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Intracellular Ca^{2+} release by flufenamic acid and other blockers of the non-selective cation channel

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We report in this paper using measurement of intracellular free Ca^{2+} with fura-2, that flufenamic acid and several related blockers of the 25 pS Ca^{2+}-activated non-selective cation channel cause release of Ca^{2+} from an intracellular store other than the endoplasmic reticulum, possibly from mitochondria. A new compound, 4′-methyl-DPC, is found to be as effective in blocking non-selective cation channels as other flufenamate analogs but, like the parent compound, the non-selective cation channel blocker DPC, it does not cause release of Ca^{2+} from intracellular stores. DPC and 4′-methyl-DPC are thus the most suitable of the available blockers of non-selective cation channels for use in studies on the role of these channels in normal cell function.

Non-selective cation channel; Intracellular calcium; Flufenamic acid; Patch clamp; Fura-2

1. INTRODUCTION

Ca^{2+}-activated non-selective cation channels are found in many different tissues [1–6] although their role in normal function is conjectural [1, 7–9]. In some cells, blocker studies using diphenylamine-2-carboxylate (DPC) have suggested that a substantial fraction of the resting Ca^{2+} influx occurs via non-selective cation channels [10], but studies of this type have been impeded by the low affinity and poor specificity of DPC for the channel [10]. Recently, Gögelein and co-workers [4,11] have demonstrated that several DPC analogs, in particular, flufenamic acid, mefenamic acid and 3′,5-dichloro-DPC, have a higher affinity for the channel, being some 2–4 times more potent than DPC. In this paper, we report that these three compounds, in addition to blocking the non-selective cation channel, also evoke release of Ca^{2+} from intracellular stores, and thus are unsuitable for studies on the normal role of the non-selective cation channel. We show, however, that another previously uncharacterised DPC analog, 4′-methyl-DPC [12], has as a blocker of non-selective cation channels a potency similar to that reported for flufenamate and 3′,5-dichloro-DPC and does not cause intracellular Ca^{2+} release.

2. MATERIALS AND METHODS

The normal bath solution contained (in mmol/l) NaCl 145, KCl 5, glucose 10, HEPES 10 and $MgCl_2$ 1, adjusted to pH 7.4 with NaOH. The high- and zero-Ca^{2+} solutions contained 2 mmol/l $CaCl_2$ and 1 mmol/l EGTA, respectively.

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DPC, 3′,5-dichloro-DPC, 4′-bromo-DPC and 4′-methyl-DPC were the gift of Prof. R. Greger, University of Freiburg, Germany. Flufenamic, mefenamic and niflumic acids were purchased from Sigma (St. Louis, MO, USA). The structural formulae of these compounds are shown in Fig. 1. 2,5-Di(*tert*-butyl)-1,4-benzohydroquinone (tBuBHQ) was the gift of Prof. G.C. Farrell, University of Sidney, Australia. All compounds were made up in dimethylsulphoxide (DMSO) so that the final DMSO concentration did not exceed 0.1%, a concentration that we have found does not influence the fura-2 or patch-clamp experiments.

Cells of the neonatal mouse mandibular cell line ST_{885} were used between passages 60 to 100. They were maintained in plastic tissue culture flasks in Medium-199 mixed with fetal calf serum (10%) at 37°C in an atmosphere of 95% air and 5% CO_2. For microfluorimetry, the cells were grown as monolayers on glass coverslips (size 0). Monolayers were loaded with 10 μmol/l fura-2-AM (Molecular Probes, Oregon, USA) and incubated in Medium-199 mixed with fetal calf serum (10%) for 30 min at 37°C in a shaking water bath.

Fluorescence measurements were carried out using a Nikon Diaphot microscope with phase-contrast Fluor lenses [10]. The cells were excited at 340 nm and 380 nm alternately for 5 s intervals with the aid of a 75 Watt xenon lamp and the light emitted at 505 nm was monitored with a photomultiplier. The photomultiplier output was sampled and the ratio of the emission during excitation at 340 nm to emission during excitation at 380 nm (the fura-2 ratio) was calculated by computer. In vivo calibration was performed as described by Williams and Fay [14] using ionomycin as a Ca^{2+} ionophore. All experiments were performed at room temperature in a small chamber (approximately 0.2 ml) perfused at 1 ml/min.

