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## Reduction of the same calcium current component by A and C kinases: differential pertussis toxin sensitivity

Robert A. Gross and Robert L. Macdonald

*Department of Neurology, University of Michigan Medical Center, Ann Arbor, MI 48104-1687, U.S.A.*

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Voltage-clamp techniques were used to study the effect of forskolin, 8-Br-cyclic AMP, and phorbol 12,13-dibutyrate (PDBu) on the 3 voltage-dependent calcium current components of mouse dorsal root ganglion neurons in culture. Forskolin and 8-Br-cyclic AMP selectively reduced the transient high-threshold (N-type) calcium current component, an effect also produced by PDBu, a C kinase activator. Pretreatment of cultures with pertussis toxin prevented the reduction of calcium current by PDBu, but was without effect on the reductions produced by forskolin or 8-Br-cyclic AMP. These results support the contention that activation of both A and C kinases selectively affect calcium currents in vertebrate neurons by reducing the N-type calcium current component. While the activation of the C kinase required a G protein to exert its effect, the activation of A kinase did not.

Two second messenger systems, the phosphatidylinositol-C kinase and adenylate cyclase-A kinase systems, regulate voltage-dependent calcium channels. In response to various neurotransmitters, the membrane-bound enzyme phospholipase C generates inositol triphosphate and diacylglycerol, the latter activates C kinase [5]. The tumor-promoting phorbol esters directly activate the C kinase [11] and have been shown to increase calcium current in invertebrate neurons [3, 18] and to decrease calcium current in vertebrate neurons [14, 19, 20]. A variety of extracellular receptors are coupled via GTP-binding proteins to adenylate cyclase, which in turn generates cyclic AMP [15]. Activation of the A kinase by cyclic AMP or its analogs also enhances calcium current in invertebrate neurons [2, 12], similar effects in vertebrate neurons have also been noted [4]. The mechanisms by which either of these second messenger systems regulate calcium channel function are largely obscure. It is not known, for example, whether the A or C kinases directly phosphorylate calcium channels, or if intermediate steps are involved.

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*Correspondence:* R. L. Macdonald, University of Michigan, Neuroscience Laboratory Building 1103 F Huron, Ann Arbor, MI 48104-1687, U.S.A.

Furthermore, there appear to be at least 3 different calcium current components in vertebrate neurons, a transient low-threshold (T-type) current, a slowly inactivating high-threshold (L-type) current, and a transient high-threshold (N-type) current (e.g. ref. 6). We studied the effects of forskolin, 8-Br-cyclic AMP and phorbol 12,13-dibutyrate (PDBu) on the 3 calcium current components of mouse dorsal root ganglion (DRG) neurons in primary dissociated cell culture. We used 8-Br-cyclic AMP which was presumed to activate the A kinase directly, and the adenylate cyclase activator forskolin [17], which was assumed to indirectly activate A kinase. PDBu was used to activate the C kinase [11]. Pertussis toxin, which inactivates the GTP binding protein  $G_i$  and/or  $G_o$  by ADP-ribosylation [10], was used to investigate the role of G proteins in second messenger regulation of calcium channels.

Preparation of DRG cultures was as previously described [7]. Recording medium suppressed sodium and potassium currents, and contained in mM: Tris-base 13.0, choline chloride 67.0, KCl 5.3,  $CaCl_2$  2.0,  $MgCl_2$  0.8, glucose 5.6, tetraethylammonium chloride 100. The pH was 7.3–7.4 at 35°C. Recordings were made with micropipettes (20–30 M $\Omega$ ) filled with 3 M CsCl. A single electrode voltage clamp amplifier was used (Axoclamp 2, Axon Instruments, Burlingame, CA) that switched between voltage sampling and current injection at 6–8 kHz with a 70–30% duty cycle. Voltage commands were generated, and current traces were digitized at 1.6 kHz and stored using the program pClamp (Axon Instruments). Leak currents were estimated by using hyperpolarizing commands of magnitudes equal to the depolarizing commands used to evoke inward currents, the resulting currents were assumed to be equal but opposite in magnitude to leak currents. Forskolin (Calbiochem, La Jolla, CA) and PDBu (LC Services, Woburn, MA) were dissolved in dimethyl sulfoxide (Sigma, St. Louis, MO) and diluted into recording medium on the day of the experiment. 8-Br-cyclic AMP was dissolved in recording medium for use that day. After neurons were voltage-clamped, currents were evoked every 15 s until current magnitudes were stable for 1–2 min. A control current was then evoked, after which the drugs were applied to the neuron by pressure injection at 0.25–0.5 psi from micropipettes with tip diameters of 10–20  $\mu$ m. Thereafter, currents were evoked every 60 s. Under these conditions, the calcium current diminished no more than 15% in the 3–5 min after application of diluent.

