Ca^{2+}-dependent interaction of BAPTA with phospholipids

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Abstract Starting from a comparative study of different Ca^{2+} chelators on the G-protein-induced inhibition of the $Ca_V2.1$ Ca channels, we demonstrate that BAPTA and DM-nitrophen are able to interact, in a Ca^{2+}- and lipid-dependent manner, with phospholipid monolayers. Critical insertion pressure and sensitivity to charged lipids indicated that insertion in the lipid film may occur in biological membranes as those found on *Xenopus oocytes*. This novel property is not found for EGTA and EDTA and may participate to the unusual ability of BAPTA-related molecules to chelate Ca^{2+} ions in the very close vicinity of the plasma membrane, where most of the Ca^{2+}-dependent signalling triggered by voltage-gated Ca^{2+} currents occurs.

Keywords: P/Q type Ca channel; Ca chelator; G-protein regulation; Shuttle buffer

1. Introduction

Ca^{2+} ions play a central role in many physiological processes either directly, through Ca^{2+} specific ion channel-induced membrane depolarisation or indirectly as a second messenger spreading excitation to a vast array of Ca^{2+}-sensitive proteins leading to contraction, secretion, gene activation [1–3], etc. The role of Ca^{2+} ions in these processes has been largely studied using specific chelators and indicators. Before 1980, ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) was almost the only chelator used in biological experiments as it displayed a high specificity towards Ca^{2+}, a simple 1:1 stoichiometry and good buffering capacity. However one of the major drawbacks of EGTA was the speed of buffering, since the protonated form of EGTA, which predominates at pH 7.0, has relatively slow Ca^{2+}-binding kinetics ($K_{on} = 1.5 \times 10^6$ $M^{-1}\,s^{-1}$ [4]). In 1980, Tsien designed and synthesized a new form of EGTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA; [5]), in which the addition of benzene rings, while keeping the overall structure of the molecule, decreased the ionization below pH 6.5 and thus accelerated Ca^{2+} chelation ($K_{on} = 9.1 \times 10^8$ $M^{-1}\,s^{-1}$). Since then, BAPTA has become a useful tool to analyse the effect of fast and localized Ca^{2+} transients such as those generated by the opening of Ca^{2+} specific ion channels. Ca^{2+} chelation using BAPTA or EGTA soon appeared to be non-equivalent, and most of the differences were attributed to their different kinetics of chelation (see for example [4]). However, recently BAPTA has also been suggested to induce changes in cellular physiology unrelated to its Ca^{2+} chelating properties and designed under the term of ''pharmacological effect'' of BAPTA.

We now report a new property of BAPTA that may in part explain these different results and additional effects. The free form of BAPTA can interact with phospholipid monolayers, a widely used membrane model [6]. The critical insertion pressure (CPI, see Section 2) and phospholipid specificity are compatible with an insertion into biological plasma membranes. The Ca^{2+} bound form of BAPTA cannot associate with phospholipid monolayers, suggesting that BAPTA may function as a shuttle buffer [7,8], driving Ca^{2+} out of the membrane and thus decreasing the Ca^{2+} concentration specifically at the membrane interface. Similar results are found with DM-nitrophen but not with EGTA and EDTA.

2. Materials and methods

2.1. Materials and oocyte preparation and recordings

BAPTA, (Sigma–Aldrich, Lisle D'Abeau-Chesnes, FR), di-Br-BAPTA, di-methyl BAPTA and DM-nitrophen (Molecular Probes Inc., Eugene, OR) were prepared at 100 mM in HEPES 10 mM (pH 7.2 with CsOH) for on-line injection into oocytes. In Fig. 1, BAPTA was loaded with 20% Ca^{2+}. EGTA and EDTA were prepared at 200 mM in the same solution. Phospholipids, dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylglycerol (DOPG), were purchased from Avanti Polar Lipids (Alabaster, AL).

Xenopus laevis oocyte preparation and injection were performed as described [9]. Each oocyte was injected with 5–10 nl of a cDNA mixture containing the $Ca_V2.1 + \alpha_2\delta$ cDNAs at ≈0.3 ng/nl with a ratio of 1:2. Oocytes were kept for 2–4 days at 18 °C under gentle agitation before use.

