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Regulation by protein kinase-C of putative P-type Ca channels expressed in *Xenopus* oocytes from cerebellar mRNA

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Xenopus oocytes injected with rat cerebellar mRNA expressed functional voltage-dependent Ca channels detected as an inward Ba current (I_{Ba}). The pharmacological resistance to dihydropyridines and ω-conotoxin together with the blockade obtained with *Agelenopsis aperta* venom suggest that these channels could be somehow assimilated to P-type Ca channels. The precise nature of the transplanted Ca channels was assessed by hybrid-arrest experiments using a specific oligonucleotide antisense-derivated from the recently cloned α1-subunit of P channels (BI-1 clone). In addition, we demonstrate that exogenous Ca channel activity was enhanced by two different PKC activators (a phorbol ester and a structural analog to diacylglycerol). The general electrophysiological and pharmacological properties of the stimulated Ca channels remain unchanged. This potentiation induced by PKC activators is antagonized by a PKC inhibitor (staurosporine) and by a monoclonal antibody directed against PKC. It is concluded that P-type Ca channels are potentially regulated by PKC phosphorylation and the functional relevance of this intracellular pathway is discussed.

Xenopus oocyte; Cerebellar mRNA; P-type Ca channel; Protein kinase-C

1. INTRODUCTION

Four types of voltage-dependent Ca channels were described as T-, N-, L- and P-types according to their electrophysiological and pharmacological properties [1,2]. The recently described P channel is resistant to ω-conotoxin (ω-CgTx) and to dihydropyridines [3]. However, the activity of this channel is strongly inhibited by several purified toxins; the funnel web spider toxin [4], the ω-Aga-IVA toxin [5] and the MVIIC-conotoxin [6], used now for its structural characterization and to define its functional roles. P-type Ca channels are widespread throughout the central nervous system (for review see [7]) and subsequently they may be involved in numerous synaptic functions by increasing intracellular calcium [3,8]. These channels are preferentially located in the neurons of the cerebellar cortex where they participate especially in the generation of Purkinje cell action potential [4]. Up to the present time, while the α1-subunit of the P-type Ca channel has already been cloned [8,9] and pharmacologically studied, any regulatory mechanism of this channel by intracellular second messengers has not been described as yet. To our knowledge, this paper is the first demonstration for a possible regulation of P-type Ca channels since the present data shed light on a positive modulation of these channels by protein kinase-C. These data have been obtained on putative P-type Ca channels expressed from cerebellar mRNA in the suitable *Xenopus* oocyte model.

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2. EXPERIMENTAL

RNA was isolated from 16-day-old rat cerebellum using a modified extraction procedure derived from the technique of Chomsinski and Sacchi [10]. PolyA$^+$ were purified using a standard procedure [11]. Preparation, maintenance and injections of *Xenopus* oocytes were performed as previsouly described [12]. Electrophysiological recordings were performed using the standard two microelectrode voltage-clamp technique with a virtual ground circuit. Oocytes were impaled with these electrodes (0.1–0.5 MΩ) filled with 3 M KCl. After a stable resting potential was achieved, oocytes were voltage-clamped at −80 mV. The recording solution of Ca channel activity was a modified BaMS medium (in mM: $Ba(OH)_2$ 40, NaOH 50, CsOH 2, HEPES 5, pH 7.4). Nifedipine, ω-CgTx, funnel web spider venom from *Agelenopsis aperta* (Aga.V.), phorbol 12-myristate 13-acetate (PMA), 1-oleyl-2-acetyl-rac-glycerol (OAG) were applied directly to the superfusate. Staurosporine (STAU) was applied before electrophysiological measurements for about 10 min. Drugs were purchased from Sigma (St. Louis, USA) and Aga.V. from Latoxan, France and Spider-Farm, USA. The monoclonal protein kinase-C antibody (PKCAb, clone 1–9) was purchased from Boehringer Mannheim. It was applied intracellulary using a third micropipette. The volume injected was 5% of the entire oocyte volume estimated at 1 μl, leading to a final intracellular concentration of about 25 μg/ml. For hybrid-arrest experiments, we used the 21mers nucleotide antisense (ASP) which has been chemically

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synthetized (Eurogentecs, Belgium) in regard to the intracytoplasmic loop located between the II and III domains of the BI-1 clone recently isolated [8]. Its sequence targets the segment between the 2313 and 2333 nucleotides of the α1-subunit. ASP was co-injected with cerebellar mRNA to a final intracellular concentration of about 25 μg/ml. Experiments, data acquisition and analysis were performed using P-clamp software, version 5.5 (Axon Instruments, USA). Results are expressed as means ± S.E.M. with the number (n) of experiments.

