Scientific Research

Lidocaine-Induced Cell Growth of Human Gingival Fibroblasts. Role of Na⁺-K⁺-ATPase and PKC Activities

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Abstract

Background: Evidences have shown that local anaesthetics are clinically useful compounds that exert a pharmacological effect by blocking nerve impulse propagation and also it is able to provoke proliferation and cell growth. *Aims*: The aim of this study was to investigate the proliferation and cell growth capacity of lidocaine on human gingival fibroblast cells and the different signal pathways involved in its effect. *Method*: For this purpose *in vitro* cultures of human gingival fibroblasts were assayed and the effects of lidocaine on proliferation and cell DNA synthesis, Na⁺-K⁺-ATPase and PKC activities and K⁺ efflux were also evaluated. *Results*: Lidocaine stimulated in a concentration-dependent manner proliferation and cell growth of human gingival cells and the mechanism involve an increment in Na⁺-K⁺-ATPase and PKC activities, which led to an increase in K⁺ release. All of these effects were blocked by tetrodotoxin, ouabain and calphostin C. In addition, PMA (activator of PKC) increased *per se* the DNA synthesis of human gingival fibroblast cells. *Conclusions*: This work demonstrates that lidocaine increase human gingival fibroblasts DNA synthesis and proliferation through an activation of PKC pathway accompanied by the stimulation of Na⁺-K⁺-ATPase activity with an increase in K⁺ efflux. These results contribute to showing another action of lidocaine different to its general use as a drug that relieves odontologic pain or acts as an anti-arrithmogenic agent.

1. Introduction

Local anaesthetics are clinically useful compounds that exert a pharmacological effect by blocking nerve im-

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pulse propagation.

Recent reports indicate that local anaesthetics have other beneficial effects on a variety of cellular activities such as thrombosis, anti-inflammatory responses and wound healing [1]-[4]. Furthermore, local anaesthetics interfere with and modulate some important biochemical pathways such as gene expression [5] and apoptosis [6] [7].

The Na^+-K^+-ATPase is an energy-transducing ion pump that converts the free energy of ATP into transmembrane ion gradients. The Na^+-K^+-ATPase is also the enzyme acting as a signal transducer that modulates cell energy-transducing ion pump on the plasma membrane protein. The enzyme not only maintains the membrane potential of excitable neuro and different cells but also is involved in reabsorption of Na^+ in the kidney [8] and in salivary glands [9].

In renal epithelial cells, inhibition of Na^+-K^+-ATPase activity by catecholamines is mediated by phosphorylation of the catalytic α-subunit, and this event does not affect the catalytic properties of the enzyme while in the plasma membrane but constitutes the triggering signal for its endocytosis into endosomes via a clathrin vesicle-dependent mechanism [10].

By contrast, in lung alveolar epithelial cells isoproterenol or dopamine increases Na^+-K^+-ATPase activity and this effect is associated with an increased recruitment of active Na^+-K^+-ATPase molecules to the plasma membrane [11]. The molecular mechanisms responsible for the increase in Na^+-K^+-ATPase activity and the translocation from intracellular compartments to the plasma membrane are not yet completely understood. Moreover, lidocaine inhibited the activities of Na^+-K^+-ATPase and Mg^+-ATPase in synaptosomes prepared from rat cerebral cortex [12].

The protein kinase C (PKC) is a family of multifunctional isoenzymes involved in apoptosis, migration, adhesion, tumorgenesis, cardiac hypertrophy, angiogenesis, platelet function and inflammation [13] [14]. It also plays a vital role in the regulation of signal transduction, cell proliferation and differentiation through positive and negative regulation of the cell cycle. Biochemical studies of purified brain Na^+ channels showed that the alpha subunits are good substrates for phosphorylation by PKC and cAMP-dependent protein kinase [13] [14]. The gating of the Na^+ channel can be modulated by protein phosphorylation by either PKC or cyclic AMP-dependent protein kinase A reducing channel activation and slowing inactivation [15]. On the other hand, PKC inhibitors are of pharmacological interest because they were found to inhibit proinflammatory and immunomodulatory events in cells [16].

