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Endogenous *Xenopus*-oocyte Ca-channels are regulated by protein kinases A and C

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Calcium entry into *Xenopus* oocyte occurs mainly through voltage-dependent calcium channels. These channels were characterized as belonging to a particular type of calcium channel insensitive to dihydropyridines, ω-conotoxin, and *Agelenopsis aperta* venom, but blocked by divalent cations (Co, Cd, Ni). Intracellular injection of cAMP, or bath application of phorbol ester, induced a marked increase in calcium current amplitude and a slowing of the inactivation time-course. Despite their different pharmacology, endogenous calcium channels, like cardiac or neuronal calcium channels, could be thus regulated by protein kinases A and C.

Calcium channel; Oocyte; Phosphorylation; Protein kinase A; Protein kinase C

1. INTRODUCTION

Xenopus oocytes have been developed as an efficient expression system in the early 70's [1]. They are now widely used to express soluble proteins as well as membrane receptors and ion channels after injection of either total (or poly $(A)^+$) or in vitro transcribed RNA from cloned proteins [2]. One of the most interesting features of the *Xenopus* oocyte to study ion channels is the fact that it possesses only a few endogenous conductances, especially when follicular cells are enzymatically removed [3,4]. However several groups have reported the existence of different conductances to Cl^-, Na^+, K^+, and even Ca^{2+} [5–12]. Such conductances can constitute disturbing background ionic 'noise', when one wants to record ionic current from expressed, exogenous RNA-directed, channels. This is especially true for the voltage-dependent calcium channels (VDCC) which belong to a different class than usual Ca channels found in other tissue, as evidenced by their specific pharmacology characterized by the absence of potent inhibitors. Dascal et al. [11], Moorman et al. [10], Lory et al. [13] have reported that these Ca channels are blocked neither by dihydropyridine (DHP) nor by the usual toxins used to differentiate VDCC (ω-conotoxin). Such observations indicate that endogenous calcium channels could represent a new class of VDCC. Moreover, up to now, no study has focussed on the intracellular regulation of these channels despite the fact that regulations of Ca channels expressed from heart or brain RNA, [11,14] have been performed in *Xenopus* oocytes. It has also been reported that these oocytes possess the complete intracellular machinery for protein phosphorylation either by kinases A or C [2]. Indeed, study on the regulation of cloned channels after injection of cRNA coding for Na or K channels, and therefore without injection of messenger RNA for kinases, has already been performed [15–17]. It thus appeared that a more precise characterization of the pharmacological properties and regulation of these *Xenopus* oocyte VDCCs needed to be realized.

Abbreviations: VDCC, voltage-dependent calcium channels; DHP, dihydropyridine; PKA, protein kinase A; PKC, protein kinase C; Ag.A.V., Agelenopsis aperta venom; DAG, diacylclycerol; PMA, phorbol 12-myristate 13-acetate; OAG, oleyl-acetyl-glycerol; IBMX, 3-isobutyl 1-methyl xanthine; PDBU, phorbol 12,13-dibutyrate.

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2. MATERIAL AND METHODS

2.1. *Oocyte preparation*

Oocytes were dissected away from tricaine methane sulfonate-anesthetized female *Xenopus laevis* (from CRBM, Montpellier, France) and prepared as reported elsewhere [13]. Stage V and VI oocytes were selected for electrophysiological measurements. Oocytes could be maintained for 2–6 days at 20°C in a medium containing (in mM): NaCl 96, KCl 2, $MgCl_2$ 2, $CaCl_2$ 1.8, Na-pyruvate 2.5, HEPES 5, pH 7.4 with NaOH; and supplemented with 50 μg/ml gentamicin. Incubation medium was renewed daily.

2.2. *Electrophysiological measurements*

Electrophysiological measurements were performed using the standard two microelectrode voltage-clamp technique with the TEV-200 Cornerstone amplifier (Dagan Instruments, Minneapolis, MN). Oocytes were tested 1–5 days after isolation, no significant differences were seen during this period. In most experiments the holding voltage was −80 mV. Stimulation of the preparation, data acquisition and analysis were performed using the pCLAMP software (ver. 5.5, Axon instrument, Burlingame, CA). Oocytes were placed in a recording

chamber (200 μl) and impaled with 3 M CsCl-filled electrodes (0.1–0.5 MΩ) to suppress endogenous K current. Drugs were applied externally by addition to the superfusate (gravity-driven superfusion). For intracellular injection, oocytes were impaled with a third additional micropipette (3–10 μM in tip diameter). The injection volume was 2–5% of the entire cell volume (1 μl) and all injected compounds were made up in water. Experiments were made at room temperature.