3. RESULTS AND DISCUSSION

In oocytes injected with cerebellar mRNA, appropriate depolarizing pulses evoked a large inward barium current (I_{Ba}) in BaMS medium. The voltage-activation threshold for I_{Ba} was −32.6 ± 1.17 mV (n = 15) and the peak current was at 10.33 ± 1.79 mV (n = 15). These electrophysiological characteristics of control I_{Ba} are illustrated on I/V curves in Fig. 2A and B. I_{Ba} was resistant to both dihydropyridine antagonist (Nifedipine) and ω-CgTx (Fig. 1A). This first step gives reasonable evidence that I_{Ba} was not due to the activation of L- or N-type Ca channels. By contrast, application of crude Aga.V. markedly reduced I_{Ba} (Fig. 1B). The inhibition was more potent by lowering the external concentration of the divalent cation (Ba^{2+}). It was 61.8 ± 7.3% (n = 8)

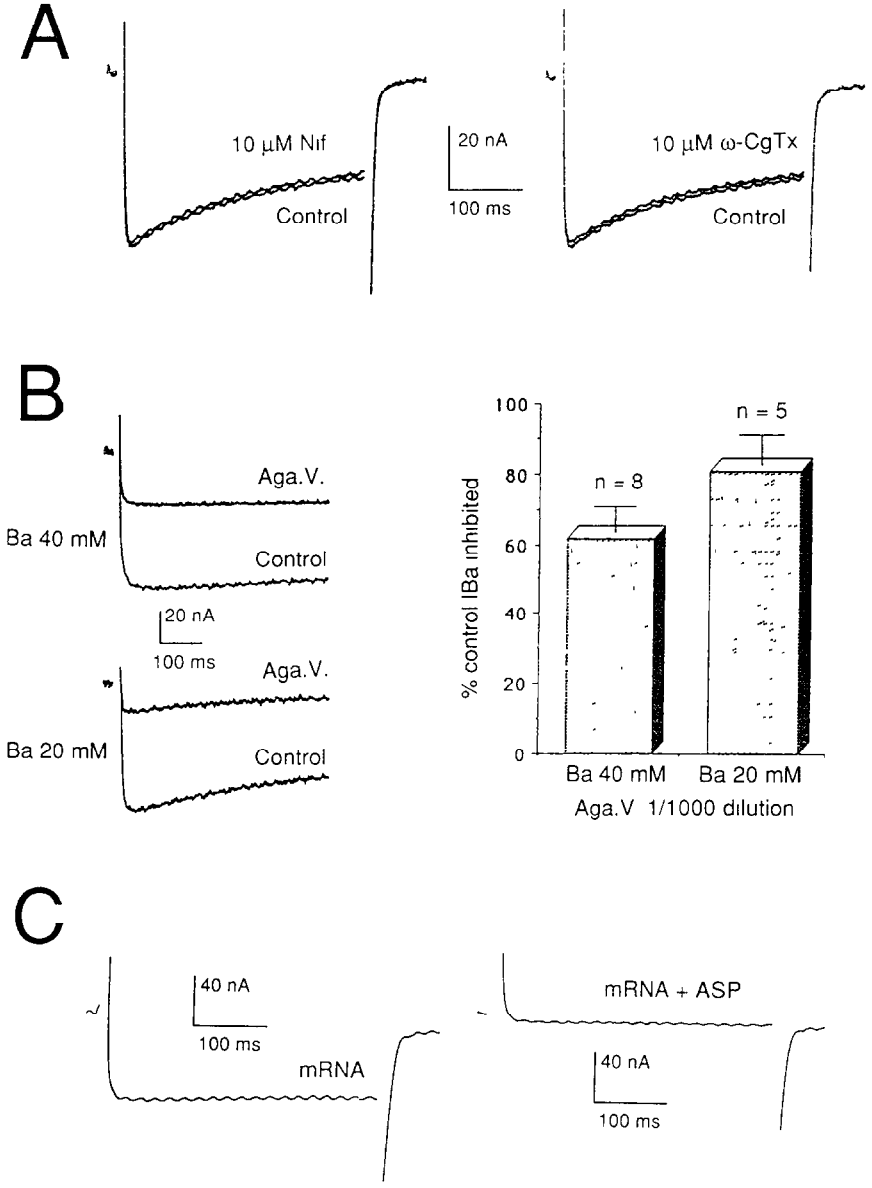

Fig. 1. Characterization of I_{Ba}. (A) I_{Ba} in control and in the presence of Nifedipine (Nif, 10 μM) or ω-conotoxin (ω-CgTx, 10 μM). (B) I_{Ba} in control and in the presence of *Agelenopsis aperta* venom (Aga.V., 1/1000 dilution) with two different concentrations of Ba^{2+} (20 and 40 mM) in the bathing solution. Current inhibited by Aga.V. was expressed as a fraction (mean + S.E.M.) of the initial control current measured in both bathing solutions. (C) I_{Ba} obtained in oocytes injected with mRNA alone or with mRNA plus oligonucleotide antisense (ASP, final concentration 25 μg/ml).

and 81 ± 8% (n = 5) in 40 mM and 20 mM Ba^{2+} concentrations, respectively. Similar results were previously reported [13] concerning I_{Ba} recorded in oocytes injected with whole brain mRNA. The results obtained by Lin et al. [13] and others [14] were considered conclusive enough to put forward the proposal that mRNA from the mammal central nervous system expressed putative P-type Ca channels. In our experiments, the inhibition of I_{Ba} in the presence of Aga.V. combined with the lack of effect of L- and N-type Ca channel blockers demonstrate the expression of such Ca