In the patch-clamp experiments, the techniques were as previously described for ST_{885} cells [5]. Both pipette and bath contained the control bath solution with 1 mmol/l $CaCl_2$. Single channel currents were filtered at 500 Hz with an 8-pole Bessel filter and sampled at 1000 Hz. In the displayed current traces, current leaving the pipette is shown as an upward deflection and all potential differences are reported with reference to the bath.

3. RESULTS

Exposure of ST_{885} cells to flufenamate or 3′,5-dichloro-DPC (both 100 μmol/l) produced reversible

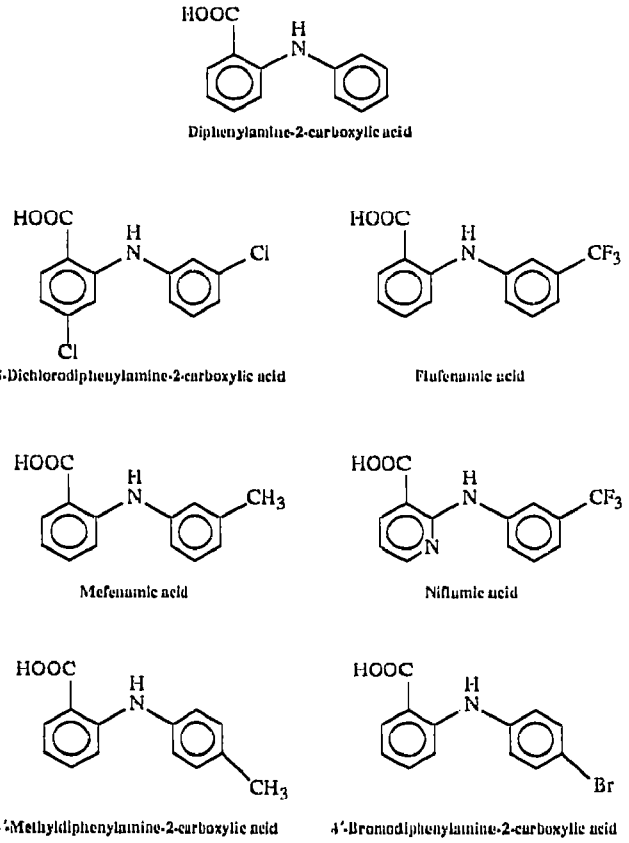

Fig. 1. Structural formulae of blockers of the Ca^{2+}-activated non-selective cation channel.

increases in the intracellular free Ca^{2+} whereas DPC did not (Fig. 2). Even when extracellular free Ca^{2+} was complexed by 1 mmol/l EGTA, the increase in intracellular free Ca^{2+} persisted for the duration of exposure to these agents and could be repeatedly evoked (Figs. 2 and 3). These findings suggest that flufenamate not only causes intracellular Ca^{2+} release but also blocks Ca^{2+} efflux from cytosol to bath. This behaviour is markedly different from that observed with 2,5-di(*tert*-butyl)-1,4-benzohydroquinone (tBuBHQ), an agent believed to cause release of Ca^{2+} from the endoplasmic reticulum [15,16]. Continuous exposure to tBuBHQ under Ca^{2+}-free conditions caused a transient increase in intracellular free Ca^{2+} and re-exposure to tBuBHQ failed to evoke a further increase (Fig. 2). The response to flufenamate was not inhibited by prior exposure to tBuBHQ (Fig. 2), suggesting that these agents are mobilizing different pools of Ca^{2+}.

The Ca^{2+}-releasing effect of flufenamate is dose-dependent over the range 10 μmol/l to 500 μmol/l (Fig. 3), the effect being half maximal at about 100 μmol/l. The maximum response of the ST_{885} cells to flufenamate was variable, but on average it increased the intracellular free Ca^{2+} from 10 nmol/l to 50 nmol/l in cells bathed in a Ca^{2+} free medium.