Whole-cell Ba^{2+} currents were recorded under two electrode voltage-clamp using the GeneClamp 500 amplifier (Axon Instruments, Union City, CA). Current and voltage electrodes (less than 1 MΩ) were filled with: 3 M KCl, pH 7.2, with KOH. Ba^{2+} current recordings were performed using the following external solution (in mM): BaOH, 10; TEAOH, 20; NMDG, 50; CsOH, 2; HEPES, 10; pH 7.2 with methanesulfonic acid. Currents were filtered and digitized using a Digidata-1200 interface (Axon Instruments), and subsequently stored on a Pentium-based personal computer using version 6.02 of pClamp software (Axon Instruments). Around 10–30 nl of each of the above-mentioned chelators (in mM: chelator, 100; CsOH, 10; HEPES, 10; pH 7.2 CsOH) were injected into the oocyte (10 psi, 150 ms) during the course of the experiment at the time indicated. Ba^{2+} currents were recorded during a 400 ms test pulse from −80 to +10 mV. Current amplitudes were measured at the peak of the current. Comparisons between experiments were made by normalizing all average amplitudes with respect to the control current amplitude set at 100%.

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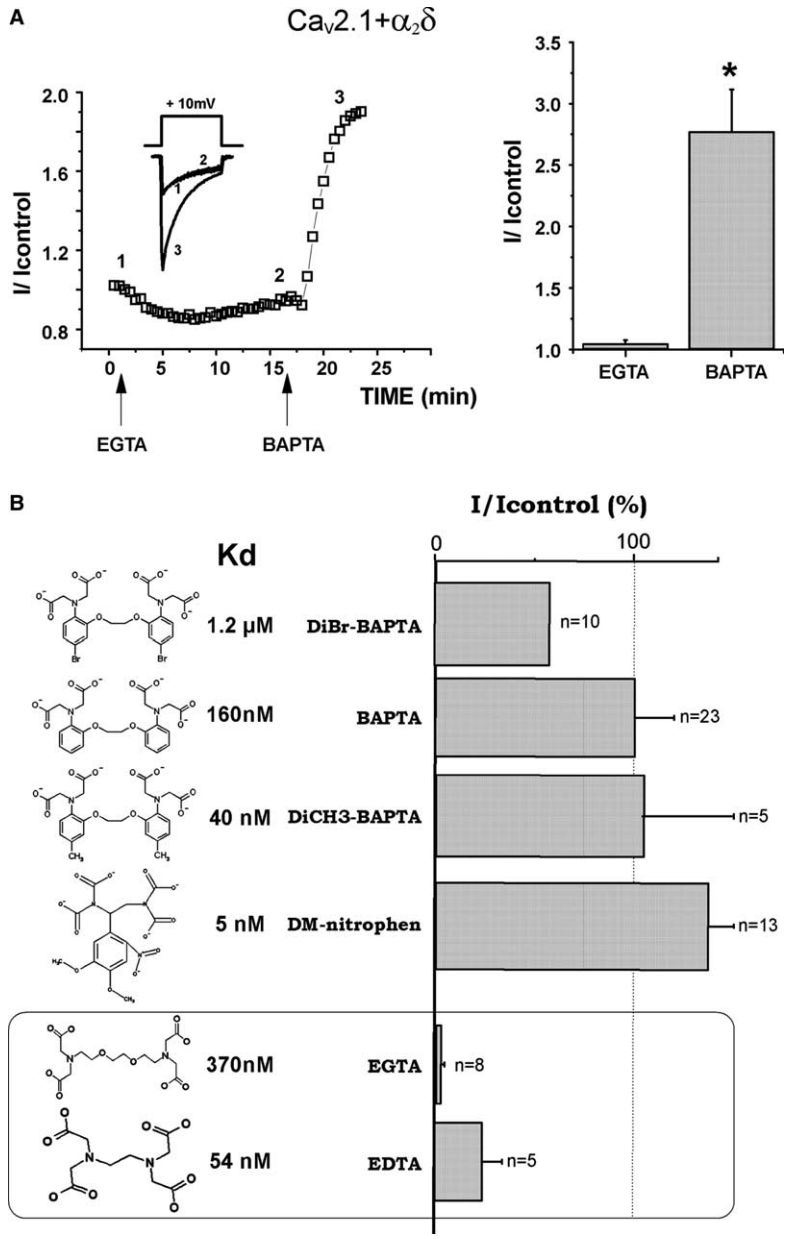

