2-APB inhibits volume-regulated anion channels independently from intracellular calcium signaling modulation

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Received 17 September 2003; revised 23 October 2003; accepted 23 October 2003

First published online 3 December 2003

Edited by Maurice Montal

Abstract **It has previously been suggested that volume-regulated anion channels (VRACs) and store-operated channels (SOCs) interact with each other according to their expected colocalization in the plasma membrane of LNCaP cells. In order to study interactions between these two channels, we used 2-aminoethoxydiphenyl borate (2-APB) as a regular SOC inhibitor. Surprisingly 2-APB reduced VRAC activity in a dose-dependent manner (IC_{50} = 122.8 μM), but not 2,2-diphenyltetrahydrofuran (a structural analog of 2-APB). This effect was also present in keratinocytes. We conclude that 2-APB is an inhibitor of the VRAC family, and is also a potent tool to study the SOC–VRAC interaction in LNCaP cells.**

Key words: Volume-regulated anion channel; Store-operated channel; 2-Aminoethoxydiphenyl borate; Calcium; Capacitative entry

1. Introduction

The volume-regulated anion channel (VRAC) is a ubiquitous type of ion channel that plays a pivotal role in regulatory volume decrease (RVD), a process by which cells control their volume (for reviews, see [1–3]). One of the most important discrepancies between the data on VRACs studied on different cellular models concerns how these channels are regulated by Ca^{2+} ions [4–6].

In our previous study [7] we addressed the hypothesis of a functional link between plasma membrane VRACs, which carry Cl^- current activated by hypotonic cell swelling ($I_{Cl,\,swell}$), and the store-operated channels (SOCs), which are activated in response to the depletion of intracellular Ca^{2+} stores in prostate cancer epithelial cells. Indeed, the activation of SOCs resulted in a significant inhibition of $I_{Cl,\,swell}$. We concluded that interaction between VRACs and Ca^{2+} occurs in the microdomains of the inner surface of the membrane, which are not accessible to changes in global intracellular calcium concentration.

The original aim of the present work was to further explore the regulation of VRACs by the most universal second messenger, Ca^{2+}, using the widely used inhibitor of SOCs and inositol 1,4,5-triphosphate (IP_3) receptors, 2-aminoethoxydiphenyl borate (2-APB) [8,9].

Unexpectedly (using patch-clamp, fluorescent Ca^{2+} measurement and flow cytometry techniques), we found that 2-APB may inhibit VRACs independently of its well-known effects on intracellular calcium signaling. Our study is the first to show that 2-APB may be a direct inhibitor of VRACs. Interestingly, this effect of 2-APB seems to be specific for volume-activated chloride channels, since it was previously reported that the Ca^{2+}-activated chloride current was not affected in *Xenopus* oocytes [10]. We demonstrate this inhibitory effect of 2-APB on VRACs using two different cellular models: prostate epithelial cancer cells LNCaP, and human keratinocytes HaCaT, thereby suggesting the ubiquitous nature of 2-APB effect on VRACs. Moreover, this inhibitory 2-APB effect on VRACs was able to impair the normal RVD process. Therefore, the inhibition of VRACs described in our study is a new 2-APB property that promises to provide a powerful pharmacological tool for studying volume-regulated channels, the pharmacology of which is known to be rather poor.

2. Materials and methods

The culture of LNCaP cells and patch-clamp procedures (including intra- and extracellular solution compositions) are the same as previously described [7]. The estimations of the changes in cell volume during the RVD process were performed on a FACScalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA) with the use of 'Q Cell Quest' software for data analysis [11]. The light-scatter channels were set on linear gains. Cells in suspension in normal or Hypo-TEA solutions were gated for forward-angle scatters and 5000 particles of each gated population were analyzed.

Fluorescence $[Ca^{2+}]_{in}$ measurements were conducted on fura-2AM (5 μM) loaded (45 min) LNCaP cells using a photomultiplier-based system (Photon Technologies, Princeton, NJ, USA) and double wavelength (340 and 380 nM) excitation protocol to quantify the absolute value of Ca^{2+} concentration [12].