Fig. 4 compares the effectiveness of flufenamate, mefenamate and niflumate in producing intracellular Ca^{2+} release. All of these agents increased intracellular Ca^{2+} over the same concentration range as they inhibited the non-selective cation channel; 3',5-dichloro-DPC (see Fig. 2) and 4'-bromo-DPC also increased intracellular Ca^{2+} (data not shown).

We have found one derivative of DPC, however, which, like DPC itself, does not increase the intracellular free Ca^{2+}. This is 4'-methyl-DPC, which, in a concentration of 100 μmol/l, does not evoke any increase in intracellular free Ca^{2+} (12 experiments) as shown in Fig. 5. Consequently, we tested its effectiveness as a blocker of the 25 pS Ca^{2+}-activated non-selective cation channel, which is present in abundance in ST_{885} cells [5]. We did this by estimating channel activity (nP), the product of the number of channels present (n) and the channel open probability (P), in data samples acquired over periods of 30 s. In inside-out patches clamped at a pipette voltage of +60 mV, we found that 10 μmol/l 4'-methyl-DPC reduced nP from 1.59 ± 0.26 (S.E.M., n = 3) to 0.61 ± 0.23 (62%) and 100 μmol/l 4'-methyl-DPC reduced it from 2.25 ± 0.48 (S.E.M., n = 3) to 0.02 ± 0.01 (99%). This channel blocking effect of the drug was fully reversible. A representative experiment of this type is shown in Fig. 6. The potency of 4'-methyl-DPC, which in our hands caused a reduction in single channel activity of 62%, was evidently comparable to that reported for flufenamate, mefenamate and 3',5-dichloro-DPC. These compounds, at concentrations of 10 μmol/l, reduced non-selective cation channel activity

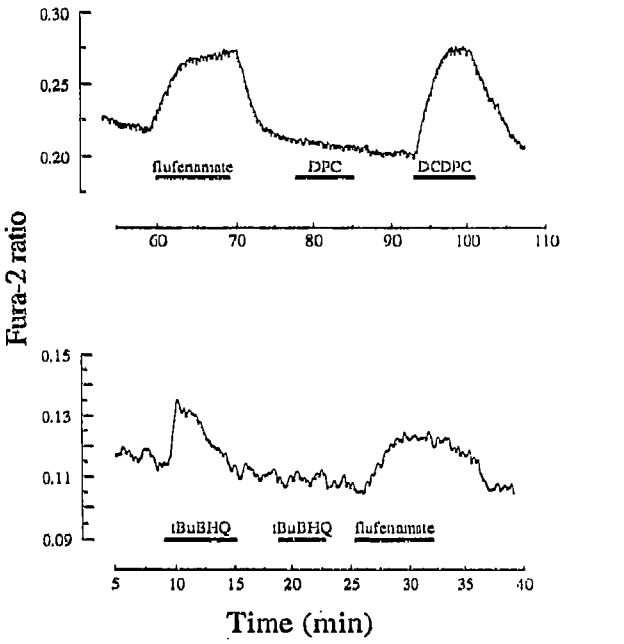

Fig. 2. Upper panel: the effects of flufenamate, DPC and 3',5-dichloro-DPC (all 100 μmol/l) on the fura-2 ratio of ST_{885} cells bathed in zero Ca^{2+}. Lower panel: comparison of the effect of tBuBHQ (40 μmol/l) and flufenamate (100 μmol/l) on the fura-2 ratio of ST_{885} cells bathed in zero Ca^{2+}.