channels. Nevertheless, Aga.V. contains antagonists of L-, N- and P-type Ca channels [15] and the real subset of Ca channel expressed cannot be precisely determined using only the crude form of the venom. Recently, Kondo et al. [14] have pointed out that oocytes injected with cerebellar mRNA displayed an I_{Ba} substantially blocked by the specific P-type Ca channel antagonist, ω-Aga.IVA. First, this last result confirms previous data suggesting that P-type Ca channels were preferentially located in the cerebellar cortex [4], and second, this strongly suggests that Ca channels expressed in the oocyte from cerebellar mRNA do belong to the P-type. Nevertheless, to further clarify the type of Ca channels expressed in our study, the hybrid-arrest method was introduced. When the oligonucleotide antisense (ASP) was co-injected with cerebellar mRNA, the expression of I_{Ba} was strongly repressed (78 ± 8% inhibition, n = 10, Fig. 1C). In oocytes, the BI clone has been shown to direct an expression of an I_{Ba} sensitive to ω-Aga.IVA [16]. Thus, the BI clone corresponds to the functional α1-subunit of P-type Ca channel. According to these data, and to obtain an oligonucleotide antisense specific to the P-type Ca channel, ASP was produced from a particular nucleotide sequence of BI-1. This sequence is highly specific since it is not found in the α1-subunit of the cardiac [17] or skeletal L-type Ca channel [18] or of the N-type Ca channel of the human brain [19]. The present results obtained with ASP point out the hypothesis that Ca channels expressed in oocytes from cerebellar mRNA fit in with the features of native P-type Ca channels. Moreover, data obtained in control experiments revealed that the expression in oocytes of kainate receptors, cloned nicotinic receptors or cardiac L-type Ca channels was not affected by ASP. Indeed, for kainate receptors, nicotinic receptors and cardiac I_{Ba}, the control current was 147 ± 27.2 nA (n = 4), 3.6 ± 0.4 μA (n = 7) and 54.5 ± 12.1 nA (n = 5), respectively versus