3. Results

3.1. $I_{Cl,\,swell}$ is sensitive to 2-APB application

As described in previous studies, the application of hypotonic external solution induced development of $I_{Cl,\,swell}$ in LNCaP cells. This current reached its maximal amplitude in 5–10 min and remained at this steady state as long as the cells were superfused with hypotonic solution [13]. In Fig. 1A, a 100 μM 2-APB containing hypotonic solution was applied after full development of $I_{Cl,\,swell}$ in a representative cell. 2-APB application induced a drastic decrease in $I_{Cl,\,swell}$ amplitude. In order to check how 2-APB modified $I_{Cl,\,swell}$ elec-

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0014-5793/03/$30.00 © 2003 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)01387-5

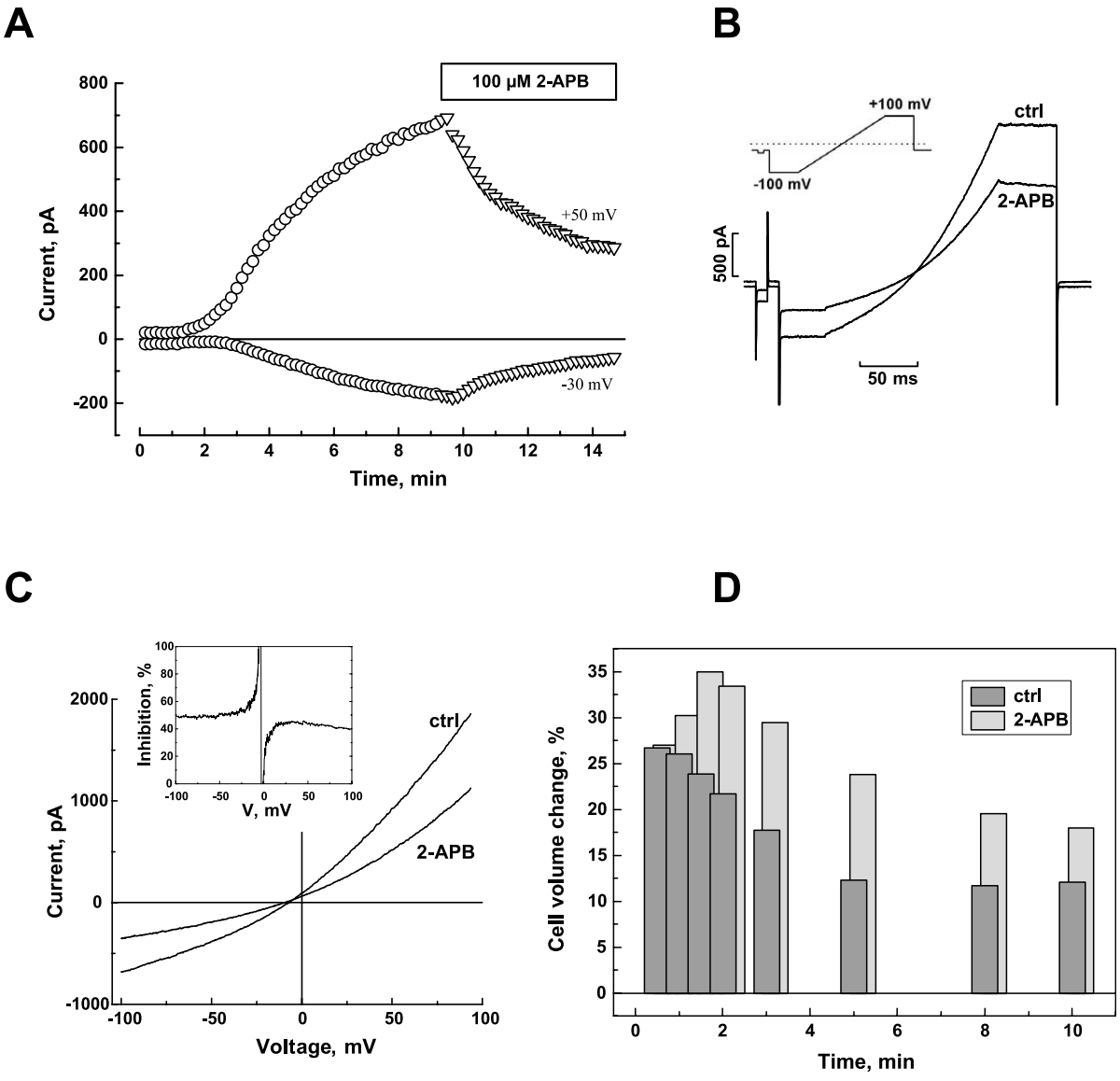

Fig. 1. 2-APB inhibits $I_{Cl,\,swell}$ in LNCaP cells. A: $I_{Cl,\,swell}$ development after exposure to hypotonic extracellular solution (at time 0) observed at +50 and −50 mV (circles), and resulting inhibition after 100 μM 2-APB exposure (down triangles) in a representative cell. B: $I_{Cl,\,swell}$ traces observed in control conditions (ctrl) or after 100 μM 2-APB application (2-APB); inset: pulse protocol. C: *I*/*V* plots derived from ramp traces shown in B; inset: voltage dependence of the inhibition. D: RVD process in LNCaP cells (5000 cells at each point) exposed to standard (dark gray bars) or 100 μM 2-APB supplemented hypotonic solution (light gray bars).

trophysiological properties, the same cell was subjected to a ramp protocol as presented in Fig. 1B (inset). Resulting currents from this protocol are presented in Fig. 1B: currents before (ctrl) and after (2-APB) 2-APB application exhibited similar outward rectification properties. As shown by corresponding *I*/*V* relationships directly derived from previous ramps, 2-APB exhibited a voltage-dependent VRAC block: the rate of $I_{Cl,\,swell}$ inhibition was slightly higher for negative potentials than for positive ones (Fig. 1C). So, at −100 mV and +100 mV, the inhibition rates by 100 μM 2-APB were 55.6 ± 6.8% and 39.2 ± 3.7% respectively ($n = 8$).