2. Aim

Therefore, taken together the role of Na^+-K^+-ATPase in different organs and cells, the aim of the present study was to determine whether lidocaine was able to induce proliferation and cell growth of human gingival fibroblast cells and the mechanism involved. Also, we demonstrated that lidocaine induced cell growth by modulation of PKC and Na^+-K^+-ATPase enzyme-stimulatory activities with an increase in K^+ efflux.

3. Methodology

3.1. Cell Culture

Pure cell cultures of normal human gingival fibroblast (2×10^6 cells/ml) were established from the gingival extraction and grown in Dulbecco's modified Eagle's medium (Gibco Invitrogen Corporation) supplemented with 10% FBS (fetal bovine serum), 2 mM L-glutamine and penicillin (100 U/ml) and streptomycin (100 μg/ml) in a 5% CO_2 environment at 37˚C [17]. The medium was replenished every 3 - 4 days. Confluent cells were cultured by detaching the monolayer with 0.25% trypsin in phosphate buffered saline solution (PBS). Only cells of passages 6 - 9 were used in the experiments and all the experiments were carried out in serum-free medium for 20 hours. Human studies have been performed in accordance with the Declaration of Helsinki.

3.2. Fibroblast Cell Treatment

To evaluate the action of lidocaine on human gingival fibroblasts, cell cultures were subjected to treatment with lidocaine (from 10^{-11} to 10^{-7} M) for 48 hours. When different enzymatic inhibitors: ouabain (1×10^{-4} M) and calphostin C (1×10^{-9} M), were used, they were added 20 min before the addition of each lidocaine concentrations. Controls included both untreated cells during 48 hours (basal value). The cell viability at the beginning of the experiments was assessed by trypan blue exclusion test (92% - 95% viable cells).

3.3. Measurement of DNA Synthesis

DNA synthesis was estimated by measuring [^{3}H] thymidine (Dupont/New England Nuclear) incorporation into trichloroacetic acid (TCA)-precipitable material. Cells growing in the absence of 10% FBS were treated with different concentrations of lidocaine for 20 hours, and [^{3}H]-thymidine (0.1 mCi/ml) was added during the last 18 hours. Cells were precipitated twice with ice-cold 10% TCA. When enzymatic inhibitors were used, they were added 15 min before lidocaine. Fibroblasts that had been serum-starved for 20 hours without any drugs were used as control. Cells were then removed with trypsin/PBS and radioactivity was determined by liquid scintillation counting. The cell cycle distribution of fibroblast used was in resting state and the percentage of confluence at the time of study for all assays was about 95% - 98%. Cell viabiality was not altered after drug treatment as assessed by trypan blue exclusion test. Results are expressed as growth stimulation in cpm absolute values.

3.4. Membrane Preparation for Na$^+$-K$^+$-ATPase Activity Determination

Fibroblast cells (2×10^6) from each treatment were washed in PBS and treat in 10 mM Tris pH 7.4, 5 mM EDTA, 1% (v/v) Triton X-100 for 20 minutes on ice**.** The cells were collected by scrapping and centrifuged for 15 min to 13000 rpm at 4˚C. The homogenates were centrifuged for 10 min at 1000 g, and the supernatants were collected and spun down for 20 min at 9000 g. The resultant supernatant was centrifuged for 60 min at 100,000 g. The pellet was then re-suspended in Tris-HCl 10 mM, EDTA 1 mM and phenylmethylsulfonyl fluoride 0.1 mM and stored at −70˚C until used.

3.5. Na$^+$-K$^+$-ATPase Activity Measurement

Membrane of gingival fibroblasts cells aliquots (approximately 10 - 20 μg of protein) were transferred to the Na$^+$-K$^+$-ATPase assay medium (final volume 172 μl, containing: 100 mM NaCl, 20 mM KCl, 3 mM MgCl$_2$, 160 mM Tris–HCl (pH 7.4), and 4 mM Na$_2$ ATP) and incubated for 30 min at 37˚C in the absence or presence of 5×10^{-4} M ouabain. The reaction was stopped by the addition of 40 μl of cold 30% trichloroacetic acid. Samples were centrifuged at 3000 g for 10 min and the inorganic phosphate liberated (total ATPase activity) was measured. Na$^+$-K$^+$-ATPase activity was calculated as the difference between the means of the total ATPase activity and the ouabain-sensitive ATPase activity and expressed as μmol Pi mg protein$^{-1} \cdot$h^{-1}.