Calcium-activated chloride currents ($I_{Cl(Ca)}$) were measured in a saline solution of the following composition (in mM): NaCl 96, KCl 2, $CaCl_2$ 1,8, $MgCl_2$ 2, HEPES 5, pH adjusted to 7.4 with NaOH. To record Ca channel activity, oocytes were routinely tested in the following BAMS medium (in mM): BaOH 40, NaOH 50, CsOH 2, HEPES 5, pH adjusted to 7.4 with methane sulphonic acid. All Ba currents were leak-subtracted using current traces recorded in the presence of Cd^{2+} (1 mM).

2.3. *Drugs*

All chemicals were from Sigma (St. Louis, MI). Bay-K 8644 and nicardipine were kindly provide by Sandoz-France, and stock solutions (10 mM) were made up in ethanol. Amiloride, ω-conotoxin and funnel-web spider venom (Ag.A.V.; from *Agelenopsis aperta*) were purchased from Sigma, RBI (Natick, MA) and Latoxan-France, respectively; stock solutions were made up in water. All final concentrations were obtained by appropriate dilution in BAMS solution. Peptidic protein kinase inhibitors, A-PKi, an 18 amino acid peptide, were synthesized at the CRBM (CNRS, Montpellier, France).

2.4. *Analysis*

Values are expressed as mean ± SE (number of different oocytes from the same donor). Current–voltage relations (Fig. 2 and 3) were normalized according to the peak current obtained in control conditions (i.e. before drug application, generally at +10 mV). Time-course of cAMP and PKC effects were normalized according to the current obtained at equilibrium to allow comparison between the two processes (Fig. 4). In both cases real current amplitudes are given in the text. Inactivation time courses were fitted to single exponential decay using non-linear regression.

3. RESULTS AND DISCUSSION

Endogeneous *Xenopus* oocyte VDCC activity could be visualized in normal saline solution by activation of a Ca-activated chloride current. This current displayed little amplitude variation when studied on oocytes from the same frog, but could vary widely on oocytes from different donors. The variation was due, at least in part, to variations in the amplitude of the endogenous Ca current which is the primary trigger of the Cl conductance. Indeed, when VDCC activity was recorded in BAMS solution, similar variations were obtained. For example, the Ba current (I_{Ba}) recorded during a test pulse to +10 mV, from a holding potential of –80 mV, varied from 12 ± 2 nA (n=7) to 33 ± 2 nA (n = 8) when obtained from oocytes of two different frogs. In 50% of the frogs, no inward Ba current could be recorded at all. Any role of the oocyte outward K^+ current in this variance can be ruled out since we used Cs^+ (instead of K^+) in the perfusing solution (BAMS), and in the recording electrodes. Moreover, these values of I_{Ba} were obtained from Cd-substracted traces. The wide range of current amplitudes probably reflects the different metabolic stages of the oocytes between different frogs. Interestingly, all Ba currents, regardless of their respective