As a further demonstration of 2-APB as an $I_{Cl,\,swell}$ inhibitor, we also investigated whether or not 2-APB would impair the RVD process. RVD is the physiological consequence of $I_{Cl,\,swell}$ activation during hypotonic challenge. This experiment is presented in Fig. 1D: the cell volume increase led to the $I_{Cl,\,swell}$ activation, which permitted the cells to recover their normal volume. Cells pre-treated for 30 min with 100 μM 2-APB and exposed to a 2-APB containing hypotonic solution exhibited a quite different response compared to control ones. 2-APB exposure was seen to be accompanied by an exaggerated volume increase, reaching the maximal value of 35% (26.7% in control) after 1.5 min exposure to hypotonic solution. After that, cells began to recover their normal value, but this process was not complete after 10 min and cell volume remained slightly higher (17.9% volume increase compared to 12% in control).

2-APB was previously shown to exhibit a strong inhibitory effect on IP_3 receptor, transient receptor potential channels and potassium channels [8,9]. However, in order to assess whether VRAC are specifically blocked by 2-APB, we used a previously described molecule, 2,2-diphenyltetrahydrofuran (DPTHF), exhibiting the same effect as 2-APB on IP_3 receptors [8]. Results of DPTHF application on VRAC are sum-

marized in Fig. 3C: perfusing the cells previously exposed to hypotonic challenge with a 100 μM and 1 mM DPTHF containing hypotonic solution led to only a minor decrease in current amplitude of 10.2 ± 4.8% for 1 mM and 100 μM, leading to a small increase in $I_{Cl,\,swell}$ amplitude of 5.6 ± 6.9%. These results have to be compared with those of 2-APB corresponding to a 44.4 ± 3 and 91.7 ± 1.6% decrease in VRAC-associated current after applications of 100 μM and 1 mM 2-APB respectively. These results argue in favor of a direct action of 2-APB: a slight structural modification of 2-APB giving DPTHF, which exhibits the same effects on IP_3 receptors as 2-APB, strongly impairing its action.