3.6. Determination of Protein Kinase C (PKC) Activity

Protein kinase c (PKC) activity was measured in human gingival fibroblast cells using the PKC Kit by Assay Designs Inc Laboratory from Ann Arbor, MI, USA, according to the procedure described by the manufacturer. Briefly, the human gingival fibroblast cells were incubated for 30 min in the absence or in the presence of lidocaine at different concentrations (from 10^{-11} to 1×10^{-6} M). Then, the cells were rinsed with PBS and afterwards lysed. The fibroblasts were collected and centrifuged for 15 min at 13,000 g at 4˚C. The results were expressed as nanograms (ng) of protein (ng/protein) per assay.

3.7. Net K$^+$ Efflux Determination

Human gingival fibroblast cells (2×10^6) were incubated in 1 ml of oxygenated Krebs Ringer bicarbonate buffer composed as follows (mM): Na$^+$ 145, K$^+$ 6.02, Ca^{2+} 1.22, Mg^{2+} 1.33, Cl$^-$ 126, HCO$_3^-$ 25.3, SO$_4^{2-}$ 1.33, PO$_4^{3-}$ 1.20, glucose 5.5 KRB at 37˚C in the presence or absence of lidocaine alone (1×10^{-8} M) or in the presence of 10^{-4} M ouabain. Aliquots of 500 μl of the medium were removed at 2, 5 min and at 10 min. At the end of the incubation period (10 min), the fibroblast cells were homogenized in the remaining KRB medium and the K$^+$ concentration in the homogenate and in the aliquots removed in the course of the experiments was measured in triplicate in an Instrumentation Laboratories Flame Photometer (Werfen Group, Bedford, USA). Net K$^+$ efflux is presented as the percentage of the total cells.

3.8. Drugs

Stock solutions of Lidocaine, Ouabain, Phorbol 12-myristate 13-acetate (PMA), Tetrodotoxin (TTX) (Sigma Chemical Co.) and Calphostin C (Tocris Cookson Inc) were freshly prepared before each experiment in the corresponding solvent and all the drugs were diluted in the bath to achieve the final concentration stated in the text.

3.9. Statistical Analyses

The Student's t test for unpaired values was used to determine the significance level. ANOVA (analysis of variance) and a post hoc test (Dunnett's method and Student-Newman-Kuels test) were used when a pair wise multiple comparison procedure was necessary. Differences between means were considered significant if $P < 0.05$.

3.10. Ethical Approval of the Study Protocol

The study was approved by the Ethics Committee of the School of Dentistry at Buenos Aires University (Buenos Aires, Argentina). The studies were conducted according to the tenets of the Declaration of Helsinki. All participants provided written informed consent to participate in the study.