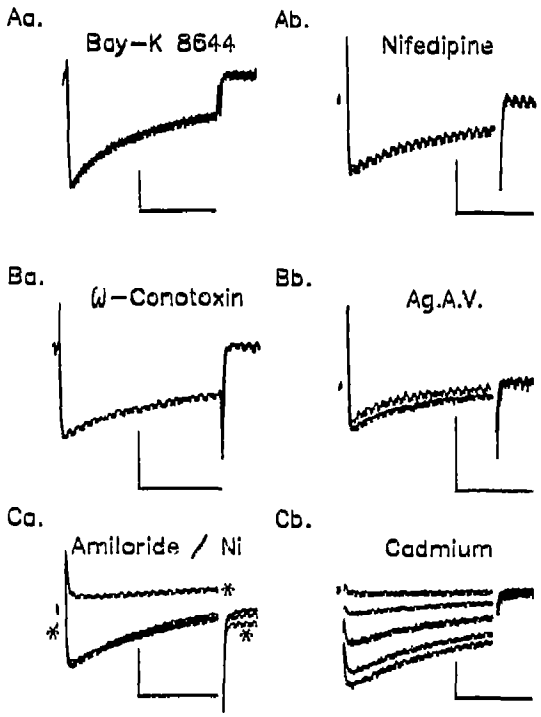


Fig. 1. Pharmacology of endogenous VDCC. (A) Absence of effect of DHP agonist (Aa; Bay-K 8644, 1 μM) and antagonist (Ab; nicardipine, 10 μM). Traces before and after drug application superimposed. (B) Endogenous VDCC was blocked neither by ω conotoxin (Ba; 1 μM), nor by crude Ag.A.V. (Bb; 1/1000 dilution, upper trace, 10% block). (Ca) Absence of effect of amiloride (10 μM), and nickel (10 μM) on control endogenous Ba current (three traces superimposed). However, the current could be blocked with higher nickel doses (1 mM, trace marked with *). (Cb) Effects of various concentrations of cadmium (1, 10, 100, 1000 μM, traces of increasing amplitude) on control Ba current (bottom trace). The dose for 50% inhibition is 10 μM. All currents were recorded in BAMS solution using voltage steps to +10 mV from a holding potential of –80 mV. Currents were cadmium subtracted, except for (Cb) where a P/4 leak-subtraction was used. Scale bar: 10 nA and 200 ms.

amplitude, always displayed the same pharmacological profile (see below), suggesting changes in the expression of a homogeneous population of VDCC, instead of different populations of channels with different pharmacological properties, as was seen in other preparations. Consistently, the inactivation time-course was not dependent upon current amplitude, and comparable inactivation time-constants were found for Ba currents of different amplitude (235 ± 48 ms, and 286 ± 57 ms, for the two batches of oocytes described previously). When all detectable endogenous Ba currents were pooled together, the mean inactivation time-constant was 250 ± 70 (at +10 mV, n=35). From these results we can state that oocytes display only a single class of calcium channel. Further characterization of the endogenous VDCC has been focussed on: (i) the effect of various 'classical' calcium channel inhibitors used to separate the different types of VDCC; and (ii) the regulation of the endogenous VDCC by protein kinases A and C.

Neither DHP antagonist (or agonist, 10 μm, Fig.

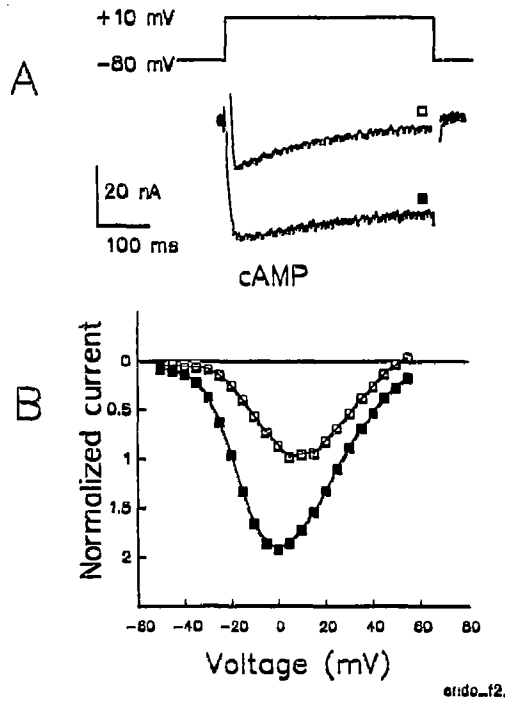


Fig. 2. Effect of intracellular injection of cAMP. (A) Current traces before (open squares) and 5 min after intracellular injection of cAMP (50 pmol, filled square). (B) Current–voltage curve for control and cAMP-stimulated Ba current. The holding potential was –80 mV, and voltage steps were generated from –50 mV to +50 mV with a 5 mV increment. Note the leftward shift of the *I–V* curve. All recording conditions were similar to Fig. 1.

