed. The inward component could be observed at $V_h = -50$ mV (Fig. 3A) in experiments in which 85% of the internal $Cl^-$ was replaced by citrate ($V_{Cl} = -50$ mV), with a mean peak current density of $4.4 \pm 3.8$ pA/pF (11 cells), while it was completely abolished when all external $Na^+$ and $Ca^{2+}$ were replaced by choline (10 cells, not shown). An inward current in response to FCS stimulation, showing the same characteristics of that recorded in Tyrode solution, could be observed in 12 cells bathed in a solution in which all $Na^+$ was substituted with choline and $CaCl_2$ was 5 mM (Fig. 3B); the mean peak current density of the inward current was $5.6 \pm 4.6$ pA/pF (7 cells). Similar results were obtained with a 150 mM NaCl, 0 mM $CaCl_2$ Tyrode solution (seven cells; data not shown). Application of 6 mM $CoCl_2$, a blocker of several types of calcium channels, to the external bath (three cells) resulted in a reversible block ot the FCS-activated inward current (Fig. 4).

As shown in Fig. 5, the time courses of the two components could be observed separately by applying to the same cell square pulses from $-95$ to 0 mV, i.e., voltages corresponding to the reversal potentials for the two currents: the upper trace (response at $V_h = 0$ mV) shows an outward component only, while in the lower trace ($V_h = -95$ mV) only an inward response is present. While the former completely vanished in less than 1 min, the latter returned to the control level in about 6 min.

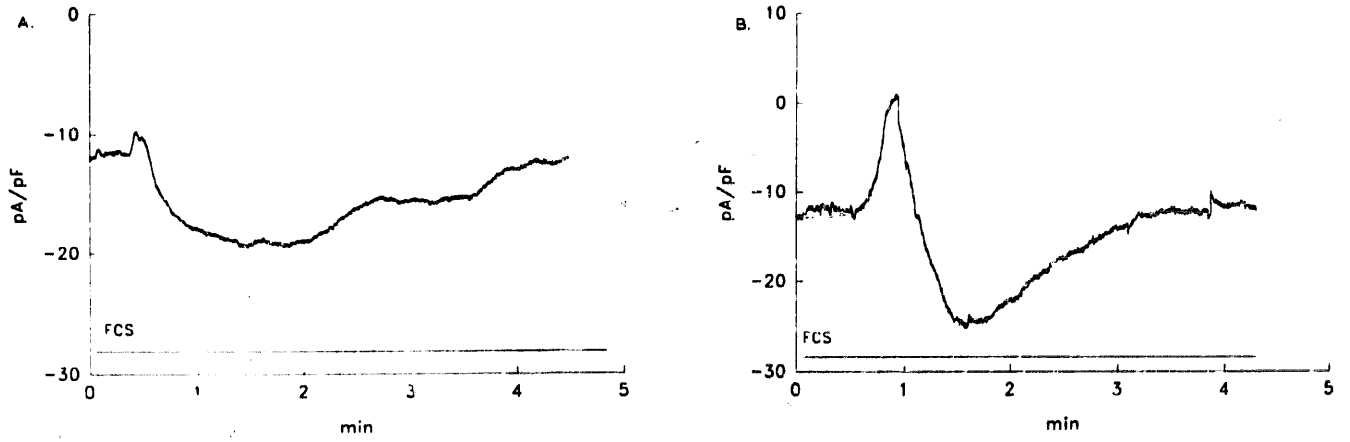


Fig. 3. Effects of ion substitutions on the inward current elicited by FCS. (A) Biphasic response observed with 85% internal $Cl^-$ substituted with citrate. (B) Biphasic response from a cell bathed in a solution containing 0 mM $Na^+$, 5 mM $Ca^{2+}$. $V_h$ was $-50$ mV in both experiments.

