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SENSITIZATION OF $InsP_3$-DEPENDENT CALCIUM SIGNALLING THROUGH STRUCTURAL MODIFICATION OF VOLTAGE-DEPENDENT CALCIUM CHANNEL: A PHYSIOLOGICAL RELEVANCE OF THE CALCIUM CHANNEL β SUBUNIT

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The expression in *Xenopus* oocytes of the human voltage-dependent Ca^{2+} channel (VDCC) β_2 subunit subtype ($h\beta_2$) enhances the endogenous Ca^{2+} channel activity. By using the native Ca^{2+}-dependent chloride conductance to monitor fast intracellular Ca^{2+} variations, we point out that the β-enhanced Ca^{2+} entry (T_1 component) is currently associated with a second delayed elevation of internal Ca^{2+} (T_2 component). Further experiments show that this additional component absolutely requires Ca^{2+} entry through the β-modulated channels although it directly derives from a Ca^{2+} release from intracellular inositol (1,4,5)-trisphosphate ($InsP_3$)-sensitive stores. Finally, our study demonstrates that $InsP_3$-evoked response in oocytes is dramatically modified since it gains a new shape of voltage dependency directly derived from the β-modified Ca^{2+} influx. The main conclusion is that the spatiotemporal pattern of $InsP_3$-dependent Ca^{2+} release may be closely influenced by the intrinsic characteristics of working VDCCs.

The *Xenopus* oocyte represents the most convenient model to study the intrinsic properties of Ca^{2+} signalling linked to $InsP_3$, since the release of intracellular stores was shown to be mediated by only one kind of store which is mobilizable by $InsP_3$ and regulated by cytoplasmic Ca^{2+} (1). This system has been widely used to express ion channels such as VDCCs either from tissue-purified RNA or from cloned cDNA. In that respect, previous studies using brain RNA-injected oocytes show that the Ca^{2+} entry which is primarily induced by activation of an acquired VDCC activity is correlated to a modulation of the Ca^{2+} release from $InsP_3$-sensitive stores (2, 3). Although these experiments demonstrate that voltage-dependent Ca^{2+} entry is required for

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this functional coupling, they do not exclude the involvement of other partners, since tissue RNA can encode an heterologous population of modulatory proteins.

VDCCs are heteromultimers composed of at least three distinct subunits α_1, α_2/δ and β (4). From several functional expression studies, it is now established that the VDCC activity is directed by the α_1 subunit, the function of which is regulated by the β subunit (5, 6). Moreover, several recent studies indicate that exogenous β subunits introduced in *Xenopus* oocyte increase the endogenous VDCC activity (5, 6, 7). Hence, the present study utilizes a VDCC β subunit as a physiological enhancer of the oocyte endogenous VDCC activity with the aim to evaluate the incidence of a direct increase in Ca^{2+} entry on the Ca^{2+} release process from $InsP_3$-sensitive stores. Our data provide strong evidence for the establishment of a functional coupling between VDCCs and $InsP_3$ receptors solely by expression of a VDCC β subunit. These results bring new items about some subsequent implication of the Ca^{2+} channel β subunit in cellular Ca^{2+} signalling.

MATERIALS AND METHODS

PCR cloning of the $h\beta_2$ subunit

One isoform of the MysB subunits (type A; 8) was directly amplified from Hela cells polyA$^+$ RNA using specific primers (5' end: CGATGGTCCAAACGGACATGTCC; 3' end: GCTCTACGTAGGCAGAGTCTGC) and Retrotherm DNA polymerase (Epicentre technology). The cycling procedure included: i) a pre-annealing to 57°C for 10 min; ii) a denaturation step to 94°C for 3 min iii) 30 cycles as follows: 57°C 1 min / 72°C 1 min / 94°C 30 sec; iv) a terminal amplification at 72°C for 10 min and v) a cooling at 4°C for overnight storage. Agarose gel electrophoresis of the PCR products showed a single 1.7 kb band which was further sequenced using the Sequenase kit (USB) according to manufacturer's instructions. The cDNA obtained was totally homologous to the MysB subunit (type A, 8). It has been named $h\beta_2$ since it shows very high similarities with the rat brain/heart β_2 subunit described by Perez-Reyes *et al.* (1992; 9). A further round of amplification was performed to add specific restriction sites required to insert the cDNA in an appropriate expression vector (primers used: 5' end: GGAATTCGATGGTCCAAACGG and 3' end: CCTTAAGGCTCTACGTAGG). The $h\beta_2$ cDNA was subcloned into the EcoRI site of the pSVK3 (Pharmacia) as reported elsewhere (7). The construct named pSVK3$h\beta_2$ was purified using Qiagen DNA purification kit which made it ready for subsequent injection in the germinal vesicle of *Xenopus* oocyte.