4. Results

Figure 1 shows the effects of lidocaine-stimulation on proliferation cells of human gingival fibroblasts, determining by cells counts and total cellular protein measurement. **Figure 1(a)** showed the morphological observation of the fibroblasts in the absence or in the presence of lidocaine (from 1×10^{-11} to 1×10^{-6} M) an increased
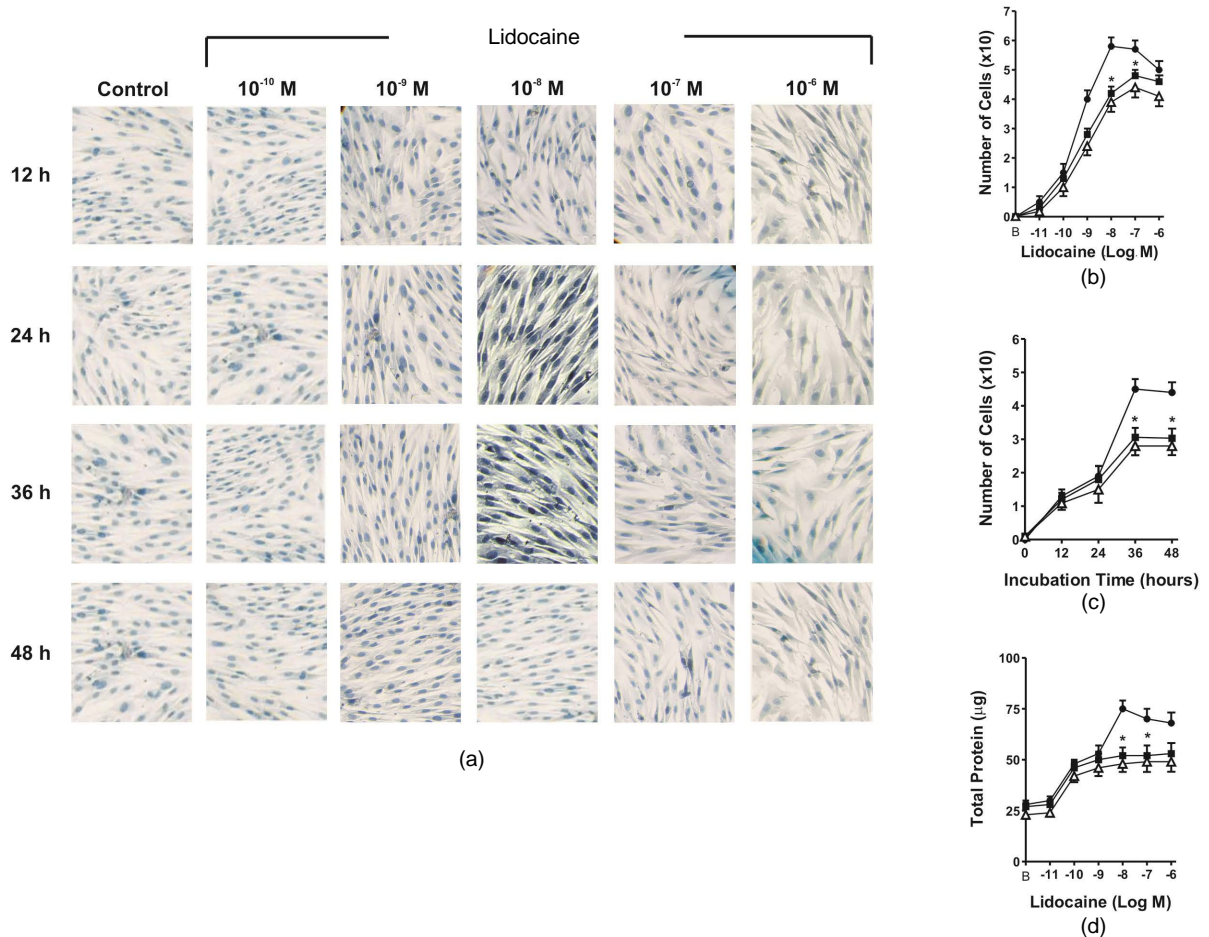

Figure 1. Concentration-response curve of lidocaine-induce proliferation on human gingival fibroblasts. (a) Morphological observations; (b) Cell counts at different concentrations of lidocaine alone. *P < 0.001 at 24 and 48 hours with lidocaine alone (●) and in the presence of ouabain (■) at 1×10^{-9} and calphostin C (Δ) at 1×10^{-8} M; (c) Cell counts at different times in hours. *P < 0.001 at 36 and 48 hours in the absence (●) and in the presence of ouabain (■) and calphostin C (Δ) plus lidocaine; (d) Total protein quantification per well. *P < 0.001 at 1×10^{-8} M lidocaine alone (●) and in the presence of 1×10^{-7} M with ouabain (■) and calphostin C (▲). Different points represent the media ± SEM of six experiments by duplicate in each concentration.

in proliferation, being 1×10^{-8} M the concentration that reach at the maximal increment as visually evident for the increase in cell number in different hours. This proliferation action of lidocaine was diminished significantly in the presence of ouabain and calphostin C. **Figure 1(b)** showed the cells counts in relationship with the different concentrations of lidocaine, in **Figure 1(c)** we can observed the total protein concentration and in **Figure 1(d)** we show a protein quantitation per well. All these increment in cells proliferation obtained by lidocaine was compared with untreated control cells.