Fig. 1. (A) Ba^{2+} currents recorded using two electrode voltage-clamp in *Xenopus oocytes* injected with $Ca_V2.1 + \alpha_2 - \delta$ subunit cDNAs during depolarizations of 400 ms duration to +10 mV from a holding potential of −80 mV every 15 s. EGTA was injected at 200 mM (2 mM final concentration) by the use of a third microelectrode. BAPTA was injected at 100 mM using a fourth microelectrode. Note that EGTA was without effect, whereas following injection of BAPTA a clear increase in current amplitude was recorded (attributed to the relief of the tonic G-protein block [13]). RIGHT. Averaged increase in Ba^{2+} current amplitude following injection of EGTA or BAPTA. *: significantly different from control current before injection. (B) The same experiment was performed using diBr-BAPTA, DiCH3-BAPTA, DM-nitrophen, EGTA or EDTA instead of BAPTA. In each case the effects on Ba^{2+} current amplitude are shown (mean of 5–20 experiments). Note that EDTA and EGTA do not have a benzene ring in their structure and were ineffective in increasing current amplitude.

2.2. Surface pressure measurements

Adsorption at the air/water interface and penetration studies into lipid monolayers were carried-out according to the Wilhelmy plate method using a Prolabo (Paris, France) tensiometer and an X-Y Kipp and Zonen BD91 recorder (Delft, The Netherlands), as previously described [10]. A solution of the lipids in chloroform/methanol (3/1, v/v) was spread on the air-buffer aqueous solution interface in order to ensure a definite surface pressure. The solvent was then allowed to evaporate and when a constant surface pressure was reached, generally 27–30 mN/m, a value corresponding approximately to the lateral pressure of biological membranes [11], aliquots of the aqueous chelator solution were injected at the desired concentration into the aqueous subphase, which was gently stirred using a magnetic stirrer, and changes in surface pressure were recorded continuously. This procedure was repeated until no further increase of the surface pressure could be detected. Adsorption measurements were thus made at equilibrium [12]. DOPC (1,2-dioleyl-sn-glycero-3-phosphocholine) and DOPG (1,2-dioleyl-sn-glycero-3-phosphoglycerol) at 100/0, 0/100 or 90/10 ratios were used to prepare monolayers of different lipid compositions.

In order to determine the CPI of the chelator into lipids, increases of surface pressure, ΔP, were recorded for different initial lipid surface pressures P_i, and the value of CPI was obtained by extrapolation of the curve for a null ΔP. This CPI corresponds to the maximum initial

pressure at which BAPTA can penetrate the lipid film and is related to the establishment of lipid–BAPTA interactions. A pH 7, 100 mM phosphate buffer (NaH_2PO_4/NaOH) was used for the subphase medium. Recordings using a 10 mM HEPES, pH 7.2, buffer solution gave similar results (not shown). All results are expressed as means ± S.E.M. of *n* independent experiments.

3. Results and discussion

Our first experimental evidence that chelation of intracellular Ca^{2+} by BAPTA is more complex than initially believed came from studies performed on P/Q type Ca^{2+} channels expressed in *Xenopus oocytes* [13]. In this model, the Ca^{2+} channels expressed without their regulatory β subunit are under a tonic inhibition produced by the excess of free Gβγ dimer [14]. This particular type of inhibition could be reversed when BAPTA was injected into the oocyte, an effect that was independent of the fast chelation kinetics of BAPTA [13]. Injection of EGTA using the same experimental protocol could not reproduce the effect of BAPTA (Fig. 1A) suggesting that the two chelators, despite their similar affinity constants for Ca^{2+}, have specific chelating properties. Injection of EGTA was not only ineffective in removing the tonic block imposed by the G-protein, but also unable to prevent the effect of a subsequent injection of BAPTA (Fig. 1A), since the increase in current amplitude recorded after injection of BAPTA was similar whether EGTA was first injected or not (2.2 ± 0.2 and 2.4 ± 0.3, fold respectively). Any pharmacological effect of BAPTA other than Ca^{2+} chelation was discarded in our previous study since pre-loading of BAPTA with Ca^{2+} strongly decreased the removal of the G-protein inhibition [13]. We thus decided to test various chelators with different structures using the same injection protocol, i.e., injection of 40 nl of a 100 mM solution of the chelator during the recording of a Ba^{2+} current elicited by test pulses of 400 ms from −80 to +10 mV every 15 s. The summary of these experiments is shown in Fig. 1B together with the chemical structure of the chelators. Although it was clear that all the chelators (injected at 100 mM, final intra-oocyte concentration 1–2 mM) that display a benzene ring in their structure were effective in increasing the Ba^{2+} current (in a roughly proportional manner with respect to their binding affinity for Ca^{2+}), EGTA and EDTA, two chelators lacking benzene rings in their structure, were almost completely ineffective, even when injected at a higher concentration (200 mM, in Fig. 1B). This structure-activity relationship lead us to propose that these chelators may share, due to their benzene rings, the capability to interact with plasma membranes and thus be able to chelate Ca^{2+} ions at the level of the inner face of the membrane. To test this hypothesis we decided to analyze the effect of these chelators on the surface pressure of lipid monolayers.