3.2. Properties of $I_{Cl,\,swell}$ inhibition by 2-APB

All previous experiments were carried out with the standard 100 μM 2-APB concentration, which is known to completely inhibit SOC. In order to assess whether $I_{Cl,\,swell}$ inhibition by 2-APB was also maximal at 100 μM, we did further experiments with various 2-APB concentrations. Our results are summarized in Fig. 2A: applications of 10, 50, 100, 200, 500, 1000, 2000 μM 2-APB led to respective decreases in $I_{Cl,\,swell}$ amplitude at +100 mV of 10.7 ± 2.5, 30.4 ± 4.3, 44.4 ± 3, 65.4 ± 3, 76.5 ± 1.9, 91.7 ± 1.6, 91.4 ± 2.6% ($n = 4$ for each concentration). We determined the concentration of half-maximal blockade (IC_{50}) and the level of maximal blockade (A_{max}) as $IC_{50} = 122.8$ μM and $A_{max} = 96.8\%$.

During our experiments we observed that increasing 2-APB concentration induced faster $I_{Cl,\,swell}$ inhibition. This observation was confirmed by plotting the time before 50% of maximal $I_{Cl,\,swell}$ decrease as a function of 2-APB concentration (Fig. 2B). So time up to 50% of maximal effect decreased from 132 ± 24.4 to 6.5 ± 0.6 s by increasing 2-APB concentration respectively from 20 μM to 2 mM. Such a result was consistent with the idea that 2-APB would act directly on VRAC rather than by an intermediate structure, according to the fast inhibitory effect registered with high 2-APB concentrations. Nevertheless, we showed that 2-APB does not change the electrophysiological properties of $I_{Cl,\,swell}$, therefore leading