1Aa, 1Ab), nor ω-conotoxin (1 μM; Fig. 1Ba) blocked (or potentiated) the oocyte endogenous VDCC. Perfusion of *Agelenopsis aperta* venom (Ag.A.V.; 1/1000 dilution) induced a slight block of the endogenous Ba current (13 ± 8%, n = 8; Fig. 1Bb), and was sometimes without effect (n=3). The same venom has been shown to block more than 70% of rat brain mRNA-directed Ca channels [18], or Ca channels expressed after the injection of RB1 cRNA, coding for a P type Ca channel [19]. However, the endogenous Ba current could be reversibly blocked by well-known inorganic Ca blockers (Cd^{2+} or Ni^{2+} at 1 mM, Fig. 1Cb; Co^{2+} not shown). Endogenous VDCC can thus be considered as a particular type of Ca channel different from the L type, N type and P type (for the pharmacology of these types see [18,20,21]). From the slow inactivation time constant and the high threshold potential for activation (see current–voltage curve Fig. 2B, for example), it seems also unlikely that it belongs to the T type. Consistently, endogenous VDCCs were not blocked by 10 μM Ni, but could be inhibited at higher (1 mM) doses, and amiloride was without effect (10 μM; Fig. 1Ca). Among the different types of Ca channels, the L and N type have been shown to be the target of various protein kinases [18]. In the case of the protein kinases A and C, functional effects of phosphorylation resulted in increased channel availability and/or open time [21,22].

When 50 pmol of cAMP are injected into a control oocyte, the inward Ba current slowly increased to a new steady level from 24 ± 7 nA to 39 ± 5 nA (n=8) in around 5 min (Figs. 2A and 4A). This potentiation occurred immediately after the injection of cAMP, and developed concomitantly to a slowing of the inactivation time-course. When fitted with a single exponential, the inactivation time constant (at +10 mV) increased from 240 ± 60 ms to 521 ± 240ms (n=10). This effect was prevented by prior injection of the specific peptidic PKA inhibitor (A-PKi; [23]) showing the involvement of the protein kinase A (not shown). The mean normalized current–voltage curves before and after cAMP injection are shown in Fig. 2B. The maximum of the current–voltage curve was shifted by 10 mV in the hyperpolarized direction after cAMP injection, and consequently, the increase was more pronounced for small depolarizations. For example, I_{Ba} was potentiated by 3-fold at –10 mV and only by 1.6-fold at +10 mV. The regulation by cAMP of the endogenous VDCC appeared similar to the regulation of expressed of native cardiac [22,24] calcium channels. Moreover, cAMP-potentiated Ba currents exhibited a pharmacology similar to unstimulated VDCC with regard to toxin, DHP inhibition and divalent cation block (not shown). Similar increase was obtained after perfusion of the phosphodiesterase (PDE) inhibitor IBMX (1 mM; not shown), suggesting that the use of the PDE-inhibitor theophylline ([11], to prevent oocyte maturation) could lead to underestimation of expressed (or endogenous) ion channel regulation by cAMP.

Another major regulatory pathway of the cardiac or neuronal VDCC is the activation of phospholipase C which leads to production of diacylglycerol (DAG), and activation of protein kinase C [22]. Such activation promotes channel phosphorylation producing an increase in the channel activity seen on macroscopic and single-channel recordings in native cells [22,25,26] or after mRNA expression in oocytes ([27]; Bourinet, E., Fournier, F., Lory, P., Charnet, P., and Nargeot, J., unpublished observations). Direct activation of PKC using extracellular application of either an analog of DAG (oleyl-acetyl-glycerol: OAG) or a phorbol ester (phorbol 12-myrystate 13-acetate: PMA) produced in both cases an increase in the endogenous VDCC activity. The potentation produced by 2 μM OAG is shown on Fig. 3Aa for a test-pulse to +10 mV. Basal Ba current increased from –24 ± 5 nA to –34 ± 3 nA (n=4) after perfusion of the drug. This effect developed with the same kinetics as with cAMP and steady-state potentation was usually obtained 5–10 min after the beginning of the perfusion. As for cAMP, the OAG-induced potentation was voltage-dependent, and a 10 mV hyperpolarizing shift of the maximum of the current-voltage curve was obtained (Fig. 3Ab). The mean current increased by 220% at –10 mV, 40% at +10 mV, while no effect was seen for potentials greater than +20 mV. Moreover, a slight slowing of the inactivation time-