We first looked at the surface activity of BAPTA at the air–buffer interface by injecting increasing concentrations of the chelator on the subphase of a lipid-free surface. Starting at 0.5 mM, BAPTA formed stable monolayers by adsoption at the interface (Fig. 2A), as indicated by the increase in surface pressure. This interfacial adsorption reached a surface pressure of 6 mN/m at 1.5 mM of BAPTA after a stabilization period of several minutes, and then remained constant, indicating the formation of a stable monolayer as expected for a surface-active compound. When injected into the stirred subphase of a lipid monolayer (DOPC) at an initial surface pressure of 27

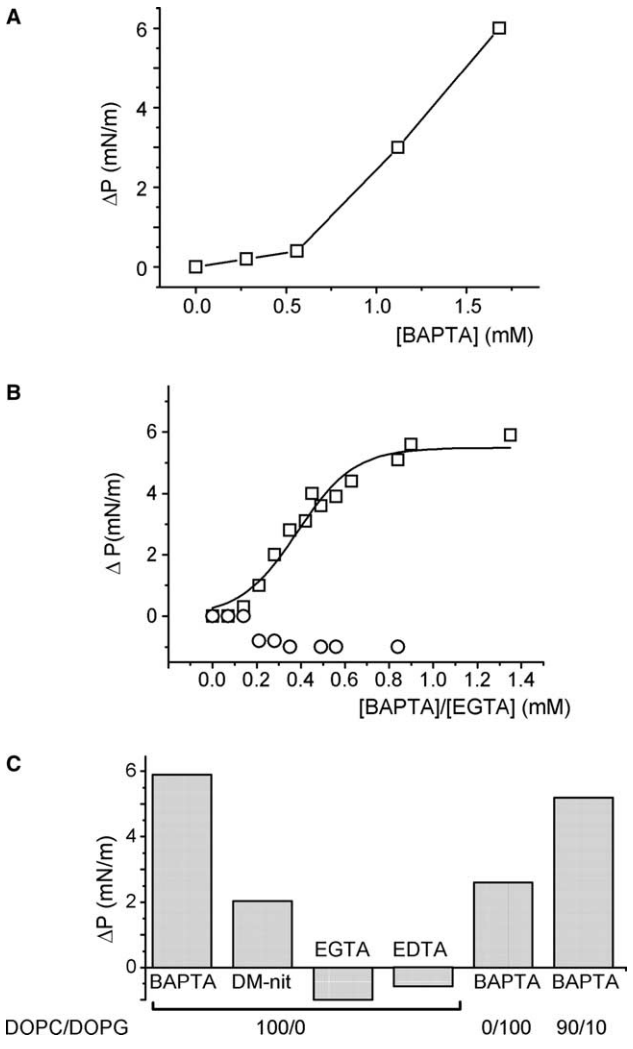

Fig. 2. (A) Adsorption of BAPTA at the air interface by injecting different concentrations of the drug into the subphase. A clear and stable increase in surface pressure was obtained for concentrations larger than 1 mM indicating the formation of a stable monolayer. (B) Injection of increasing concentrations of BAPTA into the subphase of a DOPC monolayer induced a marked increase in surface pressure. EGTA, injected in the same conditions, had no effect. (C) Similar experiments performed with 100% pure DOPC or DOPG, or a 90/10% DOPC/DOPG mixture of lipid monolayers. In the latter more physiological situation, BAPTA was clearly capable of interfacial penetration.

mM/m, BAPTA at 1 mM (close to the expected concentration in oocyte experiments) readily and spontaneously penetrated the DOPC monolayer and increased the surface pressure by about 6 mN/m (Fig. 2B). Thus BAPTA has a thermodynamic tendency to interact with lipid monolayers and to reach a new surface stability through association with lipids of the monolayer. Similar experiments performed with EGTA (1 mM) failed to reveal such surface activity (Fig. 1B), and only a small decrease in the initial pressure (~1 mN/m) was recorded after injection of EGTA in the subphase.