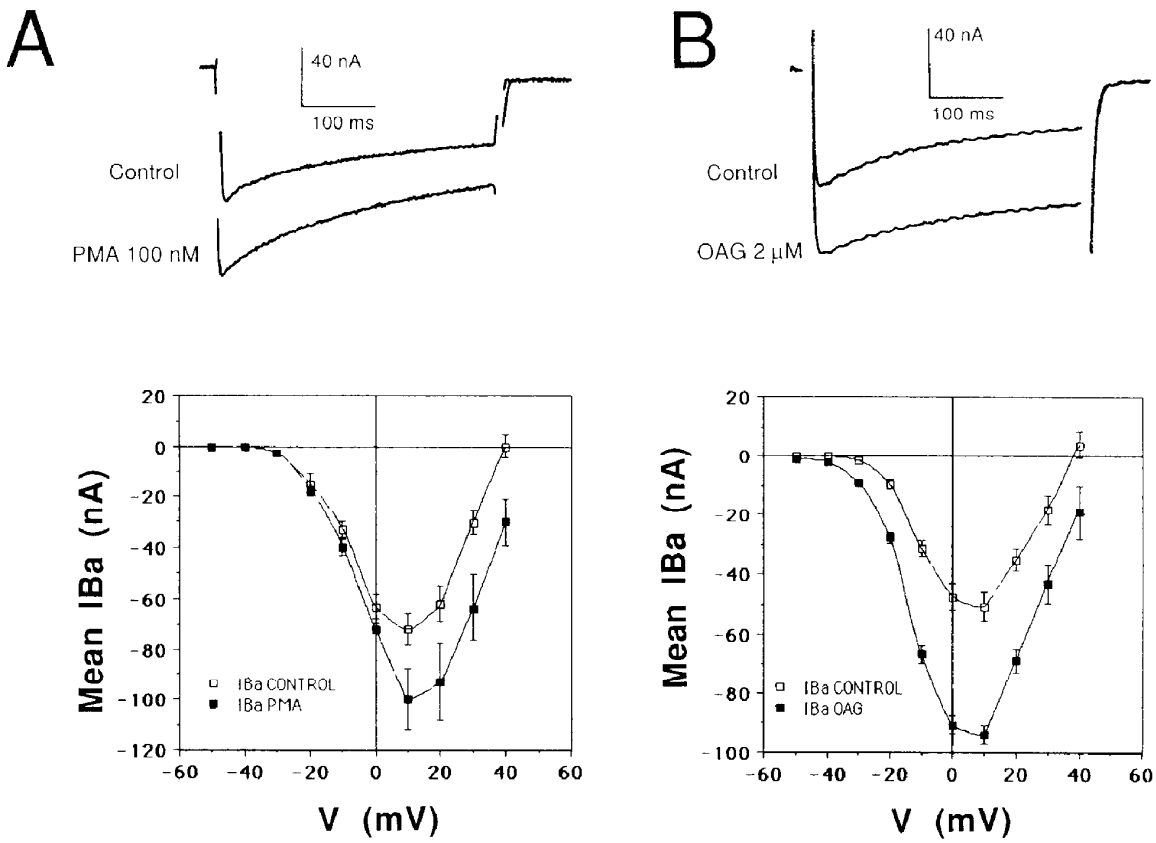

Fig. 2. Modification of I_{Ba} by PKC activators. (A) I_{Ba} in control and in the presence of phorbol 12-myristate 13-acetate (PMA, 100 nM) and current–voltage relationships (n=5). (B) I_{Ba} in control and in the presence of 1-oleyl 2-acetyl-rac-glycerol (OAG, 2 μM) and current–voltage relationships (n=4).