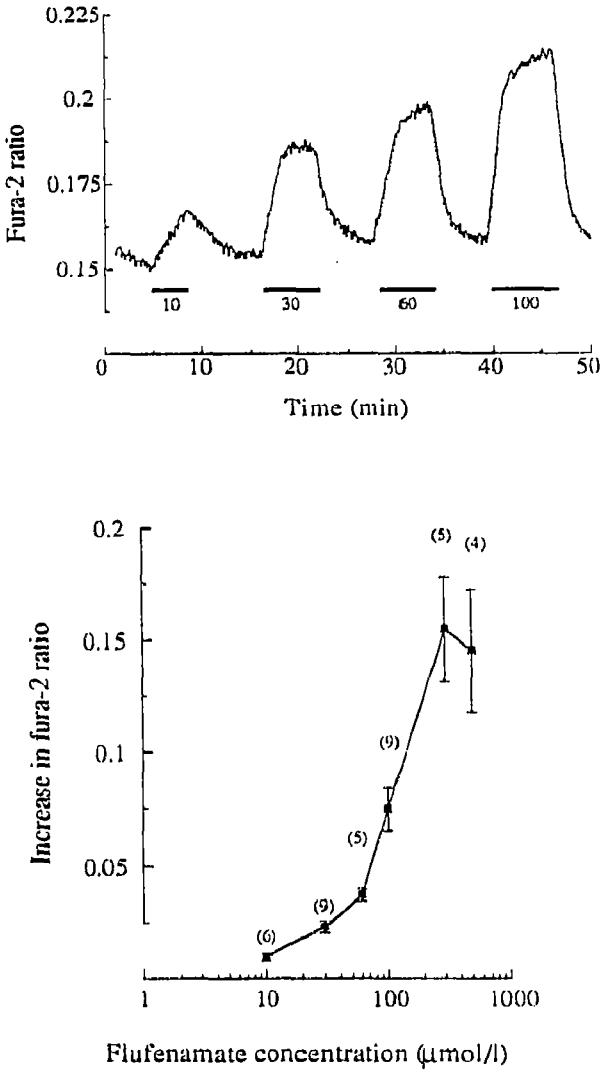

Fig. 3. Dose–response relation of the effect of flufenamate on the fura-2 ratio of ST_{885} cells bathed in zero Ca^{2+}. Upper panel: representative experiment. Lower panel: dose–response curve. The numbers in parentheses indicate the number of experiments performed; error bars indicate the S.E.M.

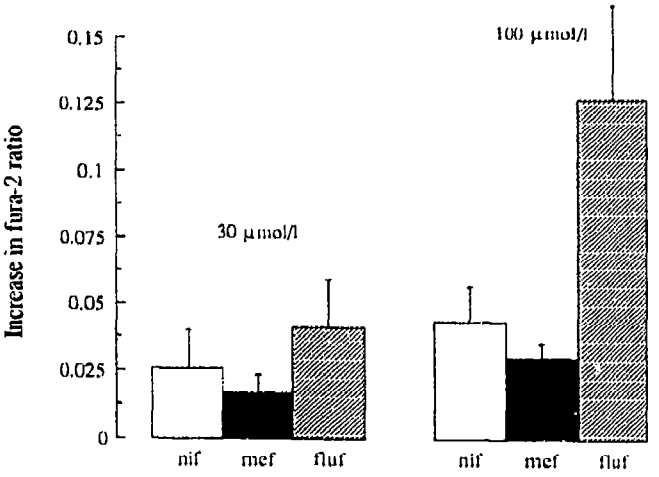

Fig. 4. The relative effectiveness of flufenamate (fluf), niflumate (nif), mefenamate (mef) in elevating intracellular Ca^{2+} in ST_{885} cells. The data are the means of 4 experiments ± S.E.M.

in the rat exocrine pancreas by approximately 35, 60, 60% respectively, compared with a block of only 20% by DPC at this concentration [4,11]. We have found, however, that in ST_{885} cells DPC appears to be as potent as the other derivatives. Addition of 10 μmol/l DPC was found to reduce the activity of the non-selective channel by 73.3 ± 11.6% (n = 3), indicating that the relative affinities of blockers of this type may vary with the cell type being studied.

4. DISCUSSION

In this paper, we show that flufenamate, niflumate, mefenamate and 3',5-dichloro-DPC all cause release of Ca^{2+} from intracellular stores and the concentration range over which they do this is the same as that over which they block non-selective cation channels. Although the present experiments were performed exclusively on ST_{885} cells, we have observed the same phenomenon in sheep parotid secretory cells, rat mandibular secretory cells, rat parathyroid cells, unfertilized mouse oocytes, cultured rat insulinoma (RINm5F) cells, and rabbit cardiac myocytes. The Ca^{2+} release evoked by flufenamate is substantial. In ST_{885} cells, the size of the Ca^{2+} increase is comparable to the elevation of Ca^{2+} produced by the action of tBuBHQ on microsomal Ca^{2+} stores. In sheep parotid cells, we find that its size is about 20–30% of the increase produced by maximum stimulation with acetylcholine and in rat insulinoma (RINm5F) cells it is comparable to the effect of depolarisation with high K^+ concentrations or extracellular exposure to alanine. DPC does not cause Ca^{2+} release and so is suitable for blocker studies on the role of non-selective cation channels [10] but it is reported to be the least potent of this group of inhibitors of the