Figure 2(a) shows the stimulation of DNA synthesis by lidocaine. The addition of different concentrations of lidocaine to human gingival fibroblast cells triggered a dose-dependent increase in DNA synthesis. The local anaesthetic effect was maximal at 1×10^{-8} M lidocaine.

To determine the nature of the mechanism which triggers the mitogenic effect of lidocaine, the action of inhibitors known to be involved in Na^+ signal transduction was explored. In the same **Figure 2(a)** it can be seen that the inhibition of the dose-response curve of lidocaine by ouabain (10^{-4} M) or by calphostin C (10^{-9} M) or by tetrodotoxin (10^{-7} M) prevented the mitogenic action of lidocaine, indicating the participation of Na^+-K^+-ATPase and PKC activities in this phenomenum.

Table 1 shows that at the inhibitors (ouabain, calphostin C and tetrodotoxin) used at the concentration cited above, *per se* did not have any effect on cell growth.

To verify if human gingival fibroblast cell Na^+-K^+-ATPase and PKC activities are involved in the action of lidocaine, Na^+-K^+-ATPase and PKC enzyme activities, were measured.

As can be seen in **Figure 3(a)** lidocaine in a concentration-dependent manner evoked a stimulation of Na^+-K^+-ATPase activity in gingival fibroblast cells. Lidocaine, over the range 10^{-11} to 10^{-7} M exerted this stimulatory effect on Na^+ pump activity being 1×10^{-11} M the threshold concentration and 1×10^{-8} M is the maximal
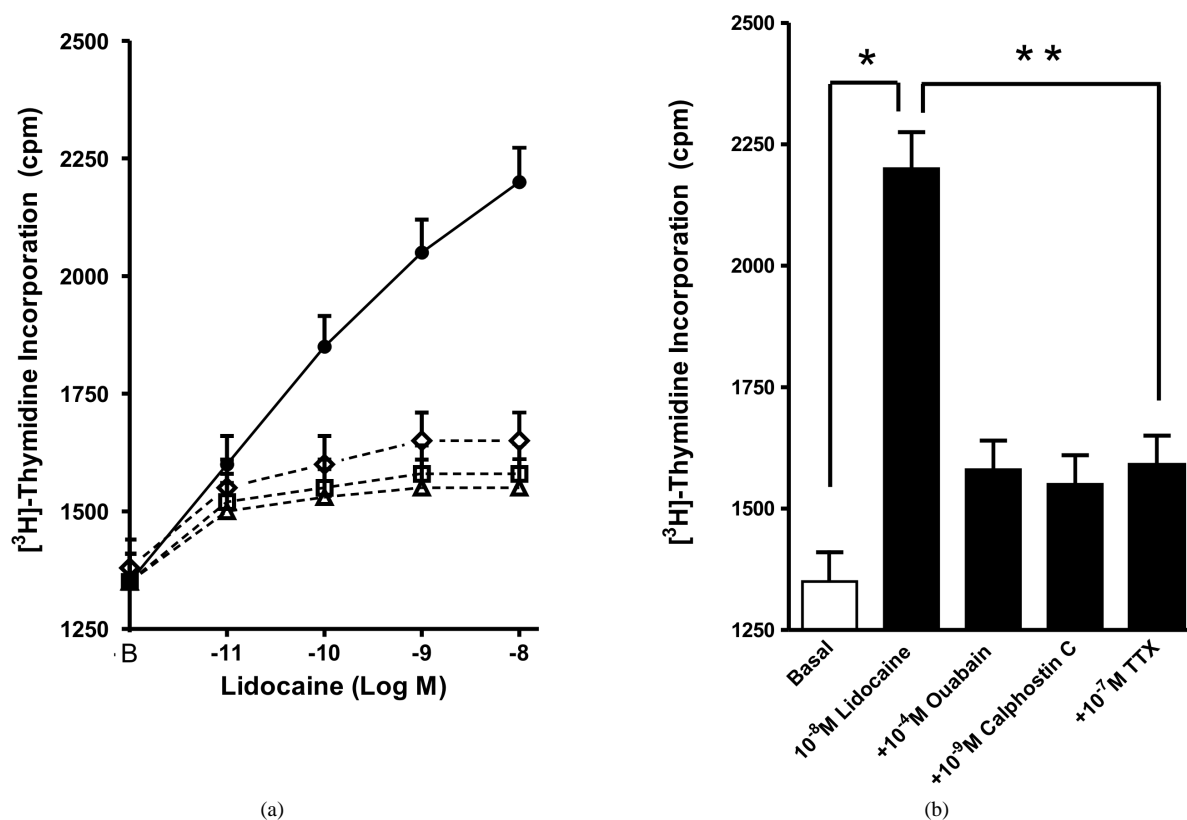

