Biochemistry. In the article “Characterization of recombinant phytochrome from the cyanobacterium Synechocystis” by Tilman Lamparter, Franz Mittmann, Wolfgang Gärtner, Thomas Börner, Elmar Hartmann, and Jon Hughes, which appeared in number 22, October 28, 1997, of Proc. Natl. Acad. Sci. USA (94, 11792–11797), a name appeared incorrectly in the acknowledgments on page 11797 due to a printer’s error. Prof. Silvia Braslavski (Max-Planck-Institut für Strahlenchemie, Mülheim) should be listed as Prof. Silvia Braslavsky.

Neurobiology. In the article “The synthesis of ATP by glycolytic enzymes in the postsynaptic density and the effect of endogenously generated nitric oxide” Kuo Wu, Chiye Aoki, Alice Elste, Adrienne A. Rogalski-Wilk, and Philip Sickevitz, which appeared in number 24, November 25, 1997, of Proc. Natl. Acad. Sci. USA (94, 13273–13278), the quality of the reproduction of Fig. 2A was poor. The figure and its legend are shown below:

![FIG. 2.](image)

Biochemistry. In the article “KSR stimulates Raf-1 activity in a kinase-independent manner” by Neil R. Michaud, Marc Therrien, Angela Cacace, Lisa C. Edsall, Sarah Spiegel, Gerald M. Rubin, and Deborah K. Morrison, which appeared in number 24, November 25, 1997, of Proc. Natl. Acad. Sci. USA (94, 12792–12796), the following correction should be noted. Due to a printer’s error, background was incorrectly added to Fig. 2A and B on page 12793, Fig. 3B on page 12794, and Fig. 4A–C on page 12795 so that multiple panels from different gels or Western blots appear as one continuous panel. Correct versions of Figs. 2, 3, and 4 and each figure legend are reproduced here and on the opposite page.

Neurobiology. In the article “β subunits influence the biophysical and pharmacological differences between P- and Q-type calcium currents expressed in a mammalian cell line” by Herman Moreno, Bernardo Rudy, and Rodolfo Llinás, which appeared in number 25, December 9, 1997, of Proc. Natl. Acad. Sci. USA 94, 14042–14047, the following correction should be noted. Due to an editorial change at PNAS, the meaning of the last sentence on page 14046 was altered. The sentence originally read as follows: On the other hand, this structure does not reproduce the pharmacological properties of either P or Q channel exactly, as the ID50 to sFTX and ω-Aga IVA for P-type channels is lower than for the α1A, α2δ, βb channels in HEK cells.
FIG. 3. The mKSR1 CA3 domain augments Raf-1 activity in a detergent-sensitive manner. *Xenopus* oocytes expressing Raf-1 alone (−) or coexpressing Raf-1 and the mKSR1 CA3 domain (+) were injected with RasV12 RNA. Immediately after (0 min) or 150 min after RasV12 injection, oocytes were lysed in hypotonic buffer and membranes were isolated. (A) Raf-1 proteins were immunoprecipitated from membrane fractions resuspended in RIPA buffer (+ detergent) or phosphate-buffered saline (− detergent), and *in vitro* kinase assays were performed using kinase-inactive MEK as a substrate (1). Phosphorylation of MEK1 on Ser-218 and Ser-222 was determined by tryptic peptide mapping analysis. (B) RasV12 and CA3 proteins were immunoprecipitated from membrane (P100) and cytosolic fractions (S100) isolated at 150 min after injection with RasV12 RNA and were examined by immunoblot analysis using Ras and Pyo antibody, respectively. The migration of processed (Pro) and unprocessed (UnPro) Ras proteins is indicated.