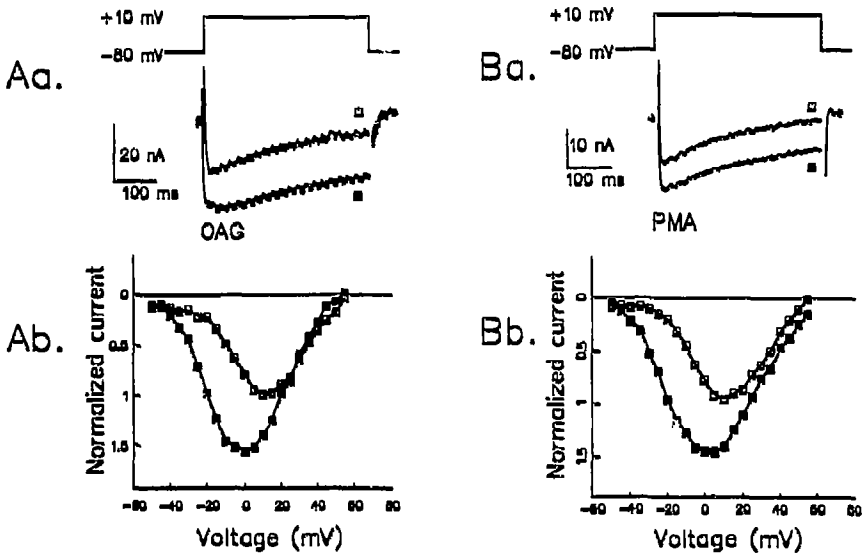


Fig. 3. Effect of PKC activation. (A) I_{Ba} stimulation induced by OAG. (Aa) Current traces obtained before (open squares) and 15 min after (filled squares) perfusion of 2 μM of OAG. (Ab) Current–voltage relationship for I_{Ba} before and after perfusion of OAG. The holding potential was –80 mV. (Ba) PMA (300 nM, filled squares) has similar effects to OAG on endogenous current (open squares). Note in both cases a slowing of the inactivation time-course. (Bb) Current-voltage relationships for I_{Ba} before (open squares), and after (filled squares) perfusion of PMA (300 nM). Note the greater potentiation for small voltage steps.

course was also noted (from 175 ± 26 ms to 285 ± 140 ms, n=4).

Using phorbol ester (PMA, 300 nM) instead of an analog of DAG to stimulate the protein kinase C produced the same effects. Basal endogenous current was potentiated from –28 ± 6 nA to –41 ± 9 nA (n=9; see Fig. 3 Ba), and inactivation was slowed (from 244 ± 77 ms to 450 ± 171 ms, n=15). A similar shift in the current–voltage relationship was obtained (Fig. 3Bb) toward hyperpolarizing potentials. The current increased by 200% when the voltage was stepped up to –10 mV, but only by 61% for voltage steps up to +10 mV. PDBU (300 nM), another phorbol ester activating PKC, produced qualitatively and quantitatively the same effects (not shown). It thus appeared that PKC activation, using phorbol esters or analogs of DAG, potentiated the oocyte calcium channel activity in a similar way to cAMP. First the kinetics of the increase were identical (see Fig. 4A), and second, both induced a slowing of the inactivation time constant. Isoprenaline (or activation of protein kinase A) has been shown to change the inactivation time constant of cardiac calcium channels when Ba ions are the charge carrier, this effect resulting from an increase in the reopening probability of the channel during the test pulse [22]. Similar observations have not yet been made during PKC stimulation of either neuronal or cardiac calcium channels [27]. cAMP-induced potentation of Ba current was on average (using oocytes from the same batch) greater than PKC-induced potentation (65% vs. 45% at +10 mV; n=9 and 10, respectively), both at supramaximal concentration. Moreover, while the stimulations were similar during the first 5–10 min, prolonged