(a)　　　　　　　　　　　(b)

Figure 2. (a) Dose-response curve of lidocaine alone (●) or in the presence of TTX (10^{-7} M) (◊), ouabain (10^{-4} M) (□) and calphostin (Δ) on human gingival fibroblast cells DNA synthesis measured by [^{3}H]-thymidine incorporation was referred as growth stimulation in absolute values (cpm) above basal (control) stimulation values; (b) Histogram shows [^{3}H] thymidine incorporation (cpm) in the presence of lidocaine alone 1×10^{-8} M or in the presence of ouabain (1×10^{-4} M), calphostin C (1×10^{-9} M) and TTX (1×10^{-7} M). Values represent the mean ± SEM of six experiments in each condition done by duplicate. *P < 0.0001 versus basal; **P < 0.001 versus lidocaine alone.

Table 1. Effect of enzymatic inhibitors on basal DNA synthesis.

Addition	[³H]-Thymidine Incorporation (cpm)	Number of experiments (n)
None	1350 ± 126	5
Ouabain 1×10^{-4} M	1289 ± 125	5
Calphostin C 1×10^{-9} M	1380 ± 130	5
Tetrodotoxin 1×10^{-7} M	1293 ± 127	6

Values are the mean ± SEM of n in each group. Cells were treated as reported in Material and Methods and DNA synthesis was measured following 24 hours culture.
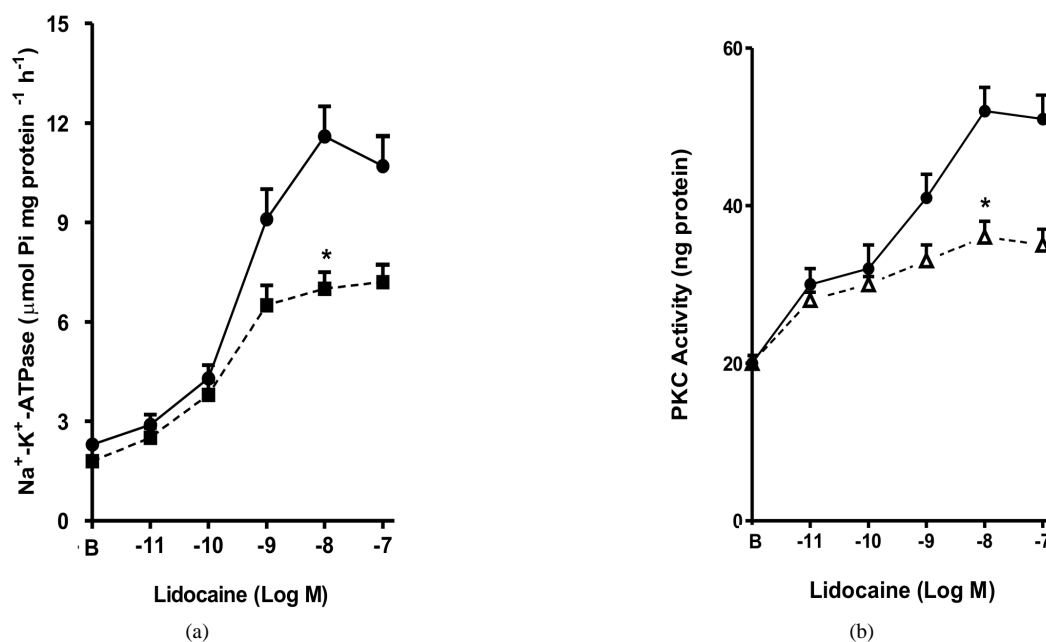