**Fig. 4.** Augmentation of Ras signaling by mKSR1 does not involve ceramide. (A) Cos cells were transiently transfected with constructs encoding wild-type (WT) or kinase-inactive (RM) mKSR1. At 60 hr posttransfection, serum starved cells were left untreated or were stimulated with 20 μM C2 ceramide for 5 or 10 min, 100 milliunits/ml sphingomyelinase (SMase) for 20 min or 10 nM tumor necrosis factor α (TNFα) for 20 min. KSR proteins were immunoprecipitated using Pyo antibody, and mKSR1 immune complex kinase assays were performed *in vitro* as described by Zhang et al. (ref. 19; Top). Immunoprecipitated mKSR1 was detected by immunoblot analysis (Middle). To observe phosphorylation of Raf-1 or modulation of Raf-1 activity, purified activated Raf-1, coexpressed in Sf9 cells in the presence of RasV12 and v-src, and kinase-inactive MEK1 were added to the mKSR1 immune complex kinase assays previously described (ref. 19; Bottom). Immunoprecipitated mKSR1 was detected by immunoblot analysis (Bottom). (B) Cos cells were treated as in A and endogenous ceramide levels, JNK activity, and MAPK activity were determined. Ceramide levels were normalized to the untreated control. C2-ceramide levels were elevated 2.9- and 3.7-fold at 5 and 10 min, respectively, and long-chain ceramide levels were elevated 12-fold by SMase and 1.9-fold by TNFα. (C) Purified brain ceramide (100 nM) (+) or diluent (−) was added *in vitro* to mKSR proteins immunoprecipitated from transfected Cos cells and immune complex kinase assays performed in the presence of activated Raf-1 and kinase-inactive MEK1 as previously described (ref. 19; Top). Immunoprecipitated mKSR1 was detected by immunoblot analysis (Bottom). (D) Oocytes preinjected with buffer or RNA encoding WT and CRM mKSR1 constructs were treated with 250 milliunits sphingomyelinase. GVBD was then scored 6 and 10.5 hr after treatment.
β subunits influence the biophysical and pharmacological differences between P- and Q-type calcium currents expressed in a mammalian cell line

(human epithelial kidney cells/voltage-gated calcium channels/coexpression/Purkinje cells)

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Contributed by Rodolfo Llinás, October 22, 1997

ABSTRACT Human epithelial kidney cells (HEK) were prepared to coexpress α1A, α2δ with different β calcium channel subunits and green fluorescence protein. To compare the calcium currents observed in these cells with the native neuronal currents, electrophysiological and pharmacological tools were used conjointly. Whole-cell current recordings of human epithelial kidney α1A-transfected cells showed small inactivating currents in 80 mM Ba2+ that were relatively insensitive to calcium blockers. Coexpression of α1A, βb, and α2δ produced a robust inactivating current detected in 10 mM Ba2+, reversibly blockable with low concentration of ω-agatoxin IVA (ω-Aga IVA) or synthetic funnel-web spider toxin (sFTX). Barium currents were also supported by α1A, β2a, α2δ subunits, which demonstrated the slowest inactivation and were relatively insensitive to ω-Aga IVA and sFTX. Coexpression of β3 with the same combination as above produced inactivating currents also insensitive to low concentration of ω-Aga IVA and sFTX. These data indicate that the combination α1A, βb, α2δ best resembles P-type calcium channels given the rate of inactivation and the high sensitivity to ω-Aga IVA and sFTX. More importantly, the specificity of the channel blocker is highly influenced by the β subunit associated with the α1A subunit.

After the initial description of P-type calcium channels in Purkinje cells (1, 2), and the subsequent designation of the Q-type calcium channels as a separate channel category (3), concern has arisen regarding their true distinctness. The issue at hand concerns the use of pharmacological or biophysical operational definitions as a sole requirement in channel characterization, especially given the functional significance of these moieties in central nervous system physiology and pathology. Indeed, the molecular elements for the native P- and Q-type channels remain unclarified (4, 5).