Oocytes preparation and maintenance

Pieces of adult *Xenopus lævis* ovary were surgically removed and dissected away in ND96 solution of the following composition: (in mM) NaCl 96, KCl 2, $MgCl_2$ 2, $CaCl_2$ 1.8, HEPES 5, pH 7.4 with NaOH. Oocytes were then treated for 2-3h with collagenase (type IA; 2 mg/ml in Ca^{2+}-free medium) to discard follicular cells. 1 to 5 ng of the pSVK3$h\beta_2$ construct was injected in the germinal vesicle of each oocyte using a Nichiryo micropipette. Oocytes were kept for 2-6 days in ND96 medium supplemented with gentamycin (50 µg/ml).

Electrophysiological measurements

Electrophysiological measurements were performed using the standard dual microelectrode voltage clamp technique. Oocytes were impaled with microelectrodes filled with 3M CsCl in a 100 µl recording chamber. Stimulation, data acquisition and off-line analysis were conducted on a personal computer running the pClamp software package (v. 5.7.1; Axon Instruments, Burlingame CA, USA). The ND96 solution was routinely used to monitor the internal Ca^{2+} variations and occasional modifications of extracellular Ca^{2+} were compensated by adequate modifications of the divalent cation concentration (Mg^{2+}). To record VDCC activity, oocytes were bathed in BaMS/TEA solution of the following composition: (in mM) $Ba(OH)_2$ 40, TEAOH 50, CsOH 2, HEPES 5, pH 7.4 with methane sulfonic acid. Therefore, the activity of

VDCCs was recorded as an inward Ba current (I_{Ba}). Leak subtraction was performed using the p/4 procedure. Drugs were applied by addition to the superfusate. An additional micropipette (3-10 µm tip diameter) was used for intracellular injection. All injected compounds ($InsP_3$, heparin) were dissolved in HEPES 5 mM, pH 7.2 with KOH.

RESULTS

The $h\beta_2$ subunit was assayed in *Xenopus* oocytes for its ability to modulate endogenous VDCC activity (10). Barium currents were recorded in the presence of 40 mM Ba^{2+} in the bath (Fig. 1A). Two days after nuclear injection, amplitude of I_{Ba} was sevenfold higher in $h\beta_2$-injected oocytes than in control oocytes (-94 ± 10 nA, n = 13 versus -13 ± 2 nA, n = 17). No change in activation threshold (-30 mV) or peak current (+20 mV) were detected following $h\beta_2$ expression (Fig. 1B). When control oocytes were bathed in normal ND96 medium, stepping the voltage from a holding potential at -100 mV to +20 mV elicited a transient outward current (T_1 component; 111 ± 7 nA, n = 12; Fig. 2A, upper traces) which is inhibited by Cd^{2+} (500 µM; figure 2A upper traces). This current has been identified as a Ca^{2+}-dependent chloride current (11). In oocytes injected with the pSVK3hβ_2 construct (Fig. 2A, lower traces) the outward current amplitude was significantly increased (873 ± 98 nA, n = 10) and an additional delayed transient component (T_2 component) could be clearly distinguished (1385 ± 239 nA, n = 10). This T_2 component spontaneously appeared in 70 % of the oocytes tested. As described earlier for brain RNA-injected oocytes (3), the outward current elicited by depolarization presents more commonly the additional delayed T_2 component. One may propose that Ca^{2+} entry through $h\beta_2$-modified endogenous VDCCs can play a pivotal role in the occurrence of T_2. Abolition of the Ca^{2+} entry either by suppressing external Ca^{2+} or applying the inorganic Ca^{2+} channel blocker Cd^{2+} (500 µM, n = 10) inhibited both T_1 and T_2 components (Fig. 2A, lower traces). In order to clearly determine the link between T_2 component and the acquired increased VDCC activity, voltage-dependence properties of both T_1 and T_2 components were investigated. As depicted in fig. 2B, T_1 and T_2 describe the same voltage-dependence properties as I_{Ba}. Furthermore, the outward chloride current inactivates in a voltage dependent manner and the inactivation properties can be adequately correlated to these of the Ba^{2+} current (data not shown).