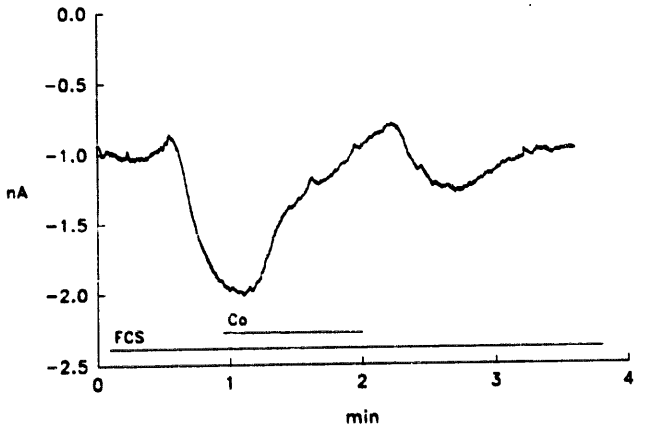


Fig. 4. Reversible block of the inward component of the response to FCS by 6 mM $CoCl_2$. $V_h = -50$ mV.

## Discussion

Several types of mitogen activated $Ca^{2+}$-permeable channels have been recently reported in fibroblasts and other preparations [5–11,17], mostly on the basis of single channel measurements. These channels show large differences in biophysical properties and mechanisms of activation. In A431 cells, at least six types of $Ca^{2+}$-permeable channels seem to be activated by EGF stimulation: $IP_3$-sensitive [5,6] and GTP-sensitive channels [6] or channels activated by hyperpolarization of the membrane potential [7] and by leucotrienes [8]. In Balb-c 3T3 cells, IGF-I and IGF-II also activate channels permeable to $Ca^{2+}$ ions [9,10]. The bradykinin (BK) activated cation channel observed in human fibroblasts [11] could be considered in this list, because it has been reported to be slightly permeable to divalent cations. In PC12 cells BK opens a channel that is more permeable to calcium than to sodium [12], but in these cells BK acts as a neurotransmitter, not as a mitogen. Other cationic channels have been observed in both non stimulated [18] and growth factor-stimulated fibroblasts [19], but none of them has been shown to be permeable to calcium ions.

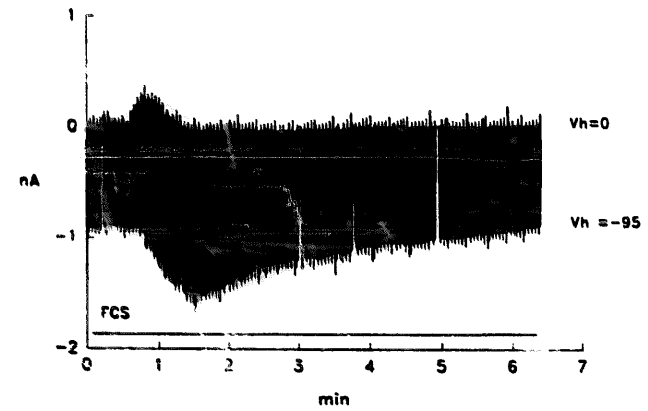


Fig. 5. Separation of the two components at two different holding potentials (−95 and 0 mV).

Despite the wide spectrum of channel types observed, their relationship to the event involved in the progression of cells into the replicative cycle has not yet been clarified. In particular, their contribution to the prolonged elevation in $[Ca^{2+}]_i$ that in many preparations has been shown to be important for mitogenesis remains to be clarified [2,3]. In fact, most of the above experiments are single channel recordings, with high $Ca^{2+}$ or $Ba^{2+}$ in the pipette, and the duration of the currents is rather short.

In the present paper, using whole-cell patch clamp recordings, we show that $Ca^{2+}$-conducting channels are involved in the response of quiescent Balb-c 3T3 cells to FCS. The first component of the response can be tentatively ascribed to the opening of $Ca^{2+}$-activated $K^+$ channels on the basis of the data presented above and by analogy with mitogen-induced outward currents in other preparations [14,16]; however, since charybdotoxin also blocks some non calcium-gated potassium channels, more detailed measurements have to be performed in order to confirm this hypothesis. Following the outward component, an inward current has been observed that can be ascribed to the opening of non specific cationic channels, permeable to both $Na^+$ and $Ca^{2+}$. The inward current and the related calcium influx are also elicited by dialyzed serum, containing the growth factors. With all $Na^+$ removed from the external solution and in the presence of 5 mM $Ca^{2+}$, the density of the inward current did not differ from that measured in the experiments with standard Tyrode solution: the channel (or channels) activated by FCS stimulation thus appears to be highly permeable to $Ca^{2+}$ ions. The $V_{Rev}$ for this current is about 0 mV, while it should be more positive for a current carried by $Na^+$ and $Ca^{2+}$ ions. To measure $V_{Rev}$, however, the current ramps were applied at the peak of the inward current, when the outward component is still present; therefore, the above discrepancy could reflect a contamination by the outward $K^+$ current. The present data do not clarify whether more than one type of mitogen activated $Ca^{2+}$-conducting channel is present in Balb-c 3T3 cells; anyway, the cationic current described in this paper does not show any voltage-dependence and thus differs from that observed in A431 cells [7].