(a)   (b)

Figure 3. (a) Concentration-response curve of lidocaine alone (●) or in the presence of ouabain $(1 \times 10^{-4}$ M) (■) on Na⁺-K⁺-ATPase activity from human gingival fibroblast cells; (b) Concentration-response curve of lidocaine alone (●) or in the presence of calphostin C $(1 \times 10^{-9}$ M) (△) on PKC activity. Values are the mean ± SEM of seven experiments performed in duplicate. *P < 0.001 versus lidocaine alone.

response. Ouabain $(1 \times 10^{-4}$ M) prevented the stimulatory action of lidocaine on Na⁺-K⁺-ATPase activity. **Figure 3(b)** showed that lidocaine is able to provoke in a concentration dependent manner an increment in the PKC activity. Also, **Figure 3(b)** shows that this increment observed in this enzyme activity is in relationship to the concentration of the local anaesthetic drug and was blunted significantly (p < 0.0001) by calphostin C 1×10^{-9} M, confirming pharmacologic way the participation of lidocaine-induced PKC activity.

To asses whether lidocaine can stimulate the activity of Na⁺-K⁺-ATPase enzyme in human gingival fibroblast cells and its influence could result in an alteration of ionic fluxes, we measurement the net K⁺ efflux with lidocaine $(1 \times 10^{-8}$ M) at the maximal concentration, as shown previously in **Figure 3(a)**.

Figure 4 shows that when lidocaine at its maximal concentration-effect on Na⁺ pump induced a significant increase (p < 0.0001) in net K⁺ release. The presence of ouabain 1×10^{-4} M or calphostin C 1×10^{-9} M impaired the action of lidocaine on K⁺ released, indicating that lidocaine participate in the activation of both enzymes (Na⁺-K⁺-ATPase and PKC, that are sensitive to ouabain and calphostin C respectively.

To assess whether lidocaine is able to provoke, in a concentration-dependent manner, the activation of PKC activity, we studied the effect of a direct activator of the enzyme as phorbol 12-myristate 13-acetate (PMA) on fibroblast DNA synthesis, obtaining an increment in the enzyme activity and also, this increment was abrogated in the presence of PKC inhibitor, calphostin C 1×10^{-9} M (**Table 2**). Also, the same **Table 2** shows the capacity of PMA to stimulate *per se* human gingival fibroblast cells proliferation.

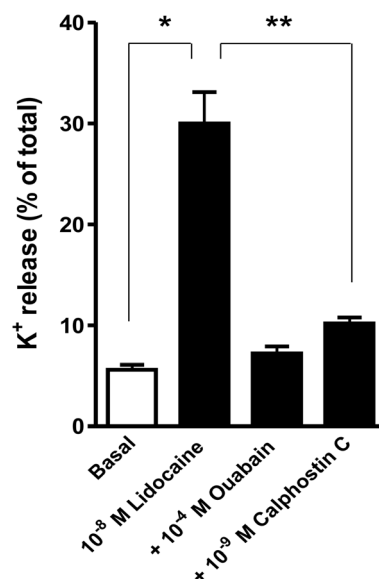**Figure 4**. Extent of net K$^+$ efflux from human gingival fibroblasts in the presence of lidocaine alone (1×10^{-8} M) or in the presence of lidocaine + ouabain (1×10^{-4} M) and lidocaine plus calphostin C (1×10^{-9} M). *P < 0.0001 vs. basal; **P < 0.001 vs. lidocaine alone. Values are the mean ± SEM of six experiments performed in duplicate.

Table 2. Effect of PMA alone or in the presence of calphostin C inhibitor on [^{3}H]-Thymidine incorporation and PKC activity.