Presently the accepted nomenclature for voltage-gated calcium channels include L, N, P, Q, R, and T. These channels are multimeric structures consisting of a main pore-forming subunit (α1), which can implement calcium ion conductance on its own in heterologous expression systems (5, 6). In addition, two other subunits are important in the functioning of the channel, α2δ and β (7, 8). It has been shown that the α2δ subunit increases the amount of α1C subunit (L-type calcium channel) at the plasmalemma, whereas β subunits increase the opening probability of the calcium channels expressed by the α1C subunit (9). In the case of P- and Q-type channels it has been proposed, based on pharmacology (5, 10), molecular dissection experiments (11), and subcellular localization (12, 13), that the α1A subunit is the pore-forming component. Given that this subunit does not reproduce the functional properties of P and Q calcium currents on its own, we tested whether different β subunits in the presence of the constant α2δ subunit produced any differences in the electrophysiological and/or pharmacological properties of α1A currents in a mammalian expression system.

MATERIALS AND METHODS

Preparation of Human Epithelial Kidney (HEK) Cells Expressing Channel Proteins. For transient expression of channel proteins, HEK 293T cells were grown in DMEM (GIBCO) supplemented with 10% fetal bovine serum, 100 units/ml of penicillin G, and 100 µg/ml of streptomycin (GIBCO), and plated at 50% confluence. The appropriate cDNAs (α1A, α2δ, βb, β2a, β3, and green fluorescence protein) were subcloned into the mammalian expression vector pcDNA 3.1 (Invitrogen). All calcium channels subunits were a kind gift of T. Snutch (University of British Columbia, Vancouver). Green fluorescence protein was obtained from CLONTECH. Cells expressing channels were prepared by using DOTAP (Boehringer Mannheim) to introduce the recombinant vectors (1 µg each) by lipofection according to the manufacturer’s protocols. After transfection, cells were allowed to reach 70% confluence and then plated at a 1:5 ratio and recorded 8–24 hr later.

Electrophysiological Analysis. Whole-cell recordings (14) were obtained from green fluorescent cells, which were observed with an excitation wavelength of 488 nm and emission of 515 nm at room temperature by using an Axopatch 200-A (Axon Instruments, Foster City, CA). Cells were maintained in an extracellular solution containing 135 mM NaCl, 3.5 mM KCl, 1.5 mM CaCl₂, 1.0 mM MgCl₂, 5.0 mM glucose, and 10 mM Hepes (pH adjusted to 7.35 with NaOH). For the recording of barium currents the external solution contained 142 mM tetraethylammonium (TEA)-Cl, 10 mM BaCl₂, and 10 mM Hepes-CsOH (pH 7.35) “standard solution.” Experiments with high extracellular barium used 80 mM BaCl₂, 12 mM TEA-Cl, 10 mM Hepes (TEA), 290 Osm w sucrose, and liquid junction potential was measured and subtracted for generation of I–V’ plots (15). The patch clamp pipettes contained a solution of 110 mM CsMeSO₄, 4.5 mM MgCl₂, 10 mM EGTA, 8 mM ATP, and 10 mM Hepes (pH adjusted to 7.35 with CsOH) and showed resistances of ~3–7 MΩ (typically 4 MΩ). Calcium currents were obtained in an extracellular solution of 40 mM CaCl₂, 80 mM TEA-Cl, 10 mM Hepes-CsOH (pH 7.35), 290 Osm. For these experiments pipette solution contained 2.0 mM EGTA, otherwise identical to that described. Seal resistance was typically 10 GΩ. Recordings were obtained with partial series resistance compensation (60–80%), and most of the cell capacitance canceled. The currents were low pass-filtered at 2–5 kHz by using an eight pole Bessel filter.

Abbreviations: HEK, human epithelial kidney cells; sFTX, synthetic funnel-web spider toxin; ω-Aga IVA, ω-agatoxin IVA.
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(Frequency Devices, Haverhill, MA) and digitized at 2.5–5 kHz. Subtraction of leak and remaining capacitance was obtained by using a P/4 protocol. For data acquisition and analysis and voltage clamp protocols we used the PCLAMP software (Axon Instruments). Drugs, as well as recording extracellular solutions, were applied locally by means of a blunt pipette (gravity flow). sFTX (1, 16, 17) was freshly prepared before using, and α-Aga IVA (a gift from Pfizer) was stored at −20°C as stock solution (100 μM).