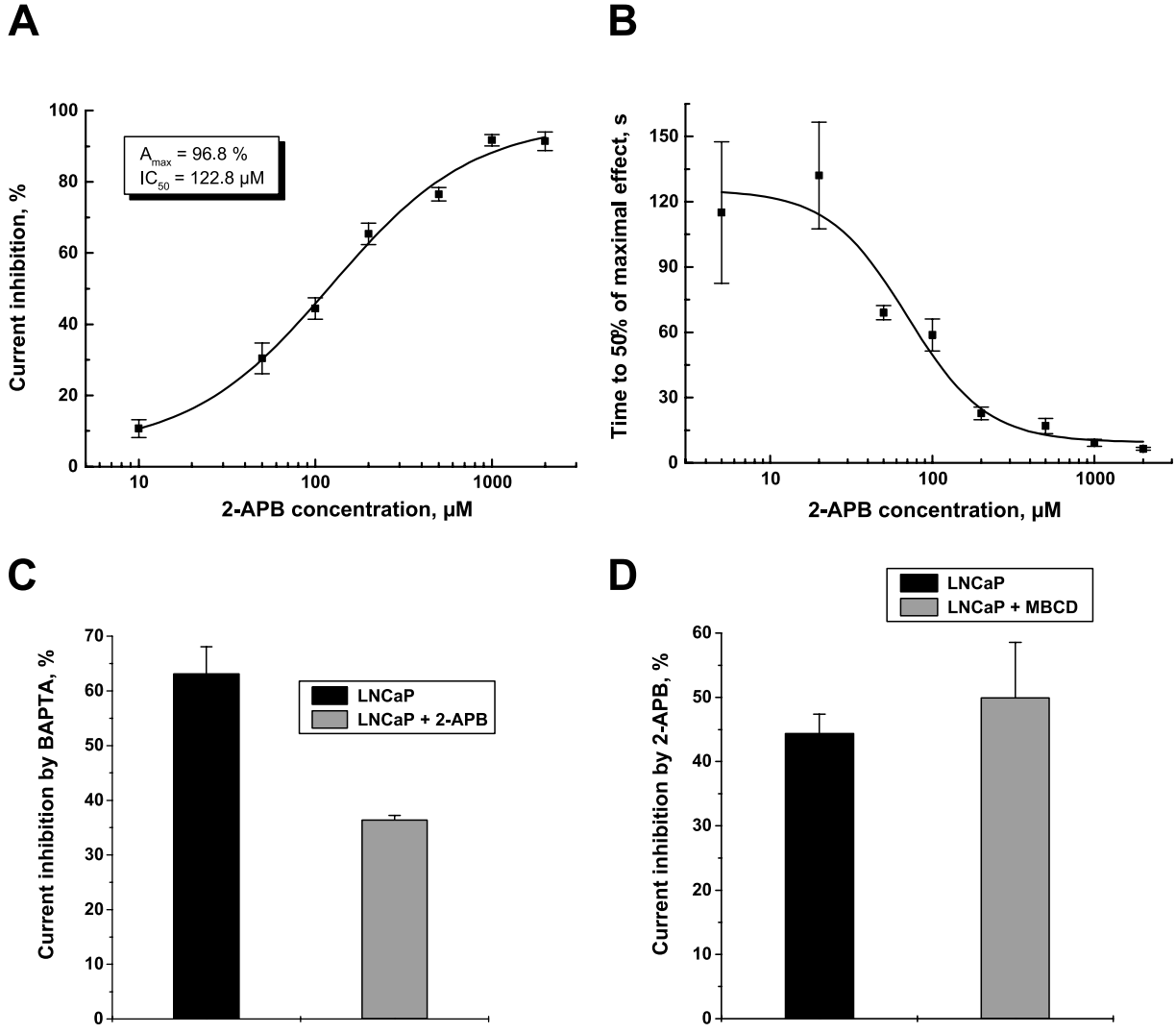

Fig. 2. $I_{Cl,\,swell}$ inhibition by 2-APB is a dose-dependent process. A: Dose–response relationship for the 2-APB-induced $I_{Cl,\,swell}$ inhibition at +100 mV with a superimposed fit of the data points ($n = 4$) by Langmuir's isotherm (smooth curve) and the corresponding A_{max} and IC_{50}. B: Dose–kinetics relationship for $I_{Cl,\,swell}$ inhibition. Each time corresponds to the mean delay before observing 50% of the maximal $I_{Cl,\,swell}$ decrease at the corresponding 2-APB concentration ($n = 4$). C: Effect of 0.5 mM BAPTA application on $I_{Cl,\,swell}$ at +100 mV in LNCaP cells ($n = 5$) and in LNCaP cells previously exposed to 100 μM 2-APB ($n = 3$). D: Histogram summarizing the effect of 100 μM 2-APB application on $I_{Cl,\,swell}$ at +100 mV in LNCaP cells ($n = 4$) and in LNCaP cells pre-treated (30 min at 37°C) with 10 mM MBCD ($n = 4$).

us to investigate whether it could change other properties of $I_{Cl,\,swell}$ in LNCaP cells. First, we tested the sensitivity of remaining $I_{Cl,\,swell}$ after a 100 μM 2-APB application, and further exposure to 0.5 mM BAPTA (Fig. 2C). In this case, BAPTA application after the 2-APB effect led to a 36.5 ± 0.7% decrease in the remaining $I_{Cl,\,swell}$ at +100 mV ($n = 3$), i.e. nearly a two-fold decrease in the normal effect recorded when BAPTA was added alone (63.2 ± 4.9% decrease, $n = 5$). As a second step, we investigated 2-APB inhibitory effect in cells exposed to methyl-β-cyclodextrin (MBCD), a disrupter of caveolae. We previously demonstrated that MBCD was able to disorganize the SOC–VRAC interaction by uncoupling the two channels, leading to the desensitization of VRAC during capacitative Ca^{2+} entry [14]. Application of 100 μM 2-APB to fully developed $I_{Cl,\,swell}$ induced a 44.4 ± 3% decrease ($n = 4$). The corresponding decrease in cells pre-treated with MBCD was not significantly different from the one in control cells (49.9 ± 8.6% decrease at +100 mV, $n = 4$), indicating that disruption of the SOC–VRAC interaction was without effect on $I_{Cl,\,swell}$ inhibition by 2-APB (Fig. 2D).