Addition	[^{3}H]-Thymidine Incorporation (cpm)	PKC activity (μg of protein)	Number of experiments (n)
None	1380 ± 129	0.024 ± 0.001	5
PMA 18 nM	2410 ± 211	0.064 ± 0.004	4
PMA 18 nM + calphostin C 1×10^{-9} M	1552 ± 154	0.028 ± 0.002	4

Values are the mean ± SEM of n in each group. Cells were treated as reported in Material and Methods. [^{3}H]-Thymidine incorporation and PKC activity were measured following 24 hours culture.

5. Discussion

In this paper we provide new evidence for an innovative physiologic role linking the local anaesthetic lidocaine in human gingival fibroblast cells DNA synthesis besides its role in odontological pain relief.

These effects are related to the capacity of lidocaine to stimulate Na$^+$-K$^+$-ATPase activity since the inhibitors of the enzyme activity blunted the lidocaine-DNA synthesis increment.

Also, the rate of human fibroblast cells growth is depending on the concentration of lidocaine used. Previously we reported [18] that lidocaine is able to provoke a stimulatory action on human gingival fibroblast cells apoptosis at 1×10^{-5} M involving the activation of adenylate ciclase system with an accumulation of cAMP and the inhibition of PKC through the stimulation of caspase-3 activity [18].

The participation of Na$^+$-K$^+$-ATPase on mitogenic effect of lidocaine was demonstrated by the ability and the effectiveness of the local anaesthetic drug to provoke an increase in the K$^+$ flux. Thus, there is evidence indicating that intracellular potassium concentration influenced macromolecular synthesis and cell division. Low potassium concentration impaired proliferation [19] inversely when potassium concentration is increased in the medium the cell proliferation is restored [20]. Moreover, when lidocaine blocks the Na$^+$ channel, reducing nerve conduction in the oral cavity, and hence provides pain relief. Furthermore, ouabain at concentrations that inhibit membrane bound Na$^+$-K$^+$-ATPase blocks proliferation of lymphoblasts [21] and fibroblasts [22]. In addition, preincubation of bone narrow cells with ouabain at concentration considered necessary to inhibit the Na$^+$-K$^+$-ATPase activity, suppressed the erythropoietin mouse haematopoietic progenitor cell proliferation [23], indicat-

ing that functional membrane-bound Na^+-K^+-ATPase is also required for the induction of cell proliferation.

By the other hand, PKC involvement in this lidocaine-DNA synthesis and PKC increase enzyme activity effects was confirmed by the fact that PMA, a direct activator of PKC, was able to mimic lidocaine effect on fibroblast cell growth. It is reported previously [24] that the activation of PKC by PMA, provoked protein phosphorylation, inhibited Na^+ current channels as was observed in neuroblastoma cells.

Is has been reported [25] that the Na^+-K^+-ATPase actively participates in the stimulation of skin keratinocytes proliferation and was also confirmed that this action was abrogated by ouabain. Furthermore, the modulation of Na^+-K^+-ATPase activity is thought to be regulated by PKC-mediated changes in the phosphorylation and dephosphorylation state of Na^+-K^+-ATPase *in vivo* and *in vitro* [26]. However, PKC was also shown to be regulated by stimulation of growth factors and neurotransmitters as activator or deactivator of its intrinsic activity to be translocate the enzyme and to activate Na^+-K^+-ATPase. This activation in turn, regulate and stimulate the cell proliferation in a variety of cell types *i.e.* cultured coronary artery smooth muscle cells [27], rat astrocytes [28], vascular smooth muscle cells [29] and rat cardiomyocytes [30].

6. Conclusions

On the basis of our results, we postulate that the activation of PKC activity by lidocaine initiates the pharmacological effect of the local anaesthetic on human gingival fibroblasts inducing an increased DNA synthesis under the regulation and/or stimulation of Na^+-K^+-ATPase activity.

In conclusion, this work demonstrates that lidocaine increases human gingival fibroblasts DNA synthesis through activation of PKC pathway involving stimulation of Na^+-K^+-ATPase activity including phosphorylation of Na^+ channels.

Acknowledgements

Conflict of Interest

All authors declare that there is no any conflict of interest.

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