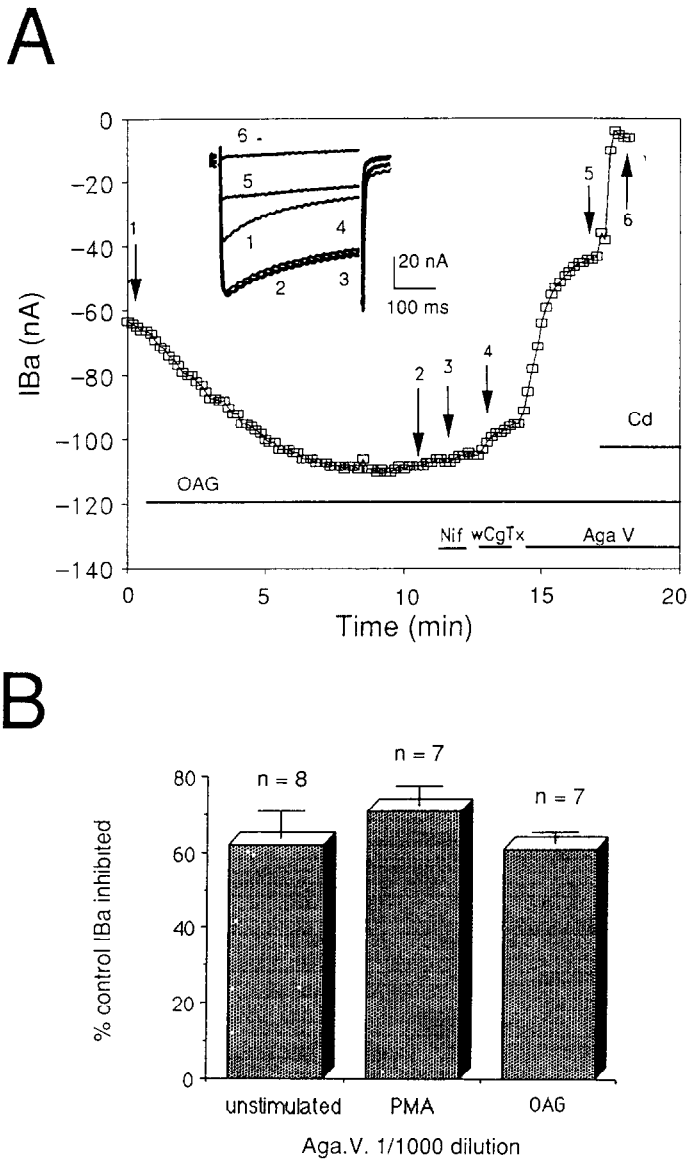

Fig. 3. Effects of calcium blockers on the I_{Ba} modified by PKC activators. (A) I_{Ba} in control (1), in the presence of OAG (2, 2μM), OAG plus Nif (3, 10μM), OAG plus ω-CgTx (4, 10μM), OAG plus Aga.V. (5, 1/1000 dilution) and OAG plus Cd^{2+} (6, 300μM). (B) I_{Ba} inhibited by Aga.V. was expressed as a fraction of the initial current measured in control condition (unstimulated), in the presence of PMA (100 nM) or in the presence of OAG (2 μM).

133.3 ± 45.3 nA (n = 3), 3.7 ± 0.6 μA (n = 7) and 51 ± 13.3 nA (n = 5) with ASP. This indicates that the α1-subunit RNA of P-type Ca channels is the specific target of ASP.

Among the various type of Ca channels, L- and N-types are known to be regulated by different protein kinases (for review, see [20]). Phosphorylation by protein kinases A (PKA) or C (PKC) induces modifications of the channel availability. In addition, the pore forming α1-subunit of the P-type Ca channel presents numerous PKC phosphorylation sites [8]. These observations led us to characterize an eventual PKC-mediated regulation of Ca channels routinely expressed in oocytes injected with cerebellar mRNA. In a first step, the effects of PKC activators were analyzed. Application of either PMA (100 nM) or OAG (2 μM), a structural diacylglycerol (DAG) analog, induced a significant increase in the I_{Ba} amplitude. This increase was 43.7 ± 2.1% (n = 42) with PMA and 94.3 ± 11.5% (n = 10) with OAG (Fig. 2A and B). The current–voltage relationships and the current kinetic properties remained unchanged in the presence of PKC activators (Fig. 2A and B). The potentiation of I_{Ba} by PMA or OAG was transient and was generally followed by an irreversible and progressive depression of the I_{Ba} amplitude (see Fig. 4B for PMA). These results indicate that P-type Ca channels expressed in oocytes could be potentially regulated by PKC. The pharmacological proper-

ties of the I_{Ba} stimulated by PMA or OAG were similar to those described for the initial control I_{Ba}. Indeed, the OAG stimulated I_{Ba} was still insensitive to dihydropyridine ($n = 7$) and to ω-CgTx ($n = 7$), but was inhibited by Aga.V. (Fig. 3A). Similar results were also obtained on PMA or phorbol dibutyrate-stimulated I_{Ba} (not shown). Moreover, Aga.V. produced an inhibition of the PMA- or OAG-stimulated I_{Ba} similar to that of the unstimulated current (Fig. 3B). The inhibition was 61.8 ± 7.3% ($n = 8$) for unstimulated I_{Ba}, 70.8 ± 5.1% ($n = 7$) for PMA-stimulated I_{Ba} and 61 ± 3% ($n = 7$) for OAG-stimulated I_{Ba}. No modification of the Cd-insensitive outward current was ever recorded in the presence of PKC activators. Combined, these data suggest that PKC could act directly on Ca channels without modifications of other membrane conductances. To confirm the involvement of PKC in PMA- or OAG-mediated effects on the I_{Ba} current, oocytes were preincubated with STAU (10 μM, external concentration), a well known potent PKC inhibitor. In this case, the stimulation of the I_{Ba} by PMA or OAG was strongly reduced (Fig. 4A). Indeed, in the presence of STAU, the poten-

