Commentary

Specificity emerges in the dissection of diacylglycerol- and protein kinase C-mediated signalling pathways

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Diacylglycerol- and Ca^{2+}-mediated activation of protein kinase C (PKC) is regarded as a key triggering step in numerous cellular processes, from the regulation of gene transcription to the modulation of stimulus secretion coupling in hormone and neurotransmitter release. However, because of the ubiquitous and often redundant expression of the known PKC isoforms, the large number of identified PKC regulators and substrates, and the presence of alternative diacylglycerol and Ca^{2+} receptors that are also activated by commonly used PKC activators, the precise role of PKC activity in these processes and the identity of the relevant PKC isoforms and substrates have often remained elusive.

Particularly in stimulus secretion coupling and the related process of synaptic transmission, the identification of steps at which PKC is involved has been difficult. This is because in many studies the physiological readout is a signal which is several steps downstream of the one modulated by PKC, and, at least until recently, reagents used to stimulate or block PKC have often been quite unspecific. In the analysis of synaptic transmission, for instance, many preparations allow only to record postsynaptic responses to presynaptic nerve stimulation. If a PKC effect is observed in such a system, a first question to be answered is whether postsynaptic sensitivity has changed or else presynaptic transmitter release. Even if in a suitable experimental paradigm an observed effect is shown to be caused by presynaptic PKC action, a key question remains: does PKC act on the electrical excitability of the nerve terminal or on the transmitter release process? Furthermore, in case of an answer in favor of the latter possibility, one would have to ask whether it is actually the release machinery of a given secretory vesicle that is altered or rather the regulation of the availability of release-ready vesicles, i.e., the vesicle recycling and priming processes.

Some more recent studies (1–6) have advanced to the point where they provided answers to the last question, but the identified processes regulated by PKC were different in different experimental model systems. Two papers in this issue of PNAS, both addressing the regulation of release of hormones from dense-core granules, go one step further in that they demonstrate a specific action of PKC on a subpool of release-ready vesicles (7) and provide quantitative data on the degree of sensitization of the release apparatus (8), with interesting consequences.

Yang *et al.* (7) used bovine adrenal chromaffin cells to identify a small pool of secretory granules with extra-high sensitivity to $[Ca^{2+}]$. This pool is not changed in its sensitivity to $[Ca^{2+}]$, but is dramatically increased in size by application of phorbol esters, which activate most PKC isoforms (but also multiple other C_1 domain proteins). The phorbol ester effect is blocked by bisindolylmaleimide, a moderately specific PKC inhibitor, indicating that it is mediated by PKC. It had previously been shown that releasable granules in chromaffin cells are increased in number by phorbol ester treatment (9) as well as by increased basal levels of $[Ca^{2+}]$ (10), but the degree of these general pool size changes was smaller than that observed by Yang *et al.* (7). The importance of the new finding lies in the fact that the highly Ca^{2+}-sensitive pool of granules has the right properties to be released by moderate changes in global $[Ca^{2+}]$, which occur during the release of Ca^{2+} from intracellular stores. Thus, tight coupling between the release apparatus and Ca^{2+}-channels, which is important for release triggered by single action potentials, is not obligatory for hormone release from this pool of granules.

Release of hormones by moderate overall changes in $[Ca^{2+}]$ is also the final outcome of PKC activation studied in the second paper on stimulus-secretion coupling in this issue (8). However, the mechanism by which this is achieved in the pituitary gonadothrophs studied in this context is different from the one identified by Yang *et al.* (7) in chromaffin cells. The pool size of readily releasable vesicles (i.e., vesicles which can be released by a step-like elevation in $[Ca^{2+}]$ within a fraction of a second) is not changed. However, the sensitivity of the entire vesicle pool to $[Ca^{2+}]$ is increased by a factor of 2–3. This results in hormone release at resting $[Ca^{2+}]$ levels and, particularly, during $[Ca^{2+}]$ transients of 2–3 μM amplitude, as they are induced by gonadotropin-releasing hormone (GnRH) action on its cognate receptors, followed by activation of phospholipase C, generation of inositol 1,4,5-trisphosphate (IP_3), and release of Ca^{2+} from intracellular stores. Also, substantial exocytotic activity is observed between depolarizing stimuli after phorbol ester treatment, whereas before phorbol ester treatment, release is restricted to depolarizing episodes. The latter effect indicates that before phorbol ester treatment, hormone release depends on relatively high $[Ca^{2+}]$ levels that only occur in the vicinity of Ca^{2+} channels while these are open.