Calcium currents evoked at potentials positive to  $-20$  mV from holding potentials negative to  $-60$  mV contained the T-, N- and L-type current components [7]. The T-type calcium current component was isolated by using very negative holding potentials (negative to  $-80$  mV) to fully remove steady-state voltage-dependent inactivation, and evoking the current at  $-60$  to  $-50$  mV.

For most experiments, however, the holding potential was  $-65$  mV, i.e. near the resting membrane potential. Currents were evoked with 300 ms depolarizing voltage commands to  $-5$  mV, and consisted primarily of the N- and L-type calcium current components. Using this paradigm, most evoked currents declined to near-plateau levels by 300 ms. Application of 100  $\mu$ M forskolin reduced the peak calcium current with little effect on the late current (Fig. 1A1). This effect was maximal 1–2 min after a 2 s application and was reversible after 5–10 min (not shown). Application of 8-Br-

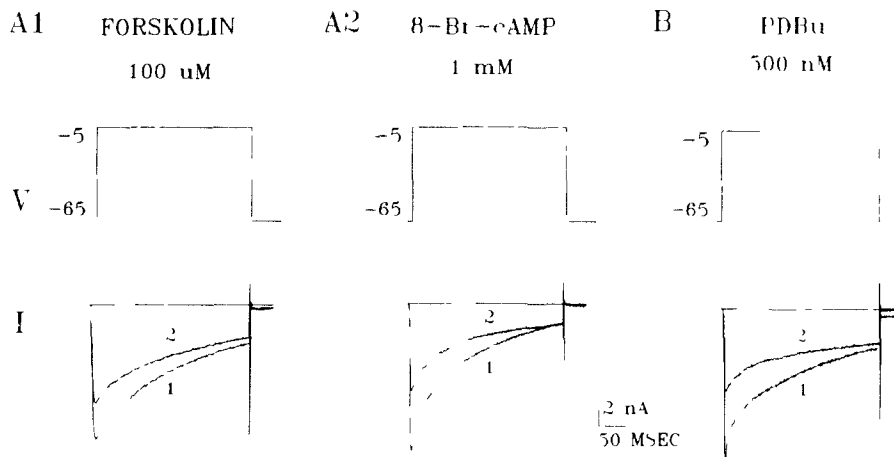


Fig 1 Forskolin 8-Br-cyclic AMP and PDBu decreased calcium current In this and other figures, illustrative voltage traces were drawn above the current traces to show the holding potential ( $V_h$ ) and the voltage step Inward currents are downward All current traces were digitized and leak-subtracted In this figure calcium current traces from each of 3 neurons are shown superimposed, traces are shown before (trace 1) and 4 min after (trace 2) a 2 s application of drug A1 effect of 100  $\mu$ M forskolin on calcium current A2 effect of 1 mM 8-Br-cyclic AMP on calcium current B effect of 500 nM PDBu on calcium current  $V_h$  was  $-65$  mV with 300 ms voltage steps to  $-5$  mV

cyclic AMP (1 mM) produced a similar reduction in calcium currents The effect was maximal 2–5 min after application but was rarely reversible within 10 min (Fig 1A2) The mean reduction of peak calcium current was  $28 \pm 2.1\%$  (S E M,  $n = 16$ ) for forskolin and  $24 \pm 6.6\%$  ( $n = 4$ ) for 8-Br-cyclic AMP Application of 500 nM PDBu also produced a similar reduction in calcium currents evoked at  $-5$  mV from a holding potential of  $-65$  mV (Fig 1B) After 2 s applications, the effect was maximal at 1–3 min and was reversible after 5–10 min The maximal peak calcium current reduction was  $33.9 \pm 3.0\%$  ( $n = 16$ )

In separate experiments, there was no effect of PDBu or forskolin application on the isolated T-type calcium current component (not shown)