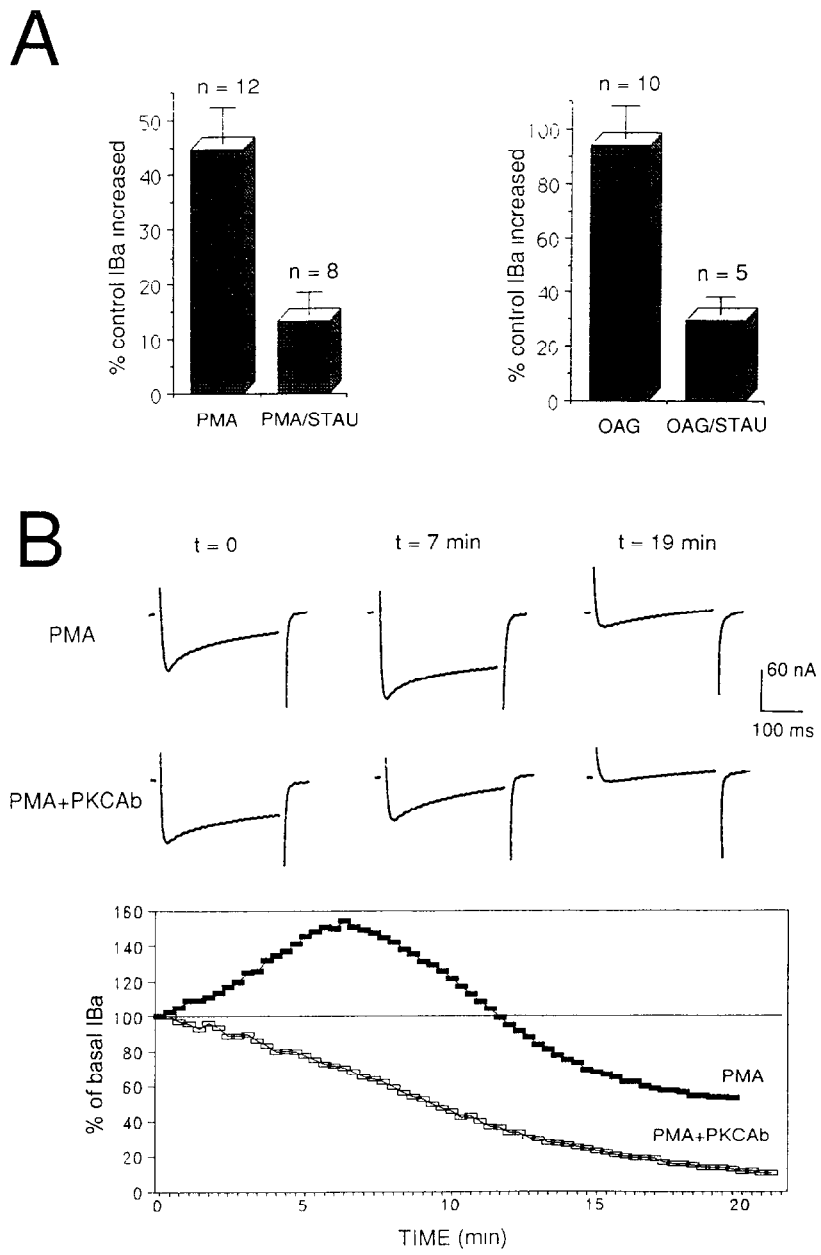

Fig. 4. Effects of a putative PKC inhibitor and of a PKC antibody on the I_{Ba}. (A) I_{Ba} increased by PMA (1 μM) or by OAG (2 μM) was expressed as a fraction of the initial control I_{Ba}. Effect of staurosporine (STAU, 10 μM in the bathing solution) was expressed as a fraction of the stimulated I_{Ba} by PMA (PMA/STAU) or by OAG (OAG/STAU). (B) Suppression of the effect of PMA (1 μM) on I_{Ba} by intra-oocyte injection of a PKC antibody (PKCAb). The final concentration in the oocyte of PKCAb was 25 μg/ml.