3.3. 2-APB allows separate study of VRAC and SOC

We repeated the experiments described in our previous work [7] by applying a fully developed $I_{Cl,\,swell}$ 1 μM thapsigargin (Tg) to a 2 mM Ca^{2+} containing extracellular solution. This experiment was shown to decrease $I_{Cl,\,swell}$ amplitude by 37 ± 6%. Fig. 3A presents the results obtained in the presence of 2-APB: after complete $I_{Cl,\,swell}$ development, we first added extracellular solution 100 μM 2-APB, which induced a two-fold decrease in $I_{Cl,\,swell}$ amplitude as described above. After steady-state inhibition by 2-APB, we applied a 100 μM 2-APB+1 μM Tg solution to the remaining $I_{Cl,\,swell}$, which appeared to be without effect. By using Ca^{2+} imaging, we proved 100 μM 2-APB was sufficient to completely block Tg-induced capacitative Ca^{2+} entry (Fig. 3B), thereby leading us to conclude that using 2-APB at the appropriate concentration could be a very useful tool to study the VRAC–SOC interaction.

3.4. VRAC inhibition by 2-APB is not restricted to human prostate cancer cells

In order to assess whether the 2-APB effect on VRAC was limited to human prostate cancer cells, we did further experiments with a well-defined keratinocyte model for which VRACs have been previously characterized, namely the HACaT cell line [15]. Like LNCaP cells, HACaT exhibits an outwardly rectifying chloride current when exposed to hypotonic solution (Fig. 4A, left panel). These *I/V* traces show that the current possesses, as in LNCaP cells, a slight inactivation for potentials above +60 mV. Moreover, a 100 μM 2-APB application induced a dramatic decrease in $I_{Cl,\,swell}$ (Fig. 4A, right panel). As illustrated in Fig. 4B, the 2-APB effect is not voltage dependent and for the same dose (100 μM) 2-APB exhibits the same inhibitory effect on $I_{Cl,\,swell}$ in LNCaP cells and HACaT: 44.4 ± 3%/52.3 ± 8.3% and 53.1 ± 8.6%/63.2 ± 6.1% at +100/−100 mV respectively (Fig. 4C).