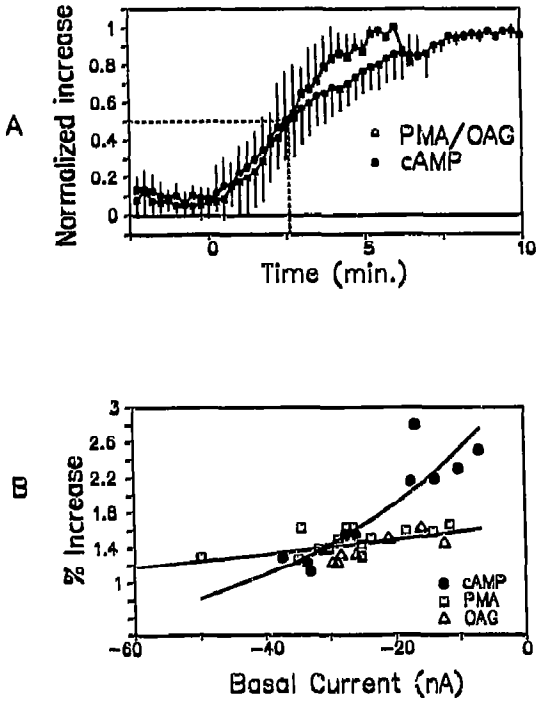


Fig. 4. (A) Time-course of protein kinase induced-stimulation. Effect of cAMP (50 pmol injected, filled circles), OAG (2 μM) or PMA (300 nM, open squares) on IBA amplitude at various times after application. The PKA and PKC stimulation produced a similar potentiation, with a half-maximal effect reached in about 2–3 min. All currents were normalized according to the maximal current obtained at the steady-state effect of the drug. (B) cAMP-induced potentiation is dependent upon basal current amplitude. The percentage of increase induced by PKA (cAMP, filled circles) and PKC (OAG, open triangles, and PMA open squares) activators is plotted against the current amplitude before drug application. Note the variability of the control current, and the relation between cAMP-induced stimulation of the size of the control current. PKC-induced stimulation did not vary with current amplitude. Lines are drawn through the points to illustrate the trends.

application of PKC agonists induced a marked run-down of the Ba current not obtained with long application of PKA agonist (not shown). A similar run-down has been observed on heart RNA-injected oocytes (Bourinet et al., unpublished observations) and on cardiac myocytes [26], and is attributed to a down-regulation of the protein kinase C, and/or membrane internalization [30]. We have also found that effects of PKA and PKC were additive, i.e. prior injection of cAMP produced an increase in calcium entry evidenced by an increase in $I_{Cl(Ca)}$, and subsequent application of PMA gave rise to an additional potentation of the chloride conductance (not shown).

It could be asked whether such regulations have any physiological relevance. It is noteworthy that the cAMP effect on endogenous VDCC seemed to be related to the amplitude of the basal Ba current: large currents were less sensitive to cAMP, whereas PKC-stimulation resulted in a rather constant increase, regardless of the basal current amplitude (Fig. 4B). Indeed cAMP concentration has been shown to play a critical role during oocyte maturation, and its level is regulated by G-protein-coupled progesterone receptors. At rest, intraoocyte cAMP concentration is 1–2 pM per oocyte [28,29], with large fluctuations from one frog to another. This could explain the variation of the amplitude of the basal Ba current in oocytes from different animals. We observed that the absolute amplitude I_{Ba} can reach in the presence of cAMP (using supramaximal concentration) was not dependent on the value of the basal current (Fig. 4B), as if the total number of channels available for opening after cAMP action was similar on all oocytes.

4. CONCLUSIONS

Xenopus oocytes possess a variable pool of endogenous VDCC which pharmacologically is clearly different from the previously-described T, L, N and P type calcium channels ([10,13] and this work). However, these endogenous (high threshold) channels share some similarities with the L type when PKA- or PKC-dependent regulation is considered. Basal PKA activity seems to control the level of Ca entry into stage VI *Xenopus* oocytes. To conclude, it should be noted that such regulation of endogenous VDCC could constitute a drawback to study the regulation of expressed Ca channels. However, our work has been conducted on oocytes displaying unusually large (>10 nA) endogenous Ba currents. Most of the oocytes did not possess such Ca channel activity, even after cAMP injection. In some batches of oocytes, devoid of Ba current, prior injection of cAMP (50 pM), purified protein kinase A holoenzyme (0.25 μg/oocyte), or both, did not produce any current (n=5; not shown), suggesting that another regulation at the transcriptional and/or translational level may exist.

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