tiation of I_{Ba} induced by PMA (1 μM) was 14.3 ± 4% (n = 8) vs. 44.6 ± 6.5% (n = 12) without STAU and that of I_{Ba} induced by OAG (2 μM) was 29.6 ± 6.5% (n=5) vs. 94.3 ± 11.5% (n = 10) without STAU. Although STAU is one of the most effective PKC inhibitors, it also inhibits other protein kinases [21]. Therefore, oocytes were challenged with a specific antibody (PKCAb) directed against PKC. PKCAb reacts with membrane-bound and cytosolic PKC. It selectively inhibits PKC but not cAMP-protein kinases and not Ca-Calmoduline kinase. This PKCAb inhibits intact PKC and its catalytic fragments and recognizes an epitope of the catalytic domain. PKCAb cross-reacts with all PKC-isoenzymes [22]. In oocytes preinjected with PKCAb 2 to 3 min before recording of I_{Ba}, PMA did not give rise to the potentiation of I_{Ba} (Fig. 4B, n=4). However, the typical 'run-down' observed with phorbol esters was still present, indicating that this phenomenon may be due to PMA itself without any participation of PKC. This declining phase has already been observed in the modulation by PMA of Ca channels expressed in oocytes injected with cardiac mRNA [12]. It was suggested that this phase could be due to a non-specific internalization of membrane channels and receptors especially induced by phorbol esters but also in a less extent by diacylglycerol.

In most batches of tested oocytes, the endogenous I_{Ba} was never detected. Thus, the I_{Ba} recorded in injected oocytes was certainly related to the expression of newly synthetized Ca channels from cerebellar mRNA. In this case, the potentiation of the I_{Ba} observed in the presence of PKC activators appeared to be mainly due to the phosphorylation of these exogenous Ca channels. In our study, oocytes injected with cerebellar mRNA expressed only P-type Ca channels. Similar results have been provided with mRNA isolated from different parts of the mammal central nervous system [23]. The important density of P channels in the cerebellum could partly explain our observations but it remains unclear why oocytes injected with other brain mRNA preferentially expressed P-type Ca channels over L- or N-types. However, the oocyte vector may be the most suitable heterologous expression assay concerning the analyzis of the properties of P-type Ca channels. L- and N-type Ca channels have been shown to be the target for PKC regulation [2,20] but this is the first time that a similar regulation is described at the P-type Ca channel level. Therefore, in addition to previous results showing that activity of P-type Ca channels could be regulated by an intracellular pathway involving a cAMP-dependent protein kinase [24], the present data demonstrate that these channels could also be sensitive to other intracellular pathways such as PKC phosphorylation.

Like L- and N-type Ca channels, P-type Ca channels have a wide distribution in the nervous system. This was supported by recent experiments of immunolocalization and by electrophysiological studies [3,7,25]. P-type Ca channels may be involved in a variety of functions: neurotransmitter release, neuronal excitability and plasticity [3,4,8]. In this context, all cellular pathways leading to a Ca entry potentiation mediated by a positive regulation of P channels could potentially modulate these functions. For example, ω-Aga.IVA has been shown to inhibit the release of an amino acid neurotransmitter from hippocampal tissue [26].

The cerebellum represents the structure of the nervous system where the FTX sensitive Ca channels are highly expressed [25]. Recent reports demonstrate that induction of long-term depression (LTD) in cerebellar Purkinje cells needs an elevation of the intracellular Ca^{2+}. The primary source of this calcium signal is an influx of Ca through voltage-gated Ca channels [27] but activation of metabotropic glutamate receptors, and then participation of PKC, is also necessary for the induction of LTD [28]. Although a correlation between P-type Ca channel activity and induction of LTD has to be directly demonstrated, it is tempting to propose that these channels are involved in this post-synaptic mechanism. Indeed, our results suggest a PKC regulation of P-type Ca channels that could occur in situ via activation of metabotropic glutamate receptors.