RESULTS

The Pharmacological Profile of the Current Expressed by α1A Subunit Varies Depending on its Associated β Subunit. The effects of sFTX and α-Aga IVA on expressed calcium currents were examined in HEK 293T cells transiently transfected with α1A, α2δ cDNAs with and without different β subunits cDNAs. Green fluorescence protein was used to identify transfected cells. Expression of the different combinations resulted in Ba²⁺ currents with characteristic electrophysiological properties (Fig. 1) in whole-cell mode (see also ref. 5). When α1A subunits were coexpressed with α2δ and βIb they produced a fast-activating inward Ba²⁺ current sensitive to submillimolar concentrations of sFTX. The dose–response curve (Fig. 2A) shows a IC₅₀ of 410 μM with a saturating maximum block around 1.5 mM. Interestingly, higher extracellular Ba²⁺ concentration (80 mM, concentration commonly used in single-channel studies) shifted the sFTX dose–response curve (Fig. 3C and D). These results were consistent with studies that report Ba²⁺ ions concentration dependence

![Graph of sFTX and α-Aga IVA effects on Ba²⁺ currents](image)

**FIG. 1.** Global properties of α1A calcium channel subunit expressed by itself and in combination with different β subunits in HEK (293T) cells. Current-voltage relations for α1A, α2δ, βIb (■), α1A, α2δ, β2a (▲), and α1A, α2δ, β3 (●). Currents were recorded in “standard” extracellular solution. (A) Macroscopic whole-cell barium currents of transiently transfected HEK cells expressing different calcium channel subunits combinations were observed. The barium currents elicited in response to depolarizing steps from a holding potential of −90 mV. α1A in 80 mM extracellular barium step to +20 mV (B), α1A, α2δ, βIb in “standard solution” step to +10 mV (C), α1A, α2δ, β2a (D), and α1A, α2δ, β3 (E) currents in response to the same protocol as in C. Tails currents of α1A, α2δ, and two different β subunits were generated from a pulse to +10 mV and then step to −110 mV in 10 mV increments every 20 sec, shown are the recordings of tails generated at −80 mV for a α1A, α2δ, β2a (three averaged currents) (F) and α1A, α2δ, βIb (G).
Similar at low and high extracellular Ca\(^{2+}\) at a concentration higher than 50 nM (Fig. 4A). For instance, 500 nM produced a 90% inhibition of this current (Fig. 4B). The inhibition was reversible in 60% of the cells reaching a 26 \pm 10% recovery 8–12 min after toxin withdrawal, which increased to 40 \pm 15% after high depolarizing prepulses as illustrated in Fig. 4B.

The response to sFTX of the currents obtained by \(\alpha 1A, \alpha 2\), and \(\beta 3\) was similar to that of the \(\alpha 1A, \alpha 2\delta\), and \(\beta 2\) combination (Fig. 4A), with similar block characteristics as those observed for the other subunit combinations tested. \(\omega\)-AgA IVA at 100 nM did not produce any significant effect on the current. In the absence of \(\beta\) subunits \(\alpha 1A\) currents were detectable only in high extracellular Ba\(^{2+}\), and local application of sFTX produced a block only at high millimolar concentrations (\(\sim 30%\) at 5 mM). No significant effects were observed at lower concentration. On the other hand, 200 nM \(\omega\)-AgA IVA produced a 30-80% block of \(\alpha 1A, \alpha 2\delta\) calcium currents at 20 mV with a peak current at \(-10\) mV with a fast-activating kinetics (time to peak \(= 2.4\) + /− 0.4 msec \(n = 12\) ) (Fig. 1A and C). The potential at which half of the channels were activated was calculated from a smooth curve fitted to the raw \(-I-V\) data \((I = G(xE)\{1+exp[\{V-V_{1/2}\}/K]\} \quad V_{50} = 6.92 + /− 1.2\) mV and \(e\) fold value \(= 2.8\)). Inactivation was studied by single long trace pulses as well as by instantaneous onset currents as shown in Fig. 5C and D. Inactivation was best-fit by a single exponential in both protocols, which generated similar results, although the rate of inactivation was rather variable between cells \((\tau\) inact \(= 3 + 20\) mV \(= 225 + /− 64\) msec \(n = 12\) ) (Fig. 5C and D). \(\alpha 1A, \alpha 2\delta, \beta 1b\) calcium currents were relatively stable, and in some cases no rundown was observed (rundown \(20 + /− 14%\) \(n = 12\) ). Recovery from inactivation demonstrated a fast partial recovery, which was little affected over periods of seconds, reaching a 68 + /− 8% of initial value at 8 sec (Fig. 5B).