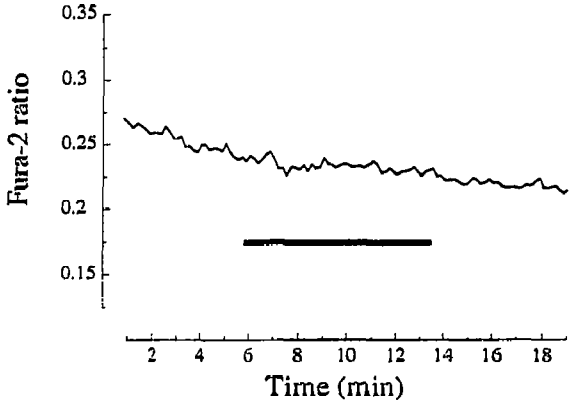

Fig. 5. The effect of 4'-methyl-DPC (100 μmol/l) on the fura-2 ratio of ST_{885} cells bathed in zero Ca^{2+}. The period of exposure to 4'-methyl-DPC is shown by the black bar.

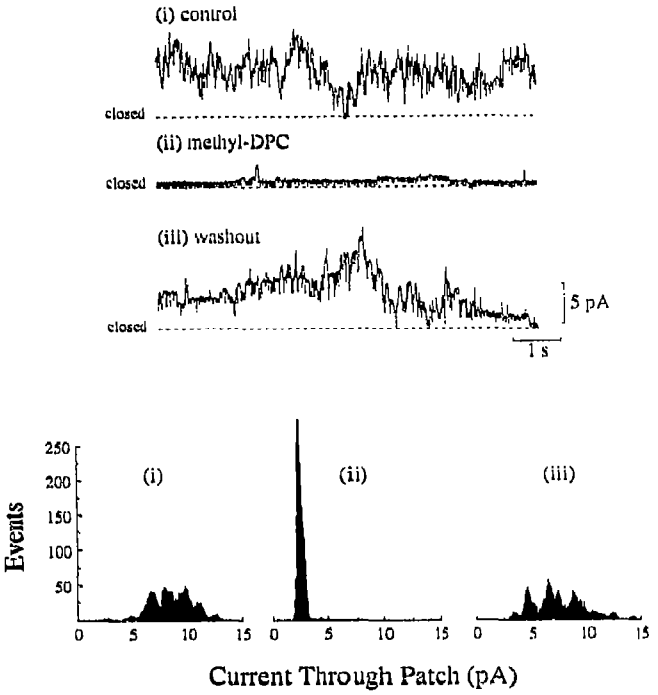

Fig. 6. Upper panel: representative recording showing the effect of 100 μmol/l 4'-methyl-DPC on individual non-selective cation channels. The arrows indicate the current level when all channels are closed. The data were obtained from inside-out excised patches clamped at a pipette potential of +60 mV. Lower panel: Current histograms taken from 30-s stretches of data from the same experiment, confirming that 4'-methyl-DPC is a blocker rather than an activator.

non-selective cation channel [4,11]. 4'-methyl-DPC is shown to be equally as effective as flufenamate and 3',5-dichloro-DPC in blocking non-selective cation channels and does not cause Ca^{2+} release. Thus either DPC of 4'-methyl-DPC are the most suitable of the currently known blockers of non-selective cation channels. Since non-selective cation channels may differ slightly in their affinity for these DPC derivatives, the choice between DPC and 4'-methyl-DPC will depend on their relative potencies for the channel being studied.

The inability of tBuBHQ to deplete the Ca^{2+} store released by flufenamate suggests that flufenamate is not acting on microsomes. Since flufenamate has been reported to block mitochondrial ATP synthesis leading to Ca^{2+} release from isolated mitochondria [17], it appears likely that mitochondria are the source of the Ca^{2+} release observed in this study.

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