Caffeine, a well known inhibitor of the $InsP_3$ response (5 mM external concentration; 12) typically inhibited the T_2 component of the outward chloride current recorded in hβ2-injected oocytes (Fig. 3A; n = 8). The same kind of inhibition was observed when low molecular weight heparin (50 µg/ml final concentration), a competitive inhibitor of the $InsP_3$ receptor (13) was injected into oocytes (Fig. 3B; n = 5). Basically, both caffeine and heparin dramatically abolished T_2 component while no significant effect was ever detected on T_1. Thus, T_2 component induced by Ca^{2+} entry through modified endogenous VDCCs appears to be linked to a Ca^{2+} release process from $InsP_3$-sensitive stores. In order to assess the involvement of $InsP_3$-dependent Ca^{2+} pools in the firing of T_2, ponctual $InsP_3$ injections (15 fmol.) were performed in $h\beta_2$-modified oocytes that did not spontaneously exhibit T_2. These injections immediatly resulted in T_2 appearance (Fig. 3C, n = 6). With the aim to deduce the influence of the β subunit-induced VDCC activity enhancement on the quantitative properties of the $InsP_3$ Ca^{2+} signalling, we compared the voltage-dependence of the $InsP_3$ response obtained in control and pSVK3hβ_2-

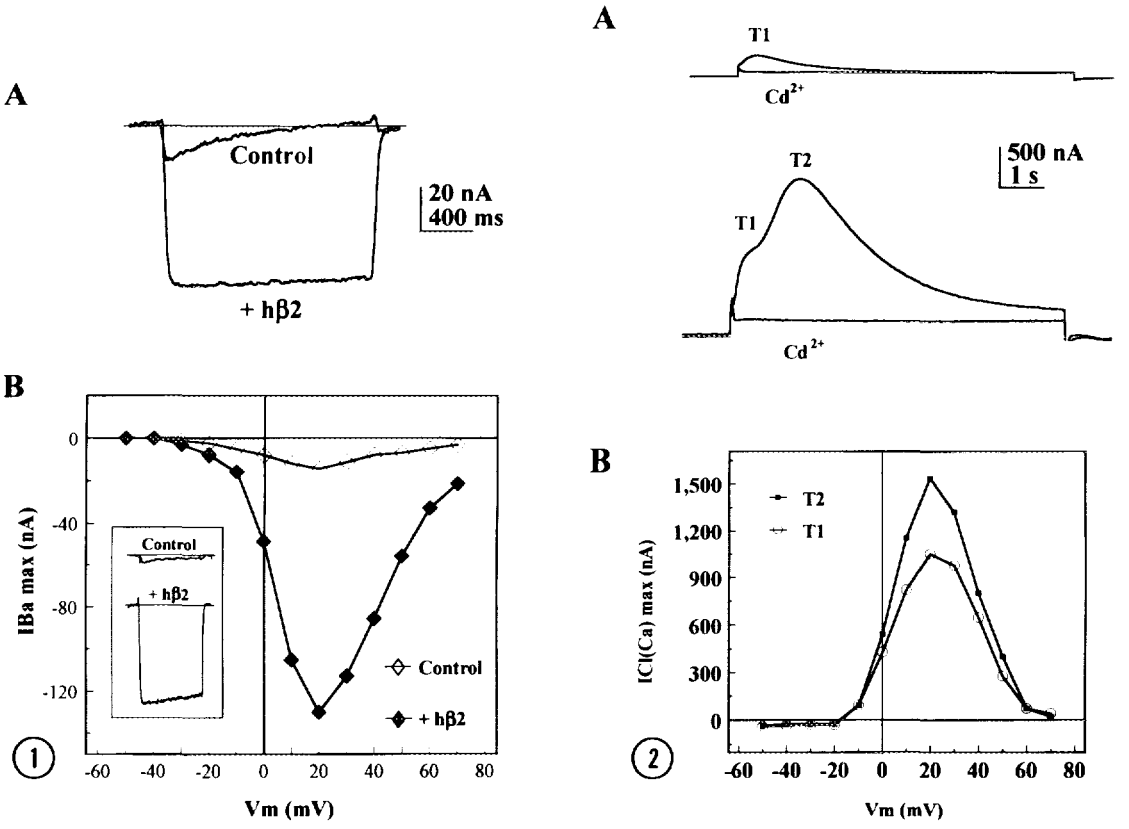

Figure 1. Functional expression of $h\beta_2$ in *Xenopus* oocytes.
Ba^{2+} currents were recorded in oocytes as described in Materials and Methods.
Leak and linear currents were subtracted using a p/4 protocol.