The two studies by Yang *et al.* (7) and Zhu *et al.* (8) once again illustrate the diversity of signaling pathways that target PKC and thereby regulate transmitter and hormone release, with very similar physiological effects being generated by quite diverse mechanisms. They describe a combination of electrophysiological and optical tools, which allow to distinguish at least four steps in the secretory pathway, which, in principle, might be altered by activation of the dual diacylglycerol/Ca^{2+} signaling pathway: several ionic conductances regulating Ca^{2+} influx, including Ca^{2+} conductances themselves, upstream steps regulating release of Ca^{2+} from intracellular stores, recruitment of vesicles to at least

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two distinct vesicle pools, and the Ca^{2+} sensitivity of the release process itself. The exciting challenge is now to use this specificity to identify the relevant molecular players. Also, given the complexity of diacylglycerol and Ca^{2+} signaling pathways in cells, the novel roles for PKCs identified in these new studies need to be further verified in the future. In both cases, the most commonly used pharmacological tools were used to interfere with PKC function: phorbol esters as PKC activators and bisindolylmaleimide derivatives as PKC inhibitors that block the ATP binding sites of many PKCs. The implication of PKCs in the processes observed by Yang *et al.* (7) and Zhu *et al.* (8) are based on the assumption that these PKC-targeted drugs are indeed specifically affecting PKC function. However, the specificity of both types of drugs has been questioned in the past. Phorbol esters are definitely not specific activators of PKCs but rather activate Chimaerins, protein kinase D, RasGRPs, Munc13s, and diacylglycerol kinase γ with equal potency (11, 12). Likewise, the use of bisindolylmaleimides has led to the implication of PKCs in certain phorbol ester- and diacylglycerol-dependent processes that were later shown to be caused by alternative diacylglycerol and phorbol ester receptors. Two examples, based on mouse genetic data, are RasGRP1, which mediates the T cell receptor-triggered and phorbol ester-induced activation of the Ras/Raf/MEK/ERK pathway in lymphocytes (13, 14), and Munc13-1, which mediates the phorbol ester-induced enhancement of transmitter release from hippocampal neurons (15).

In view of the possible caveats with phorbol ester- and bisindolylmaleimide-derived pharmacological tools, we must ask how is the functional specificity of secretory steps dissected in the two new PNAS papers by Yang *et al.* (7) and Zhu *et al.* (8) can be used to further substantiate the notion of an involvement of PKCs in the regulation of hormone release from chromaffin cells and gonadotrophs?

Fortunately, improved specificity is also emerging in the molecular tools for studying PKC. First, the relevant PKC isoforms can now be identified. Both chromaffin cells and gonadotrophs are amenable to overexpression experiments. A powerful approach in this context would be to overexpress wild-type and dominant negative forms of individual PKC isoforms (16) to interfere with the newly identified effects of phorbol esters on hormone release from chromaffin cells and gonadotrophs. Also, PKC isoform specific inhibitory or activating peptides (17) can be used to identify the relevant PKC isoforms. In addition, deletion mutant mice can serve as useful tools to study the involvement of individual PKCs. In this context, mutants lacking PKCε (18, 19) may represent a particularly useful experimental model system as this PKC isoform has been implicated in phorbol ester-induced synaptic potentiation in the calyx of Held synapse (20). A similar mouse genetic approach can help to exclude an involvement of alternative phorbol ester receptors such as Chimaerins, protein kinase D, RasGRPs, Munc13s, or diacylglycerol kinase γ.

Second, the relevant PKC targets that mediate the regulatory processes identified by Yang *et al.* (7) and Zhu *et al.* (8) in chromaffin cells and gonadotrophs can now be identified. In that respect, the two new studies provide an extremely interesting basis, allowing to focus on a particular subset of possible PKC targets involved in distinct trafficking and secretion steps (e.g., SNAREs, SNARE regulators, and particularly Ca^{2+} sensors). Admittedly, such identification of relevant PKC substrates still poses a major conceptual and experimental problem. One approach would be to overexpress phosphomimetic and phosphorylation-defective mutant variants of known PKC substrates such as SNAP-25, Synaptobrevin 2, Synaptotagmin 1, and Munc18, which all may interfere with one of the newly described phorbol ester effects or with the vesicle trafficking and secretion steps that were identified with the methods used by Yang *et al.* (7) and Zhu *et al.* (8). In the past, this approach has led to the dissection of the role of PKC-mediated phosphorylation of SNAP-25 during vesicle recruitment in chromaffin cells (21). Alternatively, a more systematic biochemical or proteomic approach can be used to find new PKC targets in chromaffin cells and gonadotrophs.

Finally, it will be important to identify the modulatory neurotransmitter systems that trigger the new PKC-mediated effects in chromaffin cells and gonadotrophs *in vivo*. In the case of gonadotrophs, the study by Yang *et al.* (7) indicates that one endogenous pathway may involve GnRH-dependent PKC activation by GnRH receptor-mediated activation of phospholipase C, followed by IP_3 production and release of Ca^{2+} from intracellular stores. In the case of the chromaffin cell study of Zhu *et al.* (8), the relevant physiological signal that triggers PKC activation remains unclear. Possible mechanisms could involve the angiotensin system, the muscarinic system, and several others.

The two new studies by Yang *et al.* (7) and Zhu *et al.* (8) describe two extremely interesting regulatory mechanisms in hormone release, both of which affect the Ca^{2+}-sensitivity of secretion. Pharmacological evidence indicates that these processes are regulated by PKCs. As is typical for fascinating novel findings, these observations set the stage for further studies that will ultimately lead to the elucidation of the intricate pathways by which PKCs regulate hormone release and other cellular processes.

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