In order to verify the relationship between the surface activity of these chelators and their ability to block the G-protein-dependent inhibition of the $Ca_V2.1$ Ca^{2+} channels, we then injected EDTA or the EDTA derivative DM-nitrophen, both at 1 mM final concentration, in the subphase of DOPC

monolayers. Fig. 2C clearly shows that while DM-nitrophen was perfectly capable of interacting with DOPC lipids, although displaying a smaller activity than BAPTA, EDTA was almost completely devoid of surface activity and no change in surface pressure was recorded after EDTA injection. We concluded that BAPTA and DM-nitrophen were able to penetrate lipid monolayers, while EGTA and EDTA were not, a property in relation with their ability to decrease G-protein inhibition. It should be noted that this insertion occurred at an initial surface pressure of 27 mN/m, suggesting it may also occur in bilayers. Moreover, the differences obtained after injection of BAPTA or DM-nitrophen in the changes in Ca^{2+} current amplitudes (Fig. 1A) and in surface pressures (Fig. 2C) suggested that the functional effect of these substances is modulated by both, their affinity for Ca^{2+} and their capability to penetrate biological membranes, this last effect being dictated by the presence of benzene rings in the EGTA (BAPTA) or EDTA (DM-nitrophen) derivatives.

We then analyzed the specificity of interaction between BAPTA and lipids by measuring the change in surface pressure recorded on lipid monolayers composed of 100% DOPC, 100% DOPG, or a DOPC/DOPG mixture of 90/10. In purely negatively charged phospholipids (100% DOPG, see Fig. 2C), the maximum surface pressure reached after BAPTA injection was clearly smaller than with the zwitterionic phosphatidyl choline DOPC (2.5 versus 6 mN/m, respectively), indicating that negatively charged lipids significantly decreased the penetration of BAPTA into the monolayer, probably via electrostatic repulsion between the negative charges of the lipids and the free form of BAPTA. However, in more "physiological" situations, when the monolayers were composed of a ratio of uncharged/charged lipids of 90/10, BAPTA was able to penetrate the lipid monolayers, inducing an increase in surface pressure close to that obtained in pure DOPC (5 versus 6 mN/N) suggesting that similar interfacial penetration should also be obtained in the Xenopus plasma membrane.

To confirm this hypothesis we measured the interfacial penetration of BAPTA at increasing initial surface pressures of DOPC monolayers, keeping a constant 1 mM BAPTA concentration. This relation allowed us to extrapolate to the abscissa the initial surface pressure at which no penetration occurs CPI. This calculated cut-off pressure is related to the lipid–BAPTA interaction and occurs at 42 mN in our conditions (Fig. 3A), a value larger than the 33 mN/m surface pressure that corresponds to the typical molecule/area of 0.7 nm^2 of fluid bilayers [15]. Together with insertion in DOPC monolayers at an initial pressure of 27 mM/m (see Fig. 2B) these results confirmed that penetration of BAPTA must also occur in lipid bilayers.

All the above experiments were performed with the free form of BAPTA without any Ca^{2+} added. However, it was interesting to analyze the surface activity of the Ca^{2+}-loaded form of BAPTA, to know if BAPTA could stabilize Ca^{2+} ions at the intracellular membrane interface, or, at the opposite, leave the membrane after Ca^{2+} binding. To test this possibility we first injected BAPTA in the stirred subphase of a DOPC monolayer, and recorded an increase in the initial surface pressure from 27 to 36 mN/m (see Fig. 3B). Then increasing quantities of $CaCl_2$ were added to the subphase and the variations of the surface pressure were recorded up to stability. As shown in Fig. 3B, increasing the total Ca^{2+} concentration of the subphase to 150 μM completely reversed the increase in pressure induced by BAPTA, suggesting that the Ca-bound form of BAPTA was not stabilized into the lipid films, leading to a complete decrease of the interfacial penetration. In similar experiments where only Ca^{2+} ions were added without BAPTA, only very small changes in surface pressure were noticed, indicating the lack significant effect of Ca^{2+} on DOPC monolayer surface pressure and stability.