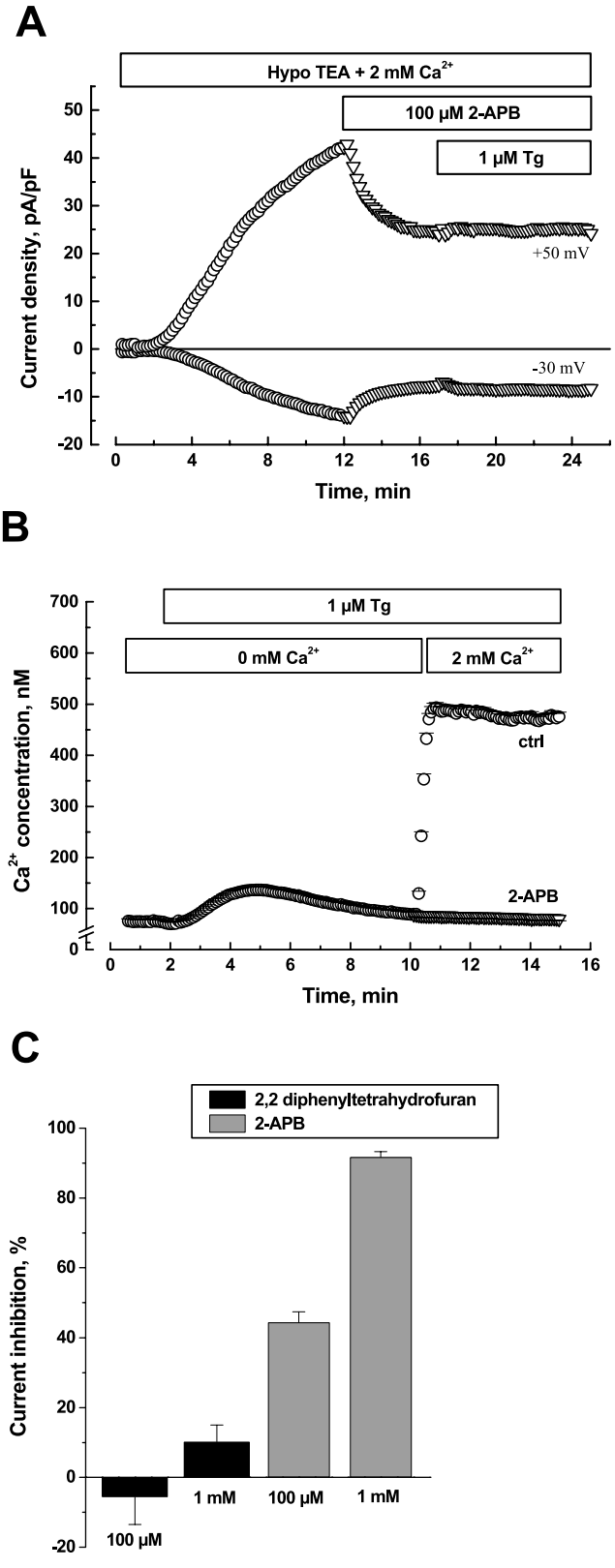

Fig. 3. 2-APB completely prevents Tg-induced capacitative Ca^{2+} entry. A: Changes of $I_{Cl,\,swell}$ (circles) initially evoked by hypotonic solution in a representative cell first exposed to 100 μM 2-APB alone and second with 1 μM Tg (down triangles). B: Intracellular Ca^{2+} variation in cells sequentially exposed to 1 μM Tg without extracellular Ca^{2+} and to a 1 μM Tg+2 mM Ca^{2+} containing isotonic extracellular solution. Resulting traces in control (circles, $n = 58$) or in presence of 100 μM 2-APB (down triangles, $n = 65$) are presented. C: Current inhibition at +100 mV in cells exposed to 100 μM and 1 mM 2,2-diphenyltetrahydrofuran ($n = 3$), or to 100 μM and 1 mM 2-APB ($n = 4$).