The inward current density, measured with physiological internal and external solutions, is an order of magnitude greater than that activated in comparable conditions by BK in PC12 cells [12]. This observation makes it a likely pathway for the mitogen-activated influx of calcium ions that gives rise to the prolonged plateau of $[Ca^{2+}]_i$ increase observed in many cell types, and that has been shown to be involved in mitogenesis [2,3,10]. Experiments with individual growth factors are

needed to identify the agonist(s) responsible for the activation by FCS of the currents observed in the present work. Yet the present findings may be of great relevance to the understanding of the relationship between changes in $[Ca^{2+}]_i$ and cell cycle control.

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## References

1 Soltoff, S.P. and Cantley, L.C. (1988) Annu. Rev. Physiol. 50, 207–223.
2 Meldolesi, J., Clementi, E., Fasolato, C., Zacchetti, D. and Pozzan, T. (1991) Trends Pharmacol. Sci. 12, 289–292.
3 Magni, M., Meldolesi, J. and Panzella, A. (1991) J. Biol. Chem. 266, 6329–6335.
4 Tsien, R.W. and Tsien, R.Y. (1990) Annu. Rev. Cell Biol. 6, 715–760.
5 Chapron, Y., Cochet, C., Crouzy, S., Jullien, T., Keramidas, M. and Verdetti J. (1989) Biochem. Biophys. Res. Commun. 158, 527–533.
6 Mozhayeva, G.N., Naumov, A.P. and Kuryshev, Y.A. (1991) J. Membr. Biol. 124, 113–126.
7 Peppelenbosch, M.P., Tertoolen, L.G.J. and De Laat, S.W. (1991) J. Biol. Chem. 266, 19938–19944.
8 Peppelenbosch, M.P., Tertoolen, L.G.J., Den Hertog, J. and De Laat, S.W. (1992) Cell 69, 295–303.
9 Matsunaga, H., Nishimoto, I., Kojima, I., Yamashita, N., Kurokawa, K. and Ogata, E. (1988) Am. J. Physiol. 255 (Cell Physiol. 24), C442–C446.
10 Kojima, I., Matsunaga, H., Kurokawa, K., Ogata. E. and Nishimoto, I. (1988) J. Biol. Chem. 263, 16561–16567.
11 Estacion, M. (1991), J. Physiol. 436, 603–620.
12 Neuhaus, R., Reber, B.F.X. and Reuter, H. (1991) J. Neurosci. 11 (12), 3984–3990.
13 Lovisolo, D., Baccino, F.M., Bonelli, G. and Tessitore, L. (1991) Pflügers Arch. 418, R129.
14 Lovisolo, D., Bonelli, G., Baccino, F.M., Peres, A., Alonzo, F. and Munaron, L. (1992) Biochim. Biophys. Acta, 1104, 73–82.
15 Miller, C., Moczydlowski, E., Latorre, R. and Philips, M. (1985) Nature 313, 316–318.
16 Pallotta, T. and Peres, A. (1989) J. Physiol. (London) 416, 589–599.
17 Gardner, P. (1990) Annu. Rev. Immunol. 8, 231–252.
18 Galietta, L.J., Mastrocola, T. and Nobile, M. (1989) FEBS Lett. 253, 43–46.
19 Frace, A.M. and Gargus, J.J. (1989) Proc. Natl. Acad. Sci. USA 86, 2511–2515.