We used the current remaining at 300 ms as an estimate of the L-type calcium current component, and the current inactivating during the voltage command as an estimate of the N-type calcium current component Since forskolin, 8-Br-cyclic AMP and PDBu all reduced the peak calcium current with little effect on the late (300 ms) calcium current, we concluded that this was most likely due to a selective reduction in the N-type calcium current component To confirm this, calcium current traces were fit using a multiexponential function using the program Asystant (Macmillan Software, New York, NY) Calcium current components could be separated, and their magnitudes determined, based on their widely different inactivation time constants Neither PDBu nor forskolin affected the magnitude of the T-type or the L-type component, but did reduce the N-type current component 15–50%

In other experiments, we applied either forskolin (100  $\mu$ M) or PDBu (500 nM) at-

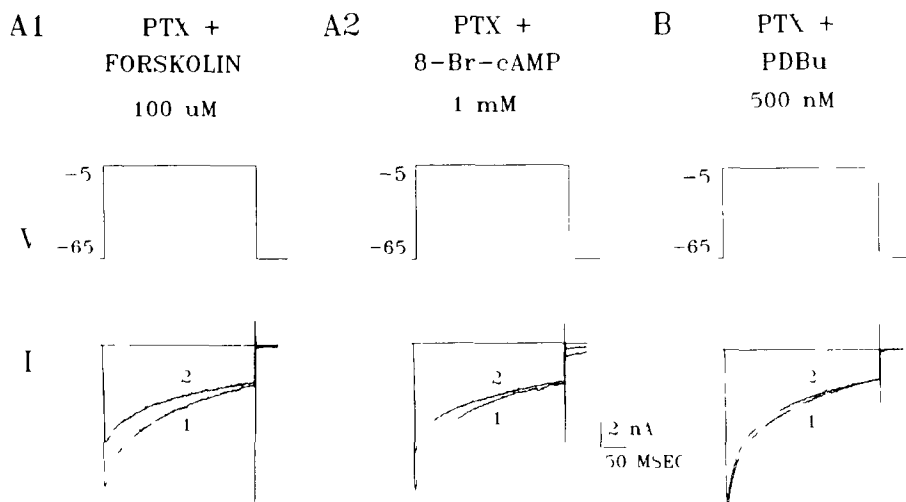


Fig 2 Pertussis toxin pretreatment blocked the effect of PDBu on calcium currents. Digitized leak-subtracted currents from three different neurons are shown. The currents were recorded from neurons in separate dishes of the same platings as in Fig 1. Pertussis toxin was dissolved in distilled water with 0.1% albumin at 50 mg/ml. Aliquots were added to cultures in growth medium to a final concentration of 100–150 ng/ml and kept at 37°C for 4 h. Pertussis toxin was also added to the recording medium at the same concentration during the experiments. Currents are shown before (trace 1) and 4 min after (trace 2) a 2 s drug application. A1: the effect of 100  $\mu$ M forskolin in the presence of pertussis toxin. A2: the effect of 1 mM 8-Br-cyclic AMP in the presence of pertussis toxin. B: the effect of 500 nM PDBu in the presence of pertussis toxin. In all cases,  $V_{\text{h}}$  was  $-65$  mV, with 300 ms voltage steps to  $-5$  mV.

ter the other drug had been applied to the neuron and the calcium current had been reduced to a stable magnitude for at least 1 min. PDBu reduced the peak calcium current  $28 \pm 2.4\%$  ( $n=7$ ), subsequent application of forskolin produced a total peak current reduction of  $42 \pm 2.5\%$ . Forskolin reduced the peak calcium current by  $29 \pm 3.1\%$  ( $n=7$ ), and subsequent application of PDBu resulted in a total peak current reduction of  $37 \pm 2.9\%$ .

We pretreated cultures with pertussis toxin to determine if G proteins were involved in the actions of the A or C kinases on neuronal calcium currents. Because drug effects varied somewhat in different culture groups, we compared the effects of forskolin, 8-Br-cyclic AMP, and PDBu on paired cultures from the same plating: one culture was pretreated with 100–150 ng/ml pertussis toxin, while the other was not. For most experiments, pertussis toxin was added to the culture 4 h before the experiment, a time sufficient to virtually eliminate neurotransmitter effects on calcium-dependent action potentials in chick DRG neurons [9]. The action of forskolin (Fig 2A1) or 8-Br-cyclic AMP (Fig 2A2) was unaffected by a 4 h preincubation with pertussis toxin. The peak calcium current reductions were  $26 \pm 2.7\%$  ( $n=6$ ) for forskolin, and  $21 \pm 3.1\%$  ( $n=6$ ) for 8-Br-cyclic AMP, neither differed significantly from the reductions recorded in the absence of pertussis toxin ( $26.0 \pm 3.1\%$ ,  $n=3$ , and  $24.0 \pm 6.6\%$ ,  $n=4$ , respectively). In contrast, in the presence of pertussis toxin, the