Attempts to study the currents with calcium as the main charge carrier produced a progressive current decay, reaching close to a complete block within \(-6\) min (Fig. 5E). Indeed, such current once having vanished in the presence of calcium, quickly returned in a Ba\(^{2+}\) environment. This result suggested that the \(\alpha 1A, \alpha 2\delta, \beta 1b\) channel protein complex undergoes calcium-induced calcium channel inhibition in a reversible fashion. Tail current analysis \((n = 6)\) demonstrated a \(\tau\) off rate to \(-80\) mV = 0.57 + /− 0.07 msec, best-fit by a single exponential (Fig. 1G). \(\alpha 1A, \alpha 2\delta, \beta 2a\) produced a fast-activating \(I_{ba}\) (time to peak \(= 2.26 + /− 0.6\) msec) (Fig. 1D).

This current also showed a high variability of the rate of inactivation; for instance, 50% of the cells \((n = 14)\) did not inactivate within 500 msec whereas the rest of the cells did inactivate at \(+20\) mV = 860 + /− 300 msec; with a peak current at \(0 + 10\) mV (Fig. 1A and D). Tail current to \(-80\) mV was best-fit monoexponentially \((\tau\) off rate at \(-80\) mV = 0.42 + /− 0.14 msec) (Fig. 1F).

Coexpression of the same \(\alpha\) subunits with \(\beta 3\) subunit produced fast-activating \((\text{time to peak} = 2.16 + /− 0.7\) msec) currents inactivated with a \(\tau\) inact to +20 mV = 214 + /− 28 msec \((n = 4)\) (Fig. 1A and E). In the three combinations studied, \(\alpha 1A, \alpha 2\delta, \beta 3\) and the different \(\beta\) (s) calcium channel subunits, high extracellular Ba\(^{2+}\) (40–80 mM) produced a shift to the right of the \(-I-V\) relationship (see Figs. 3A and C and 5A).
DISCUSSION

The present set of results address the issue of the differences between P and Q channels. The issue of what defines a channel type becomes one of the questions to be examined. Our results suggest that the P and Q channels are indeed functional variances of the $\alpha_1A$ subunit, this variance being determined by the associated $\beta$ subunits. This conclusion is based on biophysical and pharmacological measurements of the macroscopic $\alpha_1A$, $\alpha_2\delta$ supported barium currents when associated with different $\beta$ subunits. Indeed both $\omega$-Aga IVA and sFTX block preferentially the $\alpha_1A$, $\beta_\delta$ combination with an ID$_{50}$ smaller than $\alpha_1A$, $\beta_2\alpha$, and $\beta_3$, which otherwise have similar current/voltage relations. We conclude that the $\beta_\delta$-associated current is the closest to the native P/Q type current and pharmacology. However, the relatively slow inactivation differentiates it from the rapidly inactivating Q current, making the $\alpha_1A$, $\beta_\delta$, $\beta_\delta$ the most likely candidate for the P current.

**FIG. 3.** sFTX but not $\omega$-Aga IVA block of $\alpha_1A$, $\alpha_2\delta$, $\beta_\delta$ currents is affected by barium ions. $I$--$V$ relations of $\alpha_1A$, $\alpha_2\delta$, $\beta_\delta$ before (control O) and after $\omega$-Aga IVA (x) in 80 (A) and 10 mM barium (B), also shown same type of $I$--$V$ relations before and after 1 mM sFTX (x) in 80 (C) and 10 mM barium (D). All recordings were performed by using the same protocol as in Fig. 1C. Voltage steps from $-50$ to $+40$ mV in 10 mV increments were delivered every 20 sec. Recordings performed in high barium are shifted 10–20 mV to the right. This was true for all of the $\alpha_1A$, $\alpha_2\delta$, $\beta$ subunit combinations tested.