A: Current traces obtained by stepping membrane potential from -100 mV to +20 mV in non-injected (Control) and $h\beta_2$-injected oocytes ($+h\beta_2$).
B: Typical current amplitude plotted as a function of the test potential for uninjected (◇) and $h\beta_2$-injected (✦) oocytes. Final current to voltage relationships were obtained by digitally subtracting each trace by its corresponding trace recorded in the presence of Cd^{2+} (500 μM).
Inset: current traces eliciting the maximal inward Ba^{2+} current in control and $h\beta_2$-injected oocytes.

Figure 2. $h\beta_2$ expression triggers a second delayed chloride component : T_2.
A: Ca^{2+}-dependent chloride currents recorded when oocytes were depolarized from -100 mV to +20 mV. The upper traces correspond to currents obtained in control oocytes and lower traces to currents recorded in $h\beta_2$-injected oocytes. Note that both outward currents are blocked by external application of 500 μM Cd^{2+}.
B: Superimposed current to voltage relationships obtained for the two chloride components. T_1 and T_2 clearly possess the same electrophysiological characteristics since they are activated at the same voltage threshold (-20 mV) and maximal currents are recorded for the same voltage value (+20 mV).

injected oocytes. In control oocytes, the specific net $InsP_3$ response (current before $InsP_3$ injection was digitally subtracted) consisted in a passive variation of the chloride current arising at both holding and depolarizing levels (Fig. 4, "uninjected oocyte" trace; n = 4). Only an outward rectification could develop in response to membrane voltage modification demonstrating