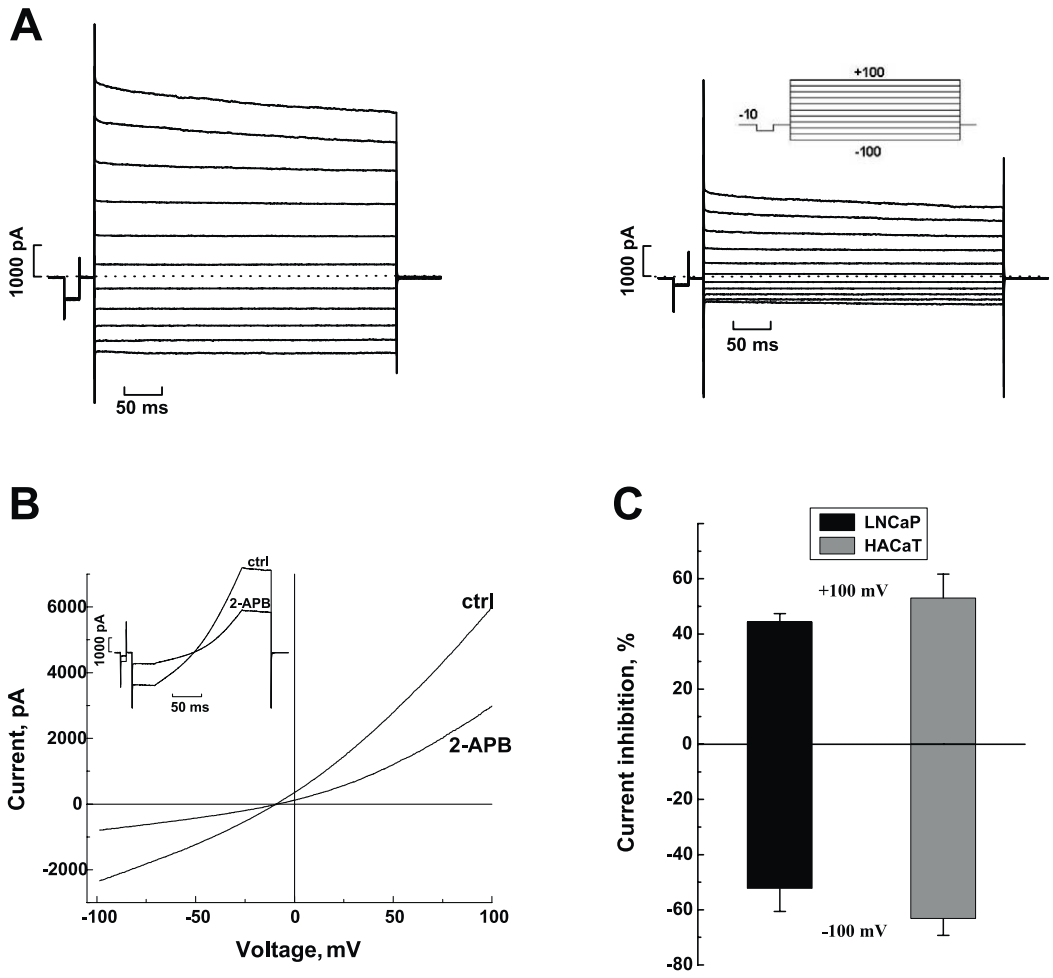

Fig. 4. 2-APB exhibits the same properties for VRAC current in keratinocytes as in LNCaP cells. A: $I_{Cl,\,swell}$ traces obtained in HACaT in control conditions (left) and after 100 μM 2-APB application (right). Pulse protocol is presented in the inset. B: *I/V* relationship derived from ramp traces shown as inset in control (ctrl) or after 100 μM 2-APB (2-APB). C: Histogram summarizing the effect of 100 μM 2-APB on VRAC current in LNCaP cells ($n = 4$) and in HACaT ($n = 6$).

4. Discussion

2-APB was firstly widely used in biological research for studying the IP_3 receptor and its action has been considered as being an exclusive inhibitor of this receptor. Further studies, however, demonstrated that 2-APB is also a potent inhibitor of the store-operated Ca^{2+} channels in human platelets [8]. The specificity of these inhibitors has to be relativized according to previous observations by Gericke et al. [16], demonstrating that the well-known VRAC inhibitor NPPB, was also a quite effective inhibitor of Ca^{2+} channels in human endothelial cells. Moreover, it was recently shown that 2-APB may block voltage-dependent potassium channels in *Limulus* ventral photoreceptors [9] and gap junctional coupling in normal rat kidney cells [17].

Our study clearly establishes that 2-APB is an inhibitor of volume-dependent chloride channels. This observation seems to be a general feature of VRACs since we observed the same inhibitory effect on VRACs using two cellular models: prostate cancer epithelial cell and keratinocytes. This effect may result either from direct interaction between 2-APB and the VRAC or from the interaction of 2-APB with an intermediate target that in turn may inhibit the VRAC activity. However, it seems very unlikely that this 2-APB inhibitory effect on VRAC may involve one of its previously reported actions. Indeed, the hypothesis that 2-APB interacts with the VRAC both directly and differentially from its other targets was confirmed by experiments using DPTHF, a compound structurally similar to 2-APB. DPTHF has indeed previously been reported to mimic 2-APB effects, in particular by inhibiting IP_3 receptors. Nevertheless, DPTHF application was shown in this study to be ineffective on VRAC current. Moreover, BAPTA, which was suggested to block VRAC activity by direct binding the channel, exhibited a reduced effect after 2-APB application. This observation is compatible with a competition between BAPTA and 2-APB to bind the VRAC.

Finally, our results indicate that $I_{Cl,\,swell}$ blockade by 2-APB is not specific to the human prostate epithelial cells, but may represent a general phenomenon independent of the cell type. We therefore suggest using 2-APB with caution, which implies carefully studying the VRAC–SOC–IP_3 receptor interactions in order to avoid any incorrect interpretations.

Acknowledgements: This work was supported by grants from INSERM, La Ligue Nationale Contre le Cancer and l'ARC (France) and INTAS-99-01248. Y.M.S. was supported by the French Ministry of Science.

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