**FIG. 4.** $\omega$-Aga IVA is a specific inhibitor of $\alpha_1A$, $\alpha_2\delta$, $\beta_\delta$ currents. (A) Comparison of the effect of 100 nM $\omega$-Aga IVA after 8 min of constant local application on $\alpha_1A$, $\alpha_2\delta$, $\beta_\delta$ (B), $\alpha_1A$, $\alpha_2\delta$, $\beta_2\alpha$ (C), and $\alpha_1A$, $\alpha_2\delta$, $\beta_3$ (D). (B) $\alpha_1A$, $\alpha_2\delta$, $\beta_2\alpha$ currents are inhibited by 500 nM $\omega$-Aga IVA in a reversible fashion, washout (wsh) is increased on strong prepulse depolarizations (wsh ++ ) (see text). (C) $\omega$-Aga IVA effect on $\alpha_1A$, $\alpha_2\delta$, $\beta_\delta$ currents before (control) and after 100 nM Aga IVA, application (Aga IVA) and on toxin withdrawal and high depolarization protocol (wsh ++ ).
Whereas the biophysical results are in agreement with previous findings (5, 9, 10) the pharmacological results are unexpected, as they demonstrate that the β subunits influence the pharmacological properties of the current. Because the β subunit is an intracellular moiety the results suggest that this subunit must induce an allosteryic change on the α1A subunit that modifies its pharmacological sensitivity. This conclusion touches on issues of channel characterization. It is apparent from present results that the association of the α1A, α2δ, βIb structure mimics the biophysical and pharmacological properties of classical P, and to a lesser extent the Q channels. Furthermore, it has been identified as P-type calcium channel of cerebellar granule neurons (19) with different functional properties from the initially described P-type calcium channel in Purkinje cells. The granule cell variant of the P channel shows slow inactivation kinetics reaching 34% inactivation at +10 mV after 720 msec. Expression of α1A, α2δ, βIb in COS cells (20) supports a slower inactivating $I_{\text{Ba}}$ $I-V$ shifted to the right as well as a lower apparent $K_D$ (11 nM) for ω-Aga IVA, than the one reported in the present work. Nevertheless, the result supports the view that the combination α1A, α2δ, βIb as best replicating the properties of the native P-type channel.

Concerning channel characterization the differences in the pharmacology and electrophysiological properties of α1A, βIb $I_{\text{Ba}}$ in various heterologous expression systems (oocytes) (4, 5), COS cells (20), and HEK cells (present work) may reflect different cellular processing mechanism. Alternatively, they may be due to post-translational modifications and/or differences in the ionic strength used in the different preparations, because expression of the same α subunits with β3 in HEK cells (21) produced an $I-V$ similar to the one found in this work.

Since the inhibition produced by ω-Aga IVA and sFTX on α1A, α2δ, β(s) currents reached a saturation level without total block and the classification of R-type calcium currents relies on the unblocked total calcium currents with a toxin cocktail, this finding opens the possibility that R-type current may represent one of the α1A, α2δ, β(s) combinations tested. This theme requires further experimentation.

In conclusion, the channels composed of α1A, α2δ, β-Ib are the most likely molecular counterpart of native P-type(s) channels. On the other hand, this structure does not reproduce the pharmacological properties of either P or Q channel exactly, as the sensitivity to sFTX and ω-Aga IVA for P-type channels is lower than for the α1A, α2δ, βIb channels in HEK...
cells, whereas in COS cells (20) the IV curve is shifted to the right. This finding indicates that other elements may be lacking in HEK cells, preventing the exact reproduction of the native channel properties.

This work was supported by National Institutes of Health Grants NS13742 to R.L. and NS30989 to B.R.