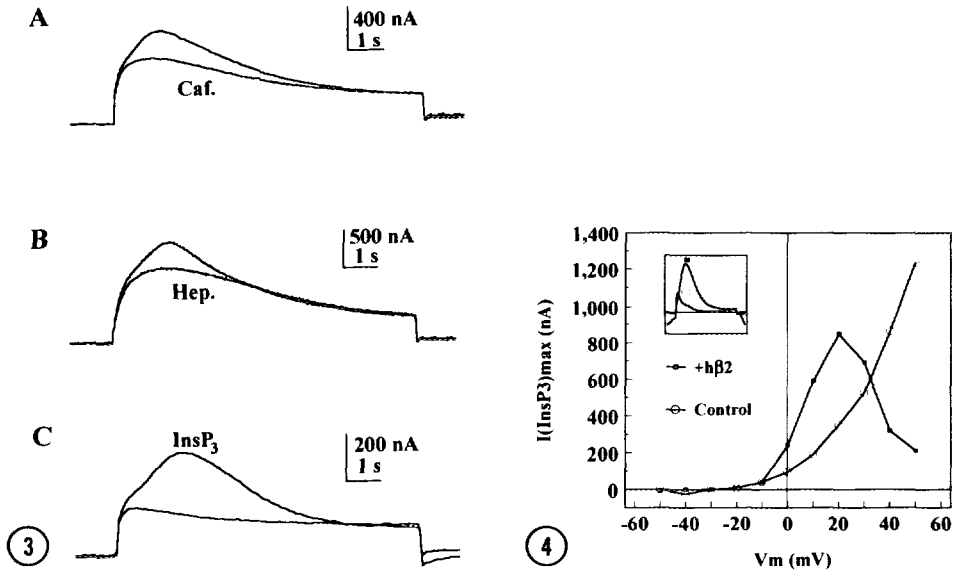

Figure 3 . Involvement of $InsP_3$-sensitive stores in the occurrence of T_2 component in $h\beta_2$-injected oocytes.
A, B: External application of 5 mM caffeine (A) and intracellular injection of heparin (B; 50 μg/ml, final concentration) both abolished T_2 component without affecting T_1 component.
C: Injection of $InsP_3$ (15 fmol.) triggered T_2 component in $h\beta_2$-injected oocytes that did not naturally elicit it.

Figure 4 . $h\beta_2$ VDCC-subunit conferes a voltage-dependent pattern to $InsP_3$ signalling.
Superimposed current to voltage relationships obtained in control and in $h\beta_2$-injected oocytes. The net $InsP_3$ response (I_{InsP_3}) was calculated by digitally subtracting the current recorded before $InsP_3$ injection (15 fmol.). The I-V curves were constructed by plotting the maximal amplitude of the $InsP_3$-elicited outward current versus potential (○: uninjected oocyte; ■: $h\beta_2$-injected oocyte).

that Ca^{2+} entry through native VDCCs had no significant influence on the $InsP_3$ response. Furthermore, no T_2 component was ever detected in control oocytes following $InsP_3$ injection as previously reported (3; data not shown). By contrast, when VDCC were structurally modified by expression of the $h\beta_2$ subunit, the increase of the rate of Ca^{2+} entry clearly affected the nature of the $InsP_3$ response. In these oocytes, the net $InsP_3$ response exclusively consisted in the appearance of T_2 and gained a new shape of voltage dependency that is closely related to that of $h\beta_2$ modified endogenous VDCCs (Fig. 4, $h\beta_2$ trace; n = 6).

DISCUSSION

To our knowledge, this is the first time that the human hβ2/MysB Ca^{2+} channel β subunit is expressed in *Xenopus* oocyte. Various β subunit subtypes have been shown to qualitatively interact with the α1 subunit of the different classes of VDCC. In that respect, the behaviour of *Xenopus* oocyte endogenous VDCCs is evidently affected by the hβ2 subunit.

The functional characteristics we observe totally corroborate these obtained with the rat β2 subunit by Castellano and Perez-Reyes (1994; 14): increasing the barium current amplitude and slowing down the inactivation kinetics.

Although the molecular properties of the $\alpha 1/\beta$ interaction are now well established, only little is known about its intracellular consequences. A recent report (3) shows that Ca^{2+} entry through VDCCs expressed in oocyte, can positively modulate the Ca^{2+} release process from $InsP_3$-sensitive stores. This Ca^{2+} release sensitization would occur only when the Ca^{2+} influx through plasma membrane is sufficiently extensive. The present study put forward the proposal that such a β subunit-mediated increase of VDCC-directed Ca^{2+} influx would constitute by itself the sufficient condition for this sensitization of the Ca^{2+} release .

Binding assays and functional reconstitution of $InsP_3$ receptors have demonstrated that binding of $InsP_3$ and opening of the Ca^{2+} channel are positively regulated by free Ca^{2+} (15). Therefore, a Ca^{2+} influx can trigger an $InsP_3$-mediated Ca^{2+} release while the concentration of $InsP_3$ remains constant. This Ca^{2+} release which occurs in $h\beta 2$-injected oocytes is attested by the development of the heparin/caffeine-sensitive T_2 component of chloride current.

An alternative hypothesis is that Ca^{2+} entry stimulates PLC generating tiny amounts of $InsP_3$ which could account for T_2. As discussed by other investigators (2), this direct activation of PLC seems to be unlikely because of the smooth-graded shape of the T_2 current and of the propagation of Ca^{2+} into the oocyte. Nevertheless, no experimental arguments allow us to withdraw the idea of a partial involvement of a direct activation of PLC.

At the light of these elements, one may speculate that the coupling between VDCCs and $InsP_3$-dependent Ca^{2+} pools may be due to an enlargement of the amount of Ca^{2+} ions in the immediate vicinity of the $InsP_3$-dependent release site. This local enhancement of the Ca^{2+} concentration is thought to be more prominent near the $h\beta 2$-modified VDCCs because of the dramatic increase in the ionic flow. Although the expected modification of inactivation parameters accounts for this coupling remains speculative, we cannot exclude that it may favorize local accumulation of Ca^{2+} by sustaining Ca^{2+} entry.

Finally, we also demonstrate that depolarization-induced Ca^{2+} influx through $h\beta_2$-modified VDCCs directly influences the qualitative properties of the $InsP_3$-dependent Ca^{2+} signalling. Indeed, the net $InsP_3$-evoked response gains a new shape of voltage dependency which reflects that of modified endogenous VDCCs, suggesting that these latter could impart their excitable features to the Ca^{2+} release process (Fig. 4).

To date, only the direct effects of the β subunit expression in regard to Ca^{2+} channels biophysical and pharmacological properties have been investigated. The present article proposes a new insight into physiological incidence of β subunit since our results clearly demonstrate that β subunit allows VDCCs and $InsP_3$-dependent Ca^{2+} stores to function in a synergistic and integrative pathway.

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REFERENCES

1. Parys, J.B., Sernett, S.W., DeLisle, S., Snyder, P.M., Welsh, M.J,. and Campbell K.P. (1992) J. Biol. Chem. 267, 18776-18782.
2. Yao, Y., and Parker J. (1992) J. Physiol. 458, 319-338.
3. Fournier, F., Navarre, P., Matifat, F., Vilbert, C., Collin, T., Guilbault, P., Brûlé, G., and Marlot, D. (1994) Cell Calcium 15, 411-422.
4. Snutch, T.P., and Reiner, P.B. (1992) Cur. Op. Neurobiol. 2, 247-253.
5. Singer, D., Biel, M., Lotan, I, Flockerzy, V., Hofmann, F., and Dascal, N. (1991) Science 253, 1553-1557.
6. Hullin, R., Biel, M., Flockerzy, V., and Hofmann, F. (1992) Trends Cardiovasc. Med. 3, 48-53.
7. Collin, T., Lory, P., Taviaux, S., Courtieu, C., Guilbault, P., Berta, P., and Nargeot, J. (1994) Eur. J. Biochem. 220, 257-262.
8. Rosenfeld, M.R., Wong, E., Dalmau, J., Manley, G., Posner, J.B., Sher, E., and Furneaux, H.M. (1993) Ann. Neurol. 33, 113-120.
9. Perez-Reyes, E., Castellano, A., Kim, H.S., Bertrand, P., Baggstrom, E., Lacerda, A.E., Wei, X., and Birnbaumer, L. (1992) J. Biol. Chem. 267, 1792-1797.
10. Williams, M.E., Feldman, D.H., McCue, A.F., Brenner, R., Velicelebi, G., Ellis S.B., and Harpold, M.M. (1992) Neuron 8, 71-84.
11. Miledi, R. (1982) Proc. R. Soc. Lond. (B) 215, 491-497.
12. Parker, J., and Ivorra, I., (1991) J. Physiol. 433, 229-240.
13. Ghosh, T.K., Eis, P.S., Mullaney, J.M., Ebert, C.L., and Gill, D.L. (1988) J. Biol. Chem. 263, 11075-11079.
14. Castellano, A., and Perez-Reyes, E. (1994) Biochem. Soc. Trans. 22, 483-488.
15. Callamaras, N., and Parker, I. (1994) Cell Calcium 15, 66-78.