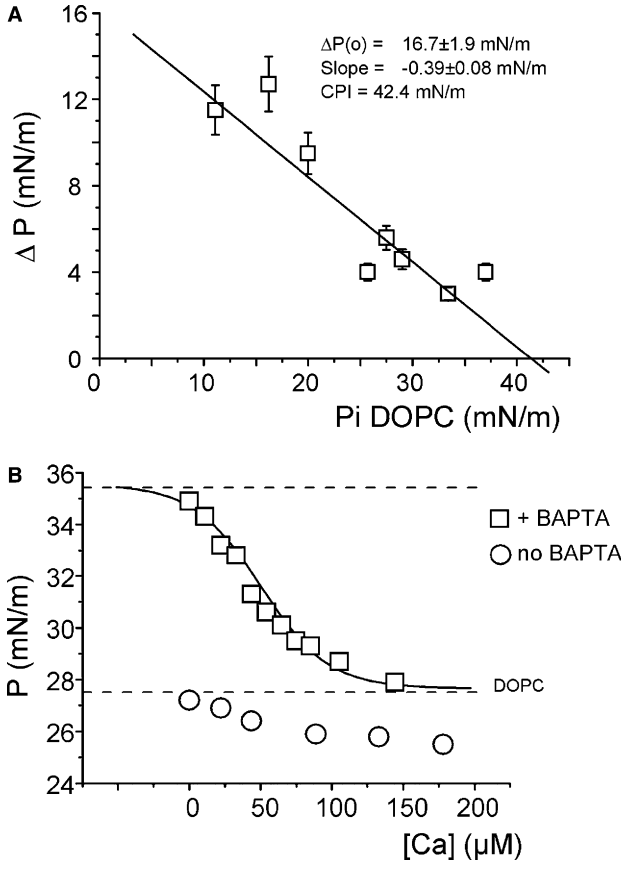

Fig. 3. (A) Dependence of the pressure increase induced by 1 mM BAPTA injection on initial surface pressure. Injection was made into the subphase of the monolayer. Estimated CPI 42.4 mN/m. (B) Changes in surface pressure of spread DOPC monolayer following addition of 1 mM BAPTA and subsequent injection of increasing concentrations of $CaCl_2$. Injection of $CaCl_2$, in the presence of BAPTA (squares), completely reversed the effect of BAPTA, while injection of $CaCl_2$, alone (circles) had no effect. This suggests that the Ca^{2+}-bound form of BAPTA was destabilized in the lipid environment and that it left the monolayer.

These results clearly show that upon binding of a Ca^{2+} ion, the molecule of BAPTA can readily leave the membrane and thus suggest that BAPTA can behave like a shuttle buffer, attached to the membrane in its free form and removing bound Ca^{2+} ion from the cytoplasmic side of the membrane. An important consequence, yet not experimentally tested, of such behavior would be a substantial decrease of the Ca concentration at the inner face of the membrane. This property is not shared by EGTA and may explain some of the specific effects of BAPTA in biological systems where Ca^{2+} compartmentalization and fast and site-specific Ca^{2+} buffering may play an important role in discrimating Ca^{2+} buffers of different biophysical properties. In

the light of these new results, and knowing that *Xenopus oocytes* plasma membranes have a ratio of charged/uncharged lipid close to 90/10 [16], we can suspect that this is what happens for BAPTA and DM-nitrophen when looking at the tonic inhibition of the $Ca_V2.1$ Ca^{2+} channel by G-proteins, where removal of the inhibition was only seen with these chelators and not with EGTA or EDTA. This would suggest that active Ca^{2+} ions for this type of regulation are located in the close vicinity of the membrane and escape the chelation by EGTA. However, we should also kept in mind the fact that specific changes in the mobility or the affinity of these chelators could occur by interactions with cytoplasmic components of large molecular weight [17] and participate to these effects. Interestingly, the effects of BAPTA and DM-nitrophen are not irreversible and some washout of the inhibition slowly occurred with time (>45 min), probably owing to the slow diffusion of injected BAPTA toward intracellular membranes, to its saturation by calcium flowing into the cytosol from Ca^{2+} channels or internal stores [18] or to interactions with cytoplasmic component [17]. A similar mechanism appears to be at work in the interaction of AP-2 proteins with membranes [19], which is specifically inhibited by BAPTA and not EGTA. This novel property of BAPTA can be of prime importance to discriminate the role of membrane-bound versus free Ca^{2+} in physiological processes.

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