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REFERENCES

[1] Porzig, H. (1990) Rev. Physiol. Biochem. Pharmacol. 114, 209–262.
[2] Scott, R.H., Pearson, H.A. and Dolphin, A.C. (1991) Progr. Neurobiol. 36, 485–520.
[3] Mintz, I.M., Adams, M.E. and Bean, B.P. (1992) Neuron 9, 85–95.
[4] Llinas, R., Sugimori, M., Lin, J.W. and Cherksey, B. (1989) Proc. Natl. Acad. Sci. USA 86, 1689–1693.
[5] Mintz, I.M., Venema, V.J., Swiderek, K., Lee, T., Bean, B.P. and Adams, M.E. (1992) Nature 355, 827–829.
[6] Hillyard, D.R., Monje, V.D., Mintz, I.M., Bean, B.P., Nadasdi, L., Ramachandran, J., Miljanich, G., Azimi-Zoonooz, A., McIntosh, J.M., Cruz, L.J., Imperial, J.S. and Olivera, B.M. (1992) Neuron 9, 69–77.
[7] Llinas, R., Sugimori, M., Hillman, D.E. and Cherksey, B. (1992) TINS 15, 351–355.
[8] Mori, Y., Friedrich, T., Kim, M.S., Mikami, A., Nakai, J., Ruth, P., Bosse, E., Hofman, F., Flockerzi, V., Furuichi, T., Mikoshida, K., Imoto, K., Tanabe, T. and Numa, S. (1991) Nature 350, 398–402.
[9] Starr, T.V.B., Prystai, W. and Snutch, T.P. (1991) Proc. Natl. Acad. Sci. USA 88, 5621–5625.
[10] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[11] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning, 2nd Edn., Cold Spring Harbor Laboratory Press.
[12] Bourinet, E., Fournier, F., Lory, P., Charnet, P. and Nargeot, J. (1992) Pflügers Arch. 421, 247–255.
[13] Lin, J.W., Rudy, B. and Llinas, R. (1990) Proc. Natl. Acad. Sci. USA 87, 4538–4542.

[14] Kondo, A., Mills, J.D. and Adams, M.E. (1992) Neurosci. Abstr. 18, 969.
[15] Mintz, I.M., Venema, V.J., Adams, M.E. and Bean, B.P. (1991) Proc. Natl. Acad. Sci. USA 88, 6628–6631.
[16] Sather, W.A., Tanabe, T., Mori, Y., Adams, M.E., Miljanch, G., Numa, S. and Tsien, R.W. (1992) Neurosci. Abstr. 18, 10.
[17] Mikami, A., Imoto, K., Tanabe, T., Niimode, T., Mori, Y., Takeshima, H., Narymiya, S. and Numa, S. (1989) Nature 340, 230–233.
[18] Tanabe, T., Takeshima, H., Mikami, A., Flockerzi, V., Takahashi, H., Kangawa, K., Matsuo, H., Hirose, T. and Numa, S. (1987) Nature 328, 313–318.
[19] Williams, M.E., Feldman, D.H., McCue, A.F., Brenner, R., Velicelebi, G., Ellis, S.B. and Harpold, N.M. (1992) Neuron 8, 71–84.
[20] Anwyl, R. (1991) Brain Res. Rev. 16, 265–281.
[21] Yanagihara, N., Tachikawa, E., Izumi, F., Yasugawa, S., Yamamoto, H. and Miyamoto, E. (1991) J. Neurochem. 56, 294–298.
[22] Mochly-Rosen, D. and Koshland, D.E. (1988) Anal. Biochem. 170, 31–37.
[23] Nargeot, J., Dascal, N.N. and Lester, H.A. (1992) J. Membr. Biol. 126, 97–108.
[24] Mogul, D.J., Adams, M.E. and Fox, A.P. (1992) Neurosci. Abstr.. 18, 1272.
[25] Hillman, D., Chen, S., Aung, T.T., Cherksey, B., Sugimori, M. and Llinas, R. (1991) Proc. Natl. Acad. Sci. USA 88, 7076–7080.
[26] Burke, S.P., Taylor, C.P. and Adams, M.E. (1992) Neurosci. Abstr. 18, 9.
[27] Konnerth, A., Dreesen, J. and Augustine, G.J. (1992) Proc. Natl. Acad. Sci. USA 89, 7051–7057.
[28] Linden, D.J. and Connor, J.A. (1991) Science 254, 1656–1659.