reduction of calcium current by 500 nM PDBu was less than that in its absence (Fig 2B). The peak calcium current reduction was  $12 \pm 1.3\%$  ( $n=39$ ) compared to  $33.9 \pm 3.0\%$  ( $n=16$ ,  $P < 0.01$ ) in the absence of pertussis toxin. The 12% reduction in peak calcium current produced by PDBu in the presence of pertussis toxin was similar to that seen after application of diluent ( $9.9 \pm 1.5\%$ ,  $n=13$ ).

In other experiments, pertussis toxin (100 ng/ml) was added to the cultures 24 h before the experiment. The results were similar to those obtained using shorter pertussis toxin preincubation times. In one experiment, for example, pertussis toxin virtually eliminated the PDBu-induced reduction of the peak calcium current: the reductions were  $32 \pm 2.3\%$  ( $n=3$ ) and 16% ( $n=2$ ) in neurons from control and pretreated cultures, respectively. In contrast, forskolin reduced the peak calcium current to the same extent in neurons from control and pertussis toxin pretreated cultures:  $29 \pm 2.4\%$  ( $n=3$ ) and  $28 \pm 3.9\%$  ( $n=6$ ), respectively.

These results show that drugs which are thought to result in the activation of A and C kinases reduced calcium currents in mouse DRG neurons, and that of the 3 calcium current components in these neurons, only the N-type calcium current component was affected. Previously reported actions of A kinase activation include an increase in calcium current in invertebrate neurons [2, 4, 12]. This invertebrate calcium current may be analogous to the dihydropyridine-sensitive L-type calcium current component in vertebrate neurons. As the associated channel appears to require A kinase-induced phosphorylation to be activated [1], it would be expected that activation of A kinase could increase the L-type calcium current component. In fact, in some neurons an increase in the magnitude of the (curve-fit) L-type current component was seen in the presence of forskolin or 8-Br-cyclic AMP. Nevertheless, the major effect of these compounds in our preparation was to reduce the N-type calcium current component in mouse DRG neurons. To our knowledge, this is the first report of vertebrate neuronal calcium current reduction by these compounds. We suggest that this common selective effect on calcium currents may be the result of A kinase activation.

PDBu similarly reduced calcium currents. Activation of C kinase has been reported to increase invertebrate neuronal calcium current [3, 18] and to decrease vertebrate neuronal calcium current [14, 19–20]. We suggest that the effect in vertebrate neurons may also be due to a selective effect on the N-type calcium current component.

Pretreatment with pertussis toxin prevented the reduction in calcium current produced by PDBu, but did not affect the reduction in calcium current produced by forskolin or 8-Br-cyclic AMP. This suggests that, while the final pool of calcium channels may be similar (less-than-additive effects of forskolin and PDBu), A and C kinases regulate calcium channels by different mechanisms. A G protein appears to be required for C kinase, but not A kinase, to reduce calcium current. These data are consistent with those obtained from other preparations. For example, phorbol esters enhanced cyclic AMP accumulation in Swiss 3T3 cells, an effect blocked by pertussis toxin [16]. It may be that C kinase inactivated  $G_i$ , resulting in greater cyclic AMP production. This hypothesis would account for similar actions of PDBu, forskolin

and 8-Br-cyclic AMP on calcium currents. Our data support the contention that C kinase acts via a G protein (see also ref. 21), but does not eliminate the possibility that the A and C kinases regulate calcium channel function by divergent pathways.

We propose that the activation of both A and C kinases selectively reduced the N-type calcium current component of mouse DRG neurons. This finding is of interest as the N-type calcium current component may be selectively reduced by neurotransmitters [7], and may also be involved in the regulation of neurotransmitter release [8, 13]. Modification of the N-type calcium current by second messenger systems could therefore be an important means of